



NANOTECHNOLOGY FOR PRECISION CANCER THERAPY: ADVANCES IN GENE THERAPY, IMMUNOTHERAPY, AND 3D BIOPRINTING

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NANOTECHNOLOGY FOR PRECISION CANCER THERAPY: ADVANCES IN GENE THERAPY, IMMUNOTHERAPY, AND 3D BIOPRINTING

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Editorial: Nanotechnology for Precision Cancer Therapy: Advances in Gene Therapy, Immunotherapy, and 3D Bioprinting

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Nanotechnology for Precision Cancer Therapy: Advances in Gene Therapy, Immunotherapy, and 3D Bioprinting

Cancer is a significant health hazard of the 21st century, and Global Observatory of Cancer (GLOBOCAN) predicts increasing cancer incidence in the coming years. Nanotechnology has sparked a rapidly growing interest in cancer nanomedicine, promising to solve several issues associated with conventional therapeutic agents, including their poor water solubility, cargo degradation, nonspecific distribution, lack of release at the tumor site, systemic toxicity, and low therapeutic index. Thus, this Frontiers Research Topic brings relevant contributions to nanobiotechnology for cancer therapies, associating biotechnologies in genetic, epigenetic, proteomic, immunotherapy and 3D-bioprinting to current nanotechnologies tools.

Circulating tumor DNA (ctDNA) represents an emerging biomarker of liquid biopsies, contributing to the development of translational medicine for early diagnosis, therapeutic effect monitoring, and patient's prognosis with cancer (Reinert et al., 2018). However, sensitive detection of ctDNA remains a challenge, due to their short half-life and low concentrations in blood samples. Miao et al. reported a new method to address this challenge by integrating cycled enzymatic DNA amplification technique and Au nanoparticle@silicon-assisted surface-enhanced Raman scattering (SERS) technique, demonstrating the ultra-high sensitivity and specificity of the method.

RNA interference (RNAi), a post-transcriptional gene regulation mechanism, has significantly obtained high attention in cancer therapy as result of its precision to multi-targeted gene silencing. However, its direct administration has not been fruitful due to inherent lower half-lives and instability in the biological systems. Swaminathan et al. highlighted different types of nanotechnology-based delivery strategies for engineering nano-RNAi-based bio drugs, which are entering the preclinical and clinical evaluation. Promising results indicate these new combinatorial therapies can successfully and safely be used to inhibit target genes in cancer patients, shaping the future for next generation cancer therapeutics. Al-Sowayan and Al-Shareeda discussed the advantages of establishing a nanogenomics-artificial intelligence-based breast cancer diagnostic approach, compared to the gold standard radiology- or histology-based methods that are currently being adapted to artificial intelligence. Furthermore, they highlighted advantages of building the diagnostic and prognostic biomolecular profiles for breast cancers based on the exosome encapsulated content, instead of the free circulating microRNA and other biomolecules.

Brar et al. focused on the recent advancement of nanotechnology to colorectal cancer (CRC) diagnosis and treatment. The application of light-sensitive photosensitizer drugs loaded gold and

silica nanoparticles can be successfully used for CRC diagnosis and targeted delivery of anti-cancer drugs to kill CRC cells. In addition, Perumal et al. discussed the presence of gut microbiota influences the route of biomarker detection and the route of the interaction of nanoparticle/drug complexes with target cells. Thereby, the development of nanoparticles with appropriate sizes, morphologies, chemical compositions and concentrations might overcome this fundamental barrier for the CRC diagnosis and treatment.

Nanotechnology can be a powerful approach to overcome the hydrophobicity and low aqueous solubility issues of anti-parasite/worm drugs such as the benzimidazole (BMZ) family. Movahedi et al. developed a nanocrystal lipid-coated calcium phosphate system, which enhance BMZ solubility, specifically targeting melanoma cells, as an optimistic anti-cancer therapy. Silva et al. established a new third-generation photosensitizer, consisting in hybrid nanostructures of lipid carriers coated with porphyrin-chitosan. They demonstrated that physical and chemical properties of nanoparticles are relevant to improve the porphyrin photodynamic activity in bladder cancer cells.

Among different cancers, lung tumors has low response to therapy and new therapeutic approaches have been developed, especially against primary tumors and secondary lung metastasis (Howlader et al., 2020). Donkor and Jones explored the pulmonary route as an attractive drug delivery pathway to treat lung tumors and discuss the potential of nanotechnology to delivery cancer vaccine vectors to prevent the seeding of tumors in the lung. Ahmad et al. reviewed the interactions between an approved cancer nanomedicine with tumor microenvironment immunology and discussed the challenges that need to be addressed for the full clinical potential of ongoing cancer nanomedicines despite the encouraging preclinical data.

Recently, spheroids and organoids have been largely explored as 3D solid tumor models for recreating *in vitro* tumorigenesis, being considered as suitable models for drug assessment and high-throughput screening (Jensen and Teng, 2020). The main advantages of 3D bioprinting are its ability to engineer complex and controllable 3D tissue models in a higher resolution (Datta et al., 2018). Although 3D bioprinting represents a promising technology, main challenges need to be addressed to improve the results in cancer research. Kronemberger et al. explored the principal cell components and extracellular matrix composition to reconstruct the solid tumor's microenvironment, the recapitulation of *in vitro* tumorigenesis through

spheroids and organoids 3D models as well as the opportunities, challenges, and applications of 3D bioprinting in cancer research.

Overall, this collection of articles covers recent advances and contributes to the research topic with innovative designs and concepts in multifunctional and intelligent nanomedicine. The bio-nano engineered approach is not only the key strategy to overcome the intrinsic limitations of nanomaterials but also providing new aspects of the nanotechnology role in diagnostic, delivering drugs for enhancing the cancer therapy, as well as reducing drugs side effects. Therefore, nanotechnology is a promising approach to offer new opportunities of patient's treatment against cancer. The editors hope that the selected articles on this Research Topic will inspire future work to further advance to drug delivery through specific nanomaterials at the tumor site, offering an efficient and personalized cancer therapy. In addition, nanotechnology could motivate basic and clinical research and industry support to accelerate the clinical trials implementation and approval in this field.

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The Proposition of the Pulmonary Route as an Attractive Drug Delivery Approach of Nano-Based Immune Therapies and Cancer Vaccines to Treat Lung Tumors

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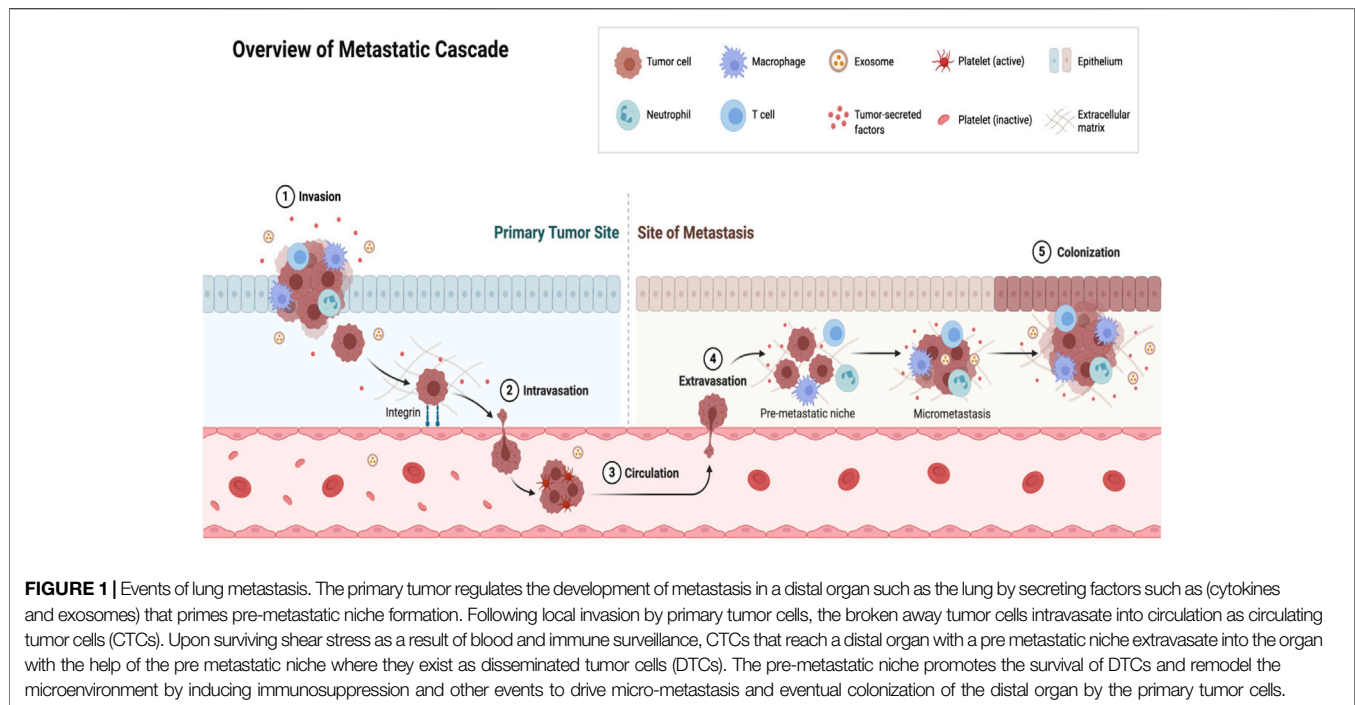
Lung cancer is the leading cause of cancer related deaths globally, making it a major health concern. The lung's permissive rich microenvironment is ideal for supporting outgrowth of disseminated tumors from pre-existing extra-pulmonary malignancies usually resulting in high mortality. Tumors occurring in the lungs are difficult to treat, necessitating the need for the development of advanced treatment modalities against primary tumors and secondary lung metastasis. In this review, we explore the pulmonary route as an attractive drug delivery approach to treat lung tumors. We also discuss the potential of pulmonary delivery of cancer vaccine vectors to induce mucosal immunity capable of preventing the seeding of tumors in the lung.

Keywords: lung, cancer, immune, therapy, nasal, delivery, pulmonary, nanotechnology

INTRODUCTION

The World Health Organization (WHO) reported lung cancer to be the most common cause of cancer deaths worldwide, contributing to 1.76 million deaths in 2018 alone (Ferlay et al., 2019). In 2020, the United States (US) estimates 228,820 new cases of lung cancer and 135,720 projected deaths; the highest among all cancer related deaths (Siegel, Miller et al., 2020). Despite advances in treatment, the 5-year survival rate for primary lung cancer remains at 19% compared to prostate cancer, melanoma of skin and female breast cancer having 5-year survival rates of 98, 92, and 90%, respectively (Lu et al., 2019, Siegel et al., 2020). The persistent low survivorship among lung cancer patients is in part due to the large proportion of patients (57%) diagnosed with metastatic disease, for which the 5-year relative survival is a meager 5% (Howlader et al., 2020). This suggests a further unmet need to improve treatment strategies that can reduce lung cancer occurrence and prevent metastasis thereby increasing survival rates. Lung cancer is broadly categorized into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the most prevalent form, accounting for about 85% of all lung cancer cases and has a 5-year survival rate of 24% (Howlader et al., 2020). NSCLC is further classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. SCLC represents the most aggressive type of lung cancer with the poorest 5-year survival rate of 6% (Howlader et al., 2020).

In addition to primary lung cancer, extra-pulmonary tumors are capable of remodeling the lung microenvironment to support the establishment and outgrowth of disseminated tumor cells to generate secondary metastatic tumors in the lung (Peinado et al., 2017) (**Figure 1**). Circulating tumor cells (CTCs) that reach the lungs directly through blood circulation *via* intravasation or through the



lymphatics are termed disseminated tumor cells (DTCs). Successful colonization of the lungs by DTCs require the initial formation of a permissive microenvironment; often referred to as a pre metastatic niche through an interplay between tumor derived components, the rich lung stromal tumor microenvironment and mobilized bone marrow derived cells (BMDCs) (Liu and Cao, 2016). The tumor derived molecular components that govern pre-metastatic niche formation include extracellular vesicles (exosomes, microvesicles and large oncosomes) and tumor derived secreted factors (growth factors, cytokines and chemokines). These induce certain changes in the lung microenvironment leading to the recruitment of BMDCs into the lung, where they act together to support metastasis. Such cross talk among various tumor derived factors, the lung stromal microenvironment and the mobilized BMDCs promotes metastasis by mechanisms including immunosuppression, inflammation, angiogenesis and lymphangiogenesis (Liu and Cao, 2016). For example, lung tropic 4,175 exosomes were identified to co-localized with S100A4-positive fibroblasts and lung epithelial cells expressing the surfactant protein C where they induced the expression of pro-inflammatory and metastatic inducible S100 genes (Hoshino et al., 2015). Small nuclear RNA enriched exosomes were reported to induce chemokine secretion in the lung and promote recruitment of neutrophils to the pre metastatic niche by activating alveolar epithelial TLR3 (Liu et al., 2016). Extracellular vesicles secreted by cancer associated fibroblasts carrying protein cargos have been implicated in the formation of pre metastatic niche in the lung by targeting lung fibroblasts through TGF- β signaling enhancing remodeling of the extracellular matrix (Kong et al., 2019). Furthermore, primary tumor derived chemokine, CCL2 induces the expression of TLR4 ligands, S100A8

and SAA3 by lung club cells to support vascular permeability in the lung and stimulate the migration of myeloid cells (Maru, 2015).

Lung metastases are identified in 20–54% of all cancer patients and spread to the lungs is usually a marker of advanced malignant disease which is associated with poor survival (Mohammed et al., 2011). **Table 1** below lists the leading primary cancers with high risk of lung metastasis and low survival rates. Consequently, prevention or successful treatment of secondary lung metastasis due to an existing primary extrapulmonary tumor is considered the gold standard to improve 5-year survival rates in cancer patients. However, tumors occurring in the lung either as a primary tumor or metastasis from an existing extra pulmonary tumor remain difficult to treat despite advancements in current treatment approaches involving surgery, chemotherapy, radiation and immunotherapy. In this review, we provide a summary of current treatments and prospective future directions to improve the management of tumors that occur in the lungs either as a primary tumor or metastasis from extra-pulmonary tumors. We focus primarily on immunotherapy as an option to prevent the seeding of tumor cells in the lungs following the occurrence of extra pulmonary tumors by highlighting the pulmonary route as an attractive strategy to deliver “prophylactic” cancer vaccines.

CONVENTIONAL TREATMENTS OF PRIMARY AND SECONDARY METASTATIC LUNG CANCER

Surgical removal is the primary course of treatment for non-metastatic lung cancers where tumors are confined in the lung as seen in stage I and II NSCLC (Lackey and Donington, 2013). The

TABLE 1 | Leading primary cancers with high risk of lung metastasis.

Cancer type	Incidence	Lung metastasis prevalence	References
Bladder	It is the 10th most common cancer, accounting for 2.1% of all cancer-related deaths worldwide	The lung is the third most common site of metastasis after the lymph node and bone	Bray et al. (2018), Ferlay et al. (2019), Saginala et al. (2020)
Breast	Approximately 12% of women in the U.S. will develop invasive breast cancer over the course of their lifetime which makes up 30% of all newly diagnosed cancer in women	21–32% of patients with breast cancer develop lung lesions with a median survival of only 25 months	Wu et al. (2017), Xiao et al. (2018), Siegel et al. (2020)
Colorectal	Second most common cause of cancer deaths in the U.S. It is estimated to contribute to over 53,000 deaths in 2020	10–15% of patients with systemic metastasis have lesions in the lung	Mitry et al. (2010), Siegel et al. (2020)
Kidney	Renal cell carcinoma, the most common type of kidney cancer makes up approximately 2% of all tumors	Lung lesions are the most common and are identified in about 45% of kidney cancer patients	Bianchi et al. (2012), Siegel et al. (2020)
Melanoma	Melanoma only makes up approximately 1% of skin cancers but most skin cancer deaths are attributed to melanoma. It is estimated to affect about some 100,350 people in the U.S. in 2020	Lung metastasis is common especially in advanced stages. 89% of patients with stage IV melanoma as described by the American Joint Committee on cancer have metastatic lung lesions	Neuman et al. (2007), Siegel et al. (2020)
Neuroblastoma	Most common malignant disease in children less than one-year old accounting for 6% of cancers that occur in children	The bone marrow, bone, lymph nodes, CNS, liver and lung are all sites of metastases	D'Ambrosio et al. (2010), Siegel et al. (2020)
Prostate	Approximately 1 in every 9 men will have prostate cancer. It is the second leading cause of cancer death among men in U.S.	More than 40% of patients are diagnosed with secondary lung metastasis	Gago et al. (2016), Siegel et al. (2020)
Wilms tumor	Most common kind of kidney cancer in children. Approximately 500–600 children are diagnosed with Wilms's tumor in the U.S. each year	Lung is the most common site of metastasis	Elayadi et al. (2020), Siegel et al. (2020)

5-year survival rate in resected stage 1 NSCLC patient is 70%, but this drops to 25% in stage III patients (Imperatori et al., 2010). Thus, surgery becomes ineffective as the tumor progresses, requiring alternative treatment modalities. In cases of lung metastases from distal primary tumors, surgery may be effective in prolonging survival in patients with metastases that is limited to pulmonary parenchyma without the involvement of other organs (Warwick and Page, 2007). By comparison, surgery is rarely an option for SCLC, the most aggressive type of lung cancer (Barnes et al., 2017; Xu et al., 2019).

Chemotherapy is the primary treatment for SCLC due its propensity to spread quickly to distal organs (Ganti et al., 2007; Karim and Zekri, 2012; Yang S. et al., 2019). Initial chemotherapy for SCLC patients usually consists of a combination of etoposide and a platinum agent (either carboplatin or cisplatin) (Ganti et al., 2007; Yang S. et al., 2019). In terms of NSCLC involving the lymph nodes, neoadjuvant chemotherapy given months before surgical resection of tumors in the lung is the standard course of action (McElnay and Lim, 2014). Chemotherapy given months before surgical procedure increases the cure rate in patients with stage III NSCLC (McElnay and Lim, 2014). Chemotherapy can also be given after surgical resection (adjuvant chemotherapy) in patients with NSCLC. This is beneficial because at the time of surgery there may be micrometastasis which may not be detected and removed as part of surgical procedure and may lead to recurrence of the tumor. Pulmonary metastasis is a sign of advanced systemic disease and systemic chemotherapy is usually part of the model of therapy (Chojniak et al., 2006). Depending on the type and nature of primary tumor and extent of lung involvement, it may be combined with surgery either as neoadjuvant or adjuvant chemotherapy to treat lung lesions and the primary tumor. For example, patients with primary osteosarcoma and pulmonary metastases (e.g., Less than eight metastatic lesions) who were

treated with neoadjuvant ifosfamide, surgical resection of both primary and metastatic tumors and high dose methotrexate, ifosfamide, doxorubicin and cisplatin had a five-year disease free survival rate of 66.7% (Harris et al., 1998).

In contrast to chemotherapy, radiation therapy is a suitable option for early stage lung cancer especially in cases where the patient is inoperable due to compromised pulmonary function or co-morbid conditions such as hypertension and diabetes (Timmerman et al., 2010). Although conventional radiation therapy in management of early stage lung cancer is disappointing, advancement in the field following the adoption of stereotactic technology has improved treatment outcomes. Stereotactic body radiation therapy (SBRT) enables the delivery of high dose radiation to tumors while sparing healthy tissues. Although radiation is highly targeted, it is not effective in eliminating tumors when it has spread from the lung. SBRT may be effective in treating patients with limited metastatic disease due to high rate of local control and minimal toxicity (Carvajal et al., 2015; Filippi et al., 2016; Rieber et al., 2016; Agolli et al., 2017).

EMERGING IMMUNE-BASED CANCER TREATMENT FOR PRIMARY AND SECONDARY LUNG METASTASIS

The Concept of Immunotherapy

The hallmark of the immune system is the ability to recognize, eradicate and establish memory defenses against pathogenic encounters and foreign substances (Schreiber et al., 2011). The immune defense system is also considered the first line against the genesis and progression of tumors (Davis et al., 2015). During tumor development, mutations occur leading to potentially immunogenic cancer cells that can be recognized and killed by

the host immune system (Pandya et al., 2016). For example, non-self-antigens expressed by tumor cells can be presented on MHC-1 molecules and recognized by cytotoxic T-cells leading to T-cell activation and killing of tumors (Speiser and Ohashi, 1998). Hence, infiltration of solid tumors by immune cells (T-cells and B-cells) has been identified as a good prognostic biomarker in most types of cancers as it correlates to favorable clinical outcomes. Therefore, immune surveillance is able to prevent the occurrence of primary tumors as well as the rise of metastasis. Yet, tumors can evade the immune system by suppressing immune cell function through various mechanisms that mimic immune tolerance to escape immune surveillance (Cacan, 2017; Claisse et al., 2017; Noguchi et al., 2017). Tumor cells achieve this through deliberate loss of antigenicity by downregulating the expression of tumor antigens required for initiation of an immune response and through mechanisms that disrupt antigen presentation, such as loss of major histocompatibility complex-1 (Schreiber et al., 2011). Tumor cells again evade immune surveillance by exploiting immune cell intrinsic mechanisms of immune tolerance by expressing PD-L1, where PD-1/PD-L1 interaction between tumors and cytotoxic T-cells have been shown to play a vital role in limiting antitumor immunity (Pardoll, 2012; Sharma and Allison, 2015). Other mechanisms include release of immunosuppressive cytokines by tumors and tumor cell intrinsic aberrations/unique metabolic pathways that lead to impaired T-cell activation and accumulation in the tumor microenvironment aiding in tumor evasion (Spranger et al., 2015; Casey et al., 2016; Koyama et al., 2016; Peng et al., 2016). For example, the activation WNT/ β -catenin signaling, PTEN loss, Myc signaling and HIF-1 α signaling are known for poor T-cell priming and reduced intratumoral accumulation of cytotoxic T-lymphocytes (Spranger et al., 2015; Zelenay et al., 2015; Casey et al., 2016; Koyama et al., 2016; Peng et al., 2016).

Immunotherapy is aimed at augmenting immune responses and/or inhibiting the suppressive activity by tumors from immune system's antitumor attack. Earlier immunotherapy drugs, such as recombinant cytokine administration, were used to activate and expand the immune system to fight against tumors. Most notable, was the administration of high dose interleukin-2 (IL-2), which is approved for management of metastatic renal cell carcinoma and melanoma (Schreiber et al., 2011). IFN- α is also one of the earliest approved immune-based adjuvant therapies used for patients with resected high-risk melanoma (Hancock et al., 2004). These are less commonly used today because of their toxic effects which warranted the development of newer advanced agents.

Current Advances in Immunotherapy

Recently more advanced approaches have arisen as the immune suppressive mechanisms exploited by tumor cells became clearer. Agents targeting the programmed death-1 receptor and its ligand (PD-1 and PD-L1) axis, termed as immune checkpoint inhibitors (ICIs) have emerged as effective therapeutic approaches in lung tumors because it restores T-cell mediated immunity (Pardoll, 2012). In 2015, nivolumab, an antibody disrupting the PD-1 and PD-L1 pathway was approved as the first immune checkpoint

inhibitor for NSCLC (Altorki et al., 2019). Since then, other ICIs have been approved to be used in different stages of lung cancer. Pembrolizumab is the standard treatment for patients having PD-L1 expression greater than 50% whereas nivolumab and atezolizumab are all available options for patients who do not respond to treatment after platinum doublet chemotherapy (Sapalidis et al., 2018; Lim et al., 2020). By extension, the immune check inhibitors may be beneficial in pulmonary metastases if the extra thoracic primary tumor expresses high levels of PD-L1. Other check point inhibitors target the CTLA-4 axis which limits the proliferation and survival of T cells. CTLA-4 antibodies turn off this inhibitory mechanism leading to the activation of cytotoxic T cells involve in killing tumors (Zhao et al., 2018). However, emerging research has shown that, the mechanism behind therapeutic CTLA-4 antibodies is their ability to cause selective depletion of Tregs in the tumor microenvironment (Liu and Zheng, 2018; Hoy et al., 2019). Some examples are ipilimumab and tremelimumab, with ipilimumab being the first drug in this category to be approved for clinical use as part of first line or second line regimen for treating advanced melanoma (Lipson and Drake, 2011; Robert et al., 2011). In May 2020, the FDA approved the combination of nivolumab, an ICI, plus Ipilimumab (anti-CTLA4 antibody) as first line regimen for patients with metastatic NSCLC cancer with PD-L1 tumor expression $\geq 1\%$. Thus, CTLA-4 responsive tumors may be efficacious in preventing pulmonary metastasis. An additional immunotherapy recently developed is the chimeric antigen receptor (CAR) T-cells therapy. It has been very successful in treating hematological malignancies but has been disappointing thus far in solid tumors due to lack of antigenic targets (Chen et al., 2019). The identification of some antigenic targets on lung cancers such as mesothelin, disialoganglioside (GD2), MUC 1, NY-ESO-1, human epidermal growth factor and epidermal growth factor receptor has begun research into testing the efficacy of CAR T-cells therapy in lung cancer (Hu et al., 2020). Currently, CAR T-cell therapy targeting the above mentioned antigens are in different stages of clinical trials as potential immunotherapy against thoracic malignancies (Kiesgen et al., 2018; Oliveres et al., 2018).

Other immunotherapy strategies include the use of monoclonal antibodies that target receptors that play crucial roles in the lung microenvironment signaling pathways to support tumor growth in the lung (Hanahan and Coussens, 2012; Pitt et al., 2016). For example, bevacizumab, a VEGF antibody inhibiting tumor angiogenesis has been approved for use in combination with chemotherapy for management of advanced, non-squamous NSCLC (Pilotto et al., 2014).

Table 2 provides a list of current immunotherapies used in primary and secondary lung cancers.

PULMONARY DELIVERY APPROACHES TO MANAGE LUNG CANCER AND SECONDARY LUNG METASTASIS

The Historical Use and Benefit

Nasal-Pulmonary Drug Delivery Systems

The pulmonary route, either intranasally or via inhalation is a common form of drug delivery. It has been used in the treatment

TABLE 2 | Current Immunotherapies to combat primary and metastatic lung cancers.

Immunotherapy	Category	Indication	References
IL-2	Cytokine therapy	Inhaled form has been shown to have some activity against secondary lung metastasis in patients with melanoma and renal cell carcinoma	Enk et al. (2000), Posch et al. (2014)
Interferon alpha	Cytokine therapy	Subcutaneous injection of IFN-alpha in combination with S-1 was effective against secondary lung metastasis from hepatocellular carcinoma	Akita et al. (2015)
Trastuzumab	Monoclonal antibody targeting HER2 protein	Went into phase II clinical trial to test efficacy against HER-2 positive non-small cell lung cancer	Hotta et al. (2018)
Ibritumomab tiuxetan and tositumomab	Radiolabeled monoclonal antibody	Effective against pulmonary metastasis in non-Hodgkin's lymphoma where the burden of lung metastasis is not high. Tositumomab was more effective	Song et al. (2007)
Cetuximab	Monoclonal antibody that targets EGFR	Cetuximab plus chemotherapy improved overall survival in patients with advanced EGFR positive NSCLC compared with chemotherapy alone in a phase III clinical trial	Pirker and Filipits (2012)
Bevacizumab	Monoclonal antibody targeting VEGF	Bevacizumab has been approved to be used with standard chemotherapy as first line treatment in patients with advanced or recurrent non squamous NSCLC.	Sandler et al. (2006), Matikas et al. (2016)
Ado-transtuzumab emtasine	Chemo labeled monoclonal antibody targeting HER2 protein	In a phase II clinical trial, ado-transtuzumab ematasine was effective in patients with HER2-mutant lung cancers	Li B. T et al. (2018)
Pembrolizumab	PD-1 inhibitor	Pembrolizumab has supplanted cytotoxic chemotherapy as first line therapy for patients with advanced NSCLC whose tumor proportion score of programmed death ligand 1 (PD-L1) is 50% or more. Currently, its efficacy and safety as a combined regimen together with chemotherapy is being investigated in metastatic non-small cell carcinoma	Gandhi et al. (2018), Lim et al. (2020)
Nivolumab	PD-1 inhibitor	Nivolumab has been approved by the FDA as second line treatment in patients with advanced NSCLC	Somasundaram et al. (2016)
Atezolizumab	PD-L1 inhibitor	Atezolizumab has been approved as part of a second drug combination for the management of people with non-squamous NSCLC with no alteration in the EGFR or ALK genes	Chen et al. (2018)
Ipilimumab	CTLA-4 inhibitor	The FDA has approved the combination of ipilimumab and nivolumab as first line regimen for patients with metastatic non-small cell lung cancer with PD-L1 tumor expression $\geq 1\%$	Mankor et al. (2020)

of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and viral infections (e.g., influenza) that affect the airways where corticosteroids, beta-2 adrenergic agonist and antiviral agents are administered directly into the lung (Levin et al., 1996; Mossad, 2003; Lee et al., 2010). The advantages are well known including accessibility, efficient high drug concentrations in the lung and hence a minimal amount of the drug is needed to achieve higher efficacy with minimal side effects in the above-mentioned conditions (Rau, 2005). Currently, there is considerable attention in formulation of drugs for pulmonary delivery for substantial reasons. Pulmonary delivery is not affected by first pass metabolism as the lungs exhibit very low metabolic activity (Pilcer and Amighi, 2010). Importantly, the lung's anatomy aids its use as an entryway of drugs. It has a large surface area (100 m²) and thin absorption membrane (0.1–0.2 μ m) that supports rapid absorption of substances including drugs and high blood flow (5 L/min) which quickly distributes molecules throughout the body. This supports the reason for the use of pulmonary route to treat systemic diseases such as diabetes (inhaled insulin) (Chan and Cheng-Lai, 2017), making it ideal to be considered as a viable option in the treatment of primary lung cancer and secondary lung metastasis.

Inhaled Chemotherapy

In recent years, pulmonary delivery has been considered an improved drug delivery approach in delivering chemotherapeutic drugs locally to the tumor site in the lungs as it offers several benefits such as increase in the amount of drug deposited in tumors, less drug concentration required to achieve efficacy and minimal toxicity due to limited systemic distribution of drugs (Zarogoulidis et al., 2012b; Zarogoulidis et al., 2012c; Goel et al., 2013; Sardeli et al., 2020). Evaluation of this delivery approach in human subjects has shown promising results. For example, inhaled 5-fluorouracil (5-FU) in human subjects led to higher drug concentrations in tumors than in surrounding tissues (Tatsumura et al., 1993). Zarogoulidis et al. also showed that inhaled carboplatin provided improved anticancer efficacy and prolonged survival of patients with NSCLC compared with patients who received intravenous carboplatin (Zarogoulidis et al., 2012b). However, the physicochemical properties of chemotherapeutics such as molecular weight and solubility, may limit their penetration and accumulation into pulmonary tumors despite improved localization of these drugs in the lung following pulmonary delivery (Minchinton and Tannock, 2006; Zarogoulidis et al., 2012a). Again, the nature of the tumor including size, cellularity and density of interstitial tissues may limit drug penetration following pulmonary administration

(Mangal et al., 2017). These characteristics ultimately decrease the anti-tumor efficacy of chemotherapeutics following pulmonary delivery and may lead to unwanted side effects due to the potential exposure of healthy cells in the lung to the toxic effects of chemotherapeutics due to their inability to penetrate and accumulate into tumors.

Nanotechnology has been used to offset similar barriers in the administration of systemic chemotherapy (Senapati et al., 2018). The success of this approach has fomented an interest in exploring the prospects of incorporating chemotherapeutics into nano-based systems such as liposomes, polymeric nanoparticles and micelles capable of pulmonary delivery. Nanoparticles are able to penetrate and accumulate in solid tumors by a phenomenon known as the enhance permeability and retention effect (Mohanty et al., 2012) due to the uniqueness of tumor tissue vasculature (poor fenestrations) and inefficient lymphatic drainage. Also, unlike free drug, nanoparticle encapsulated drug can be taken up directly by tumor cells via endocytosis leading to enhanced accumulation of the drug in tumors (Guo et al., 2018). Importantly, nanoparticle encapsulated chemotherapeutics are more efficacious and safer because they are able to accumulate more in pulmonary tumor tissues, thereby sparing healthy cells due to less systemic drug circulation. Nanoparticle encapsulating chemotherapeutic agents delivered via the pulmonary route have been shown to have increased efficacy in treatment of primary lung tumors and inhibiting metastasis from an existing extra-pulmonary primary tumor. For example, Inhaled lipid coated formulation of 5-FU achieved sustained drug release and enhanced anticancer properties (Hitzman, et al., 2006a; Hitzman, et al., 2006b). Lemarie et al. also showed that pulmonary delivery of nanoparticle encapsulating gemcitabine leads to minimal systemic cytotoxicity due to lower plasma drug concentration (Lemarie et al., 2011). In addition, nanoparticles can be surface functionalized with a targeting moiety such as molecules that are only expressed on the surface of tumors to allow for targeted delivery of drugs (Mout et al., 2012). This receptor ligand conjugation approach further enhances the concentration of drug locally with reduced doses and less cytotoxicity to healthy tissues. However, the adverse effects of pulmonary administration of chemotherapeutic drugs including its effect on the lung parenchyma needs to be properly assessed. Neurotoxicity was noted to be associated with pulmonary delivery of paclitaxel liposome aerosol as treatment to inhibit pulmonary metastasis from an extra-pulmonary tumor (Koshkina et al., 2001). Inhaled doxorubicin has been associated with severe cardiotoxicity (Hershey et al., 1999). Two phase I and phase I/II trials indicated metallic taste, mild bronchospasm and moderate reduction of pulmonary function test as side-effects associated with aerosol treatments (Zarogoulidis et al., 2012a).

Inhaled Immunotherapy

The efficacy of chemotherapeutics administered into the lungs *via* pulmonary delivery supports the potential for a strategy to be employed for the purpose of immunotherapy delivery approaches via the nasal-pulmonary tract. The potential advantages are a safe

and non-invasive alternative to current approaches that require direct injection of immunomodulators into tumors (Hamid et al., 2020) which are typically hard to reach by direct injection such as those located in the lungs. Notwithstanding, in situations that require repeated injection to maintain immune responses, the pulmonary administration offers a noninvasive approach allowing for non-invasive repeated treatment modalities that directly target the lung without the potential for systemic adverse effects. In efforts to reduce toxicities from systemic administration of IL-2, the pulmonary route has been investigated as a viable alternative where low dose IL-2 has shown some efficacy for the treatment of lung metastasis in patients with melanoma and renal carcinoma (Enk et al., 2000; Hulan et al., 2003; Posch et al., 2014). Recently in efforts to make ICIs more efficacious while reducing side effects, methods of sustained and tumor targeted delivery of ICIs are being explored. Some of the delivery strategies currently under investigation include: the use of viral vectors, delivery using bacteria and delivery of ICIs as DNA encoded monoclonal antibodies (Engeland et al., 2014; Lin et al., 2017; Perales-Puchalt et al., 2019; Reul et al., 2019; Gurbatri et al., 2020). However, most of the above delivery strategies require intratumoral or intravenous injections requiring hospitalization. This makes pulmonary delivery of ICIs an attractive alternative that requires much attention and research in treating both primary lung cancer and secondary lung metastasis as pulmonary delivery such as inhalation can easily be performed in an outpatient setting.

PULMONARY DELIVERY OF THERAPEUTIC LUNG CANCER VACCINE AS PART OF INHALED IMMUNOTHERAPY

Dendritic Cell-Based Cancer Vaccines

The immune system does not only recognize and eliminate substances that are non-self but also generates memory after eliminating these substances (Schreiber et al., 2011). This forms the basis of vaccination; the ability of the immune system to mount a robust and specific immune response upon exposure to a previously eliminated antigen. This approach of inducing immunity is being used to mobilize antitumor immunity to help patients that do not benefit enough from current immunotherapies such as checkpoint inhibitors. The direct induction of antitumor immunity by vaccination relies on dendritic cells (DC) because of the unique role they play as part of the immune system where they serve as a bridge between the innate and adaptive immunity by initiation and directing the adaptive immune response (Harari et al., 2020). There are two broad strategies in which DCs are being used to promote anticancer immunity. The first approach involves the release of tumor antigens into the body either through *in situ* vaccination approaches or direct introduction of tumor antigen carried by a vaccine vector and the *ex-vivo* approach, which relies on loading patients DCs with tumor antigen *in vitro* followed by administration of those DCs back to the patient (Disis, 2014). Although studies have shown less clinical benefits, DC-based vaccines could still be an essential part of an integrated

anticancer strategy in managing tumors occurring in the lungs. This will require the identification of a suitable tumor antigen capable of invoking robust anticancer immunity, an appropriate vaccine vector for delivery of the antigen and an appropriate delivery strategy into the body.

Cancer Vaccine Antigens

Therapeutic cancer vaccines either employs whole tumor cells or lysates which contains a broad range of antigenic repertoire or a single/few selected and validated cancer antigens in the form of peptide or recombinant proteins. Most cancer vaccines target tumor associated antigens (TAAs), which are abnormally or overexpressed proteins by cancer cells due to mutations in proteins that drive their transcription. TAAs include cancer germline antigens which are normally expressed only in trophoblast tissue and germline cells (e.g., MAGE-A1, MAGE-A3, and NY-ESO-1), cell lineage differentiation antigen, which are normally expressed by cells early in life but usually absent during adulthood (e.g., prostate specific antigen (PSA) and prostate acid phosphatase (PAP) and antigens that are present at relatively low levels on normal cells but overexpressed by cancer cells (e.g., HER2 and MUC-1). Because they are not exclusively expressed by cancer cell but are expressed by certain normal cells early on in life or at relatively low levels, immune activation to these antigens is low because T cells and B cells that strongly recognize these antigens are clonally deleted from the repertoire during negative selection. Vaccination with TAAs therefore require strong vaccine adjuvants and repeated vaccination to generate robust immune response capable of immunological memory. However, this may lead to the expansion of self-reactive T cells causing autoimmune disease. For instance, Palmer et al., showed that adoptive transfer of CD8⁺ T cells specific for melanoma associated antigen triggered severe ocular autoimmunity in mice as this antigen is also expressed in eye melanoma cells (Palmer et al., 2008). The potency and off target effects of vaccines must be critically evaluated when it involves TAAs. The etiology of approximately 10% of human cancers is viral infection. This means that vaccination to prevent or treat viral infection that induces the cancer can lead to cancer prevention. For example, vaccination against Hepatitis B Virus, a known cause of hepatocellular carcinoma is known to decrease both the rate of infection and incidence of hepatocellular carcinoma.

Neoantigens are non-self-proteins that arise due to cancer specific mutations. They could specifically be recognized by T-cell receptors specific for the antigen in the groove of MHC molecules. In this regard, neoantigens are potentially ideal targets for the construction of therapeutic cancer vaccines. Unlike TAAs, neoantigens are unlikely to mediate immune tolerance and autoimmunity when used with strong vaccine adjuvants. The expression of neoantigens is driven by mutations hence they differ in patients having same type of tumor. The benefit of neoantigen as a cancer vaccine is fully realized when personalized for each patient type where the patient tumor genome is sequenced to identify mutations from which neoantigens are expressed. The identified neoantigen can then be delivered to the patient to induce immune response

(Castle et al., 2019). The advantage of using a selected few of validate cancer antigens is the generation of a more focused immune response. However, the ability of tumors to downregulate the expression of antigenic targets may render such vaccines ineffective (Odunsi et al., 2007). This can be corrected with the use of whole tumor cell lysate that generates a strong and broad polyclonal T cell responses from its polyvalent antigens hence preventing the emergence of tumors that are immune-resistant because of their ability to downregulate the expression of some antigenic targets (Chiang et al., 2015). In addition, the use of whole tumor cell lysate does not require prior knowledge of the patient's HLA haplotype and allows for the induction of both CD4⁺ and CD8⁺ T cells (González et al., 2014). However, such mechanisms cannot be guaranteed with the use of peptides and recombinant proteins. The disadvantage of using whole tumor cell lysate is that they contain self-antigen that can mediate tolerance and immunoregulatory cytokines and factors that dampens immune response. Whole tumor cell lysate may contain IL-10 and transforming growth factor which inhibits the expression of MHC molecules and co stimulatory molecules by APCs and inhibits T-cell proliferation (Loercher et al., 1999), major histocompatibility complex class I related proteins A and B (MICA and MICB) that inhibits killing of tumors mediated by immune cells expression of NKG2D (Groh et al., 2002), Fas ligand which may cause lymphocyte death (Rabinowich et al., 1998) and VEGF which suppresses DCs function (Dikov et al., 2005). Inducing immunogenic cell death (ICD) in tumor cells through irradiation, HOCl oxidation and hyperthermia treatments prior to extraction of whole tumor cell lysate have been found to increase the immunogenicity of the lysate (Vandenberk et al., 2015). ICD leads to the expression of NKG2D ligands, heat shock proteins and calreticulin on the surface of cells and release of immunostimulatory factors that can stimulate immune effectors (Zhou et al., 2019). The priming and boost approach can also help derive the full benefit from whole tumor cell lysate (Harari et al., 2020). In this approach, cancer specific T-cell responses are first induced by administration of whole tumor cell lysate in the priming phase. The patient's immunological response is then analyzed to identify and characterize effective cancer epitopes. The validated cancer antigens are then used to prepare a personalized synthetic vaccine which is administered to the patient to sustain T cell responses in the boosting phase. Therapeutic cancer vaccine against lung cancer in the form of pulsing patients' dendritic cells *in vitro* with whole tumor cell lysate followed by administration of the dendritic cells to the patients showed mixed responses in patients (Um et al., 2010; Kamigaki et al., 2013).

Utility of the Pulmonary Route for Delivery of Cancer Vaccine Vectors—A Case for the use of Nanoparticles

The selection of an appropriate vaccine vector capable of pulmonary delivery of antigens to dendritic cells in the lung and regional lymph nodes may be helpful in generating a robust mucosal immunity in the lung that can eliminate a primary lung

tumor or prevent the seeding of an existing extra-primary tumor to the lung. Although some works have been done with regards to respiratory infections where the pulmonary administration of vaccines has been shown to induce robust mucosal immunity against infections when studied using influenza, tuberculosis and more recently Covid-19 models (Bhide et al., 2018; Hassan et al., 2020; Jiang et al., 2020), the pulmonary route has so far not been considered in delivering vaccines against lung tumors although it offers a promising alternative for generating robust respiratory antitumor immunity capable of restricting the growth of primary lung tumors and preventing the seeding of extra pulmonary tumors in the lung. So far, DC-based vaccines have been delivered to individuals through intravenous, subcutaneous, intradermal, intralymphatic and intratumoral route (Fong et al., 2001; Dohnal et al., 2007; West et al., 2009). For lung occurring tumors, the intravenous and subcutaneous route have been exploited as a way of vaccine delivery into the body to combat lung lesions (Um et al., 2010; Wurz et al., 2014). The pulmonary delivery of DCs that have been primed *in vitro* by a tumor antigen or administration of antigens to prime DCs in the lungs can lead to the sensitization of a large percentage of T-cells in the lungs. Easy accessibility, non-invasiveness and the potential of administration in the out-patient setting are advantages of vaccine delivery by the pulmonary route.

Currently, cancer vaccine vectors that have been studied include cellular vaccines, virus vector vaccine and molecular vaccine (Hollingsworth and Jansen, 2019). Cellular vaccines include the use of killed tumor cells or adoptive transfer of antigen presenting cells (APCs) from the cancer patient loaded with tumor antigens. Bacteria and yeast can be used as vectors in the formulation of cellular vaccine to deliver tumor antigens (Nascimento and Leite, 2012). Viral vaccine vectors involve the use of viruses that encode a tumor antigen to stimulate immune response against the tumor antigen (Choi and Chang, 2013). Molecular vaccine has to do with the administration of a peptide derived from the tumor antigen or DNA/RNA that encodes a tumor antigen or peptide to drive immune response. Pulmonary delivery of these vaccine vectors to induce lung specific immunity have so far been tested against respiratory infections in mice. For example, Jiang et al. showed that there was generation of specific lung and localized immune response against *mycobacterium tuberculosis* following intranasal administration of *mycobacterium* antigens carried by the vector *Listeria ivanovii* (Jiang et al., 2020). Intranasal delivery of peptides and DNA vaccine have also been proven to induce lung specific immune response in mice (Tesoro Cruz et al., 2008; Yang J. et al., 2019). Immune response against vectors (viral or bacterial) carrying a tumor antigen can neutralize the vector, limiting repeated vaccination which is required to generate immune response capable of immunological memory (Saxena et al., 2013). This can be a drawback with the use of vectors (viral or bacterial) for pulmonary vaccine delivery. Again, the administration of naked proteins, peptides and DNA are easily cleared by mucosal ciliary movement before they are able to induce immune response.

In view of this, nanotechnology is emerging as an approach to target anticancer therapies to the lung including their potential use in activation of immune responses in the lung (Marasini et al.,

2017; Al-Halifa et al., 2019; Alshweiat et al., 2019). Nanotechnology have been used to target antigens to dendritic cells either *in vivo* or *ex vivo* as a way of improving the clinical outcomes of therapeutic cancer vaccines as summarized in **Table 3**. Nanoparticles protect antigens susceptible to degradation by proteases and enhance their cellular uptake by antigen presenting cells (APCs), they allow for co-loading and co-delivery of antigens and adjuvants in a sustained released manner enhancing the priming of cytosolic T-lymphocytes (CTL) (Goldberg, 2015). During active targeting, the surface of nanoparticles can be decorated with DC receptor ligands such as ligands for Fc receptors and the C-type lectin receptor family leading to the induction of strong immune response compared to non-targeted nanoparticles conjugates encapsulating antigens (Tacken et al., 2007). These therapeutic cancer vaccines based on nano formulations can be targeted to dendritic cells in the lung via pulmonary delivery. Besides the potential to generate potent mucosal immunity due to the direct delivery of antigens and adjuvants into the lungs where proteolytic activity is low, it offers a noninvasive alternative route to deliver vaccines (Al-Halifa et al., 2019). These advantages of pulmonary deliver of nano-based vaccines make it ideal to considered as a viable model in the management of lung occurring tumors where it can serve as adjunct treatment for primary lung cancer to mobilize the body's immune system to fight the tumor following chemotherapy and/or radiation treatment especially in cases where the tumor has not spread beyond the lung draining lymph nodes. Again, protection against secondary lung metastasis from an existing primary tumor can be achieved following pulmonary administration of a nano-based vaccine against the primary tumor.

The deposition of particles in the respiratory tract following inhalation is size dependent such that particles smaller than the average mesh spacing of the airway mucus are able to penetrate it, escaping physiological mucus clearance to be deposited in the alveolar spaces of the lung parenchyma where their clearance is delayed (Schneider et al., 2017). Once particles get in the gas exchange region, the main mechanism of clearance is by alveolar macrophages. Particles less than 200 nm are less efficiently cleared increasing their retention time in the lung parenchyma compared to larger particles (Blank, et al., 2013). These make nanoparticles ideal for targeting antigens to dendritic cells in the lung parenchyma. Following the deposition of antigens, these antigens are required to be picked up by resident dendritic cells and migrate to a regional lymph node where they can activate naïve T-cells to induce anti-tumor immunity. Smaller particles, however, can easily traffic to the lymph nodes from the site of delivery where they will encounter a high proportion of antigen presenting and naïve T-lymphocytes that can be activated to generate anti-tumor immunity (Choi et al., 2010). For nanoparticles targeting dendritic cells in the lungs, size is important to ensure effective antitumor immunity. Nanoparticle size ranging from 20 to 50 nm are detected in higher quantities in the lung draining lymph node compared to larger nanoparticles (Blank et al., 2013). Again, higher frequencies of nanoparticles ranging from 20 to 50 nm are trafficked by lung parenchyma dendritic cells to regional lymph nodes where they induce stronger antigen specific

TABLE 3 | Nanoparticle platforms used in targeting tumor antigens to dendritic cells.

Nanoparticle type	Use	Properties	References
Inorganic nanoparticles			
Gold nanoparticles (AuNP)	AuNP were used to deliver antigens to dendritic cells by decorating the surface with a red fluorescent protein that served as a model antigen and CpG as a vaccine adjuvant. This construct exhibited significant antitumor efficacy in RFP expressing melanoma tumor model and allowed for vaccine tracking and detection by CT imaging	AuNPs have dual ability to serve as carriers for biomolecules as well as imaging agents to track and detect these biomolecules, it is nontoxic and biocompatible, its size can easily be manipulated to adapt to different applications and the surface can be easily be functionalized	Lee et al. (2012), Mieszawska et al. (2013)
Iron nanoparticles	Superparamagnetic iron oxide nanoparticles coated with zinc oxide (ZnO) carrying a ZnO binding peptide were linked to a tumor antigen (carcinoembryonic antigens). This modified nanoparticle led to tumor growth attenuation while allowing for the <i>in vivo</i> monitoring of dendritic cell migration when tumor bearing mice were immunized with dendritic cells that were pulsed with the nanoparticle-CEA complex In an interesting approach, perica et al., synthesized nano-scale artificial antigen presenting cells (nano-aApc) by conjugating MHC-peptide complex and co-stimulatory anti CD 28 to a paramagnetic iron-dextran nanoparticle. The iron based aAPC induced antigen specific T-cell proliferation and restricted tumor growth <i>in-vivo</i>	Have dual ability to serve as carries for biomolecules and imaging agents	Cho et al., 2011, Perica et al. (2014)
Mesoporous silica nanoparticles (MSN)	DC vaccination based on human epidermal growth factor receptor 2 (HER-2) loaded MSN produced a strong anti-tumor immunity by inducing cross presentation and eliciting type 1 interferon production MSN with extra-large pores that carried antigenic OVA and CpG ODNs delivered them efficiently to dendritic cells to induce strong anticancer immunity against OVA expressing tumors	Easy to synthesize, have large and tunable pore size making it ideal to incorporate various biological agents. They are ideal for immunotherapy because they possess an intrinsic adjuvant property	Xia et al. (2015), Wang et al. (2016), Cha et al. (2018)
Carbon dots (CDs)	PEGylated CDs carrying tumor antigens enhanced antigen uptake and maturation of dendritic cells leading to potent activation of T-cells that significantly restricted tumor growth in a melanoma tumor model	Have good water solubility, chemically stable, ease of surface conjugation and large-scale production	Wang et al. (2019), Shields et al. (2020)
Organic nanoparticles PLGA	PLGA have been used to demonstrate effective targeting to solid tumors. PLGA nanoparticles developed to deliver antigenic peptides (TRP2 and GP100) with Freud's complete adjuvant induced potent cytotoxic T lymphocyte in melanoma burdened mice In a recent development, min et al., developed PLGA nanoparticles with motifs able to selectively capture disseminated tumor antigens following tumor irradiation for dendritic cell uptake. Rutika et al., formulated a tumor antigen encapsulated nanoparticle from PLGA polymer which was surface functionalized with CpG, a vaccine adjuvant. This construct restricted the growth of primary breast tumor by inducing a potent T-cell immunity PLGA nanoparticle encapsulating adjuvants coated with cell membrane derived from cancer cells effectively delivered membrane proteins to dendritic cells leading to the generation of antitumor immunity against cancer membrane antigens	An FDA approved polymer, it is both biodegradable and biocompatible, safe and easy to synthesize	Zhang et al. (2011), Fang et al. (2014), Kokate et al. (2016), Min et al. (2017)
Polypropylene sulfide (PPS) nanoparticles	Pluronic -stabilized poly (propylene) sulfide (PPS) nanoparticles used to target adjuvants to tumor draining lymph node, presumably already bathed in tumor antigens promoted anti-tumor immunity and restricted tumor growth by facilitating uptake of antigens by dendritic cells	Resistant to degradation by most stimuli except reactive oxygen species (ROS) making it ideal for degradation in tissues producing high amount of ROS.	Lee et al. (2013), Thomas et al. (2014)

(Continued on following page)

TABLE 3 | (Continued) Nanoparticle platforms used in targeting tumor antigens to dendritic cells.

Nanoparticle type	Use	Properties	References
lipid-Calcium phosphate (LCP) nanoparticles	Multi-functionalized LCP nanoparticles used to co deliver the peptide antigen TRP2 and vaccine adjuvant, CpG induced robust T-cell antitumor immunity against primary melanoma and its associated secondary lung metastasis	Has high endosomal escape facilitating cross presentation. Has the ability to encapsulate phosphorylated moieties. These make them attractive vehicles for immunotherapy	Xu et al. (2013), Huang et al. (2018)
Liposomes	Liposomes have been used to deliver peptide antigen consisting gonadotropin releasing hormone to dendritic cells for the generation of tumor specific cytosolic T-lymphocytes against prostate cancer Liposomes coupled with the TAA—glypican -3 (GPC3) and CpG ODN as vaccine adjuvant on its surface effectively induced GPC3 specific CTLs that restricted the growth of GPC3 expressing hepatocellular carcinoma in mice following intradermal administration Cationic liposomes have been used to deliver RNA encoding neoantigens to dendritic cells to elicit robust tumor specific CTL response against melanoma	Their cell membrane like structure enables efficient cell affinity and increases cellular uptake. They can be loaded with both hydrophobic and hydrophilic molecules	Cruz et al. (2011), Iwama et al. (2016), Kranz et al. (2016)
Carbon-based nanostructures			
Carbon nanotubes (CNTs)	Multiwalled CNTs were synthesized to deliver ovalbumin (OVA), anti-CD40 and CpG ODNs to dendritic cells that led to the induction of ova-specific anti-tumor immunity	Nontoxic and easily taken up by phagocytic cells making them ideal for immunotherapy. Their high surface areas are easily accessible to attach high densities of adjuvants and antigens which increases their interactions with antigen presenting cells	Hassan et al. (2016), Shields et al. (2020)

CD4 T -cell proliferation compared to larger nanoparticle size (Nochi et al., 2010; Blank et al., 2013). However, intranasal administration of cationic liposome-hyaluronic acid hybrid with average size of 250 nm was able to induce antigen specific immunity (Fan et al., 2015).

The behavior of inhaled particles (aerodynamic behavior) is guided by their aerodynamic diameter which is the diameter of a sphere of unit density and affects how they are deposited in the lungs. Particles with aerodynamic diameter $>5\ \mu\text{m}$ are deposited predominately in the upper airways by inertial impaction due their inability to change their trajectory with tidal air (Sturm and Hofmann, 2009). Particles with aerodynamic diameter between 1 and $5\ \mu\text{m}$ are predominately deposited in the lower airways involving the bronchioles and alveoli by gravitational sedimentation (Rissler et al., 2017). Particles with aerodynamic diameter to be less than $1\ \mu\text{m}$ do not get deposited but remain suspended in the airstream leading to their likelihood to be exhaled following inhalation (Mangal et al., 2017; Rissler et al., 2017). Because nanoparticles have the tendency to aggregate to form uncontrolled sizes leading to inconsistent and unpredictable aerosolization, nanoparticles are often administered as particles/droplets with an aerodynamic diameter of $1\text{--}5\ \mu\text{m}$ (Desai, 2012) to enhance their delivery in the lung parenchyma. This is facilitated with the use of nebulizers and metered dose inhalers (MDIs) that are able to convert nanoparticle suspensions into inhalable droplets.

Although the surface charge of nanoparticles does not impact their distribution in the lung, it influences their interaction with lung dendritic cells. Positively charged nanoparticles are preferentially

taken up by lung dendritic cells while their negatively charged counterparts are engulfed by alveolar macrophages leading to their clearance (Fromen et al., 2016). This means that cationic nanoparticles are more successful at inducing stronger adaptive immune response following pulmonary delivery.

The use of nanoparticle-based vaccines for pulmonary delivery continues to show promise. For example, Nochi et al. created an adjuvant free intranasal vaccine from nanogels made from self-assembly pullulan polymers modified with cholesteryl and *Clostridium botulinum* type A neurotoxin that was able to induce both IgG and IgA antibodies response (Nochi et al., 2010). Fan et al. induced potent humoral immune responses through the intranasal delivery of cationic liposome-hyaluronic acid hybrid nanoparticles made to deliver antigens against *Yersinia pestis* (Fan et al., 2015). Li et al. have developed carbon dots nanoparticles capable of intranasal delivery that enhanced immunization efficacy by significantly increasing humoral immunity and memory T-cell formation (Li S. et al., 2018). These examples together with the recent study by Zhao and his coworkers where they synthesized an inhalable phosphatidylserine coated liposome loaded with immunostimulants (STING agonist cyclic guanosine monophosphate-adenosine monophosphate—cGAMP) that stimulated APCs activation and cross presentation and synergizes with radiation treatment to protect against secondary lung metastasis following the occurrence of a primary breast tumor after intranasal administration (Liu et al., 2019) show that nanoparticles can be used to deliver tumor antigens to induce antitumor respiratory immunity following pulmonary delivery.

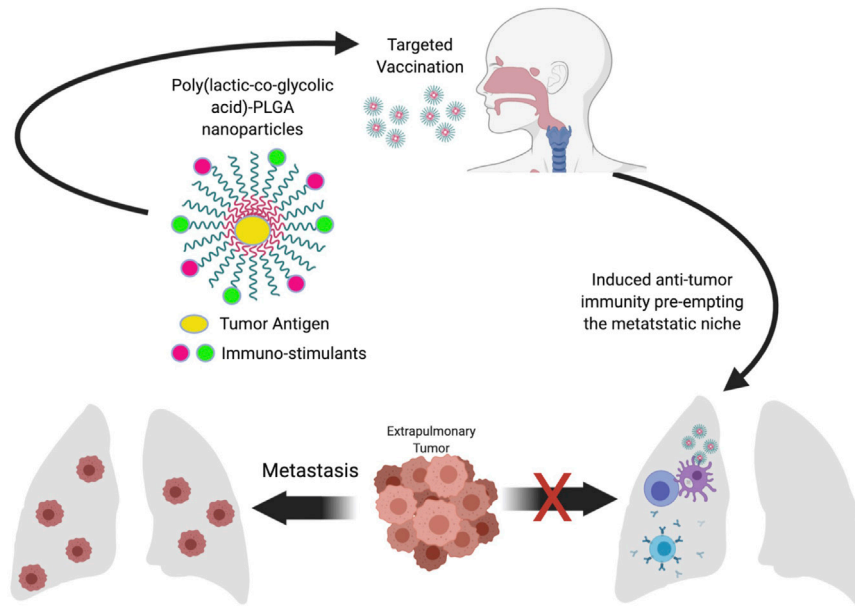


FIGURE 2 | Conceptual framework. Pulmonary administration of immunostimulant coated nanoparticles encapsulating tumor antigens induced lung specific anti-tumor immunity capable of forestalling immunosuppression by the premetastatic niche leading to the ability of immunosurveillance mediated by cytotoxic T-cells and antibodies to clear disseminated tumor cells and prevent the colonization of the lung by the existing extra-pulmonary tumor.

Host derived extracellular vehicles (EVs) such as exosomes are also emerging as attractive vehicles to target antigens to dendritic cells. Exosomes are nano-sized EVs (size ranging from 30 to 100 nm) that play important roles in cell-to-cell communication (Meng et al., 2019). Endogenous drug delivery systems such as exosomes have advantage over synthetic nano formulations because of their native biocompatibility *in vivo* which helps them enhance drug delivery and therapeutic efficiency (Batrakova and Kim, 2015; Peng and Mu, 2016). Tumor derived exosomes are rich sources of tumor rejection antigen capable of inducing tumor specific immunity (Luan et al., 2017). However, tumor cell derived exosomes can also contain immunosuppressive proteins that dampens immune response enabling tumor cells to escape immune surveillance (Xu et al., 2020). Antigen presenting cells such as dendritic cells derived exosomes have shown great promise in delivery of tumor antigens to induce antitumor immunity (Whiteside, 2016). They have the capacity to transfer peptide-MHC complexes that have been exposed to an antigen to other DCs that have not encountered similar antigens (Wahlund et al., 2017). The pulmonary delivery of exosomes loaded with a tumor antigen can serve as an appropriate drug delivery platform to treat lung tumors.

THE CONCEPTUAL FRAMEWORK

Therapeutic cancer vaccines have been used to treat tumors and prevent metastasis by targeting the primary tumor. If the seed soil hypothesis by Stephen Paget that explains metastasis is anything to go by, then targeting the soil (future metastatic sites) may serve as a means to prevent metastasis. The seed soil hypothesis

proposes that, the primary tumor secreted factors prepares the soil (future metastatic sites) for colonization by detached cells form the primary tumor (seed) that reach the distal organ either through circulation or the lymphatics. As stated in the introduction one of the events that occurs in the future metastatic sites as a result of molecular and cellular changes fashioned by the tumor derived factors is immune suppression. For the seed to successfully colonize future metastatic sites they have to evade immune surveillance by $CD8^+$ T-cells and NK cells that directly kill tumor cells. Tumor cells derived chemokines and cytokines recruits suppressive and regulatory immune cells such myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), Tumor associated neutrophils and Tregs into distal organs to support metastasis by inducing immunosuppression. Pulmonary Tregs, MDSCs and alveolar macrophages have been shown to restrain antitumor T-cells response. Targeted induction of respiratory immunity can reverse the immunosuppression induced in the lung prior to lung metastasis from an extra pulmonary malignancy by creating an immune protective microenvironment to prevent and/or minimize the seeding of tumors in the lungs. This has great potential to serve as a prophylactic approach to preclude immune evasion by CTCs that reach the lungs. Although many studies have demonstrated the promise of mobilizing antitumor immunity using vaccination strategies, its efficacy in the protection against lung metastases, particularly in the context of existing extra pulmonary primary tumor development has not been tested. The design of an appropriate vaccine capable of intranasal administration may serve as a prophylactic cancer vaccine to prevent lung metastasis from extra pulmonary malignancies by generating robust mucosal anti-tumor

immunity in the lungs. Its success will have a significant impact on the number of deaths caused by extra-pulmonary malignancies. The ease of administration, as such therapy can be self-administered will be an added advantage (Figure 2).

AUTHOR CONTRIBUTIONS

There remains current unmet need in reducing mortality associated with primary and metastatic lung cancer. This review sheds light on the past, present and future prospects of nasal-pulmonary route as an advanced approach of delivering immune-based therapeutics for the treatment of primary and metastatic lung cancer. Insights drawn from this review is expected to lead to further knowledge prompting anti-cancer research.

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GLOSSARY

NSCLC Non-small cell lung cancer

SCLC Small cell lung cancer

CTCs Circulating tumor cells

DTCs Disseminated tumor cells

BMDCs Bone marrow derived cells

MHC Major histocompatibility complex

PD-L1 Programmed death-ligand 1

PD-1 Programmed cell death protein 1

WNT Wingless-related integration site

B-catenin Beta-catenin

PTEN Phosphate and tensin homolog

HIF-1 α Hypoxia-inducible factor 1- α

IFN- α Interferon- α

ICIs Immune check point inhibitors

CTLA4 Cytotoxic T-lymphocyte-associated protein 4

Tregs Regulatory T cells

VEGF Vascular endothelial growth factor

CAR T-Cells Chimeric antigen receptor T cells

HER-2 Human epidermal growth factor receptor-2

EGFR Epidermal growth factor receptor

ALK Anaplastic lymphoma kinase

IL-2 Interleukin-2

TAA Tumor associated antigen

MAGE-A1 Melanoma-associated antigen 1

MAGE-A3 Melanoma-associated antigen 3

NY-ESO-1 New York esophageal squamous cell carcinoma 1

PSA Prostate-specific antigen

PAP Papanicolaou test

ICD Immunogenic cell death



Nanogenomics and Artificial Intelligence: A Dynamic Duo for the Fight Against Breast Cancer

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Application software is utilized to aid in the diagnosis of breast cancer. Yet, recent advances in artificial intelligence (AI) are addressing challenges related to the detection, classification, and monitoring of different types of tumors. AI can apply deep learning algorithms to perform automated analysis on mammographic or histologic examinations. Large volume of data generated by digitalized mammogram or whole-slide images can be interoperated through advanced machine learning. This enables fast evaluation of every tissue patch on an image, resulting in a quicker more sensitivity, and more reproducible diagnoses compared to human performance. On the other hand, cancer cell-exosomes which are extracellular vesicles released by cancer cells into the blood circulation, are being explored as cancer biomarker. Recent studies on cancer-exosome-content revealed that the encapsulated miRNA and other biomolecules are indicative of tumor sub-type, possible metastasis and prognosis. Thus, theoretically, through nanogenomics, a profile of each breast tumor sub-type, estrogen receptor status, and potential metastasis site can be constructed. Then, a laboratory instrument, fitted with an AI program, can be used to diagnose suspected patients by matching their sera miRNA and biomolecules composition with the available template profiles. In this paper, we discuss the advantages of establishing a nanogenomics-AI-based breast cancer diagnostic approach, compared to the gold standard radiology or histology based approaches that are currently being adapted to AI. Also, we discuss the advantages of building the diagnostic and prognostic biomolecular profiles for breast cancers based on the exosome encapsulated content, rather than the free circulating miRNA and other biomolecules.

Keywords: artificial intelligence, machine learning, exosome, cancer, screening, diagnosis

For years now, a number of application software are being utilized to aid in the screening and diagnosis of breast cancer. However, these software come with limitations related to the detection, classification, treatment, and monitoring of different types of breast tumors. Therefore, recent advancements in computer science and artificial intelligence (AI) are focusing on addressing these limitations. Unlike previous detection and diagnostic computer-aided software, AI allows the computer to employ algorithms to reach machine-based conclusions in a manner similar to the reasoning-based cognition of the human brain. Machine learning (ML) is a subdomain of AI; ML-based algorithms allow the computer to draw new interfaces based on the available training

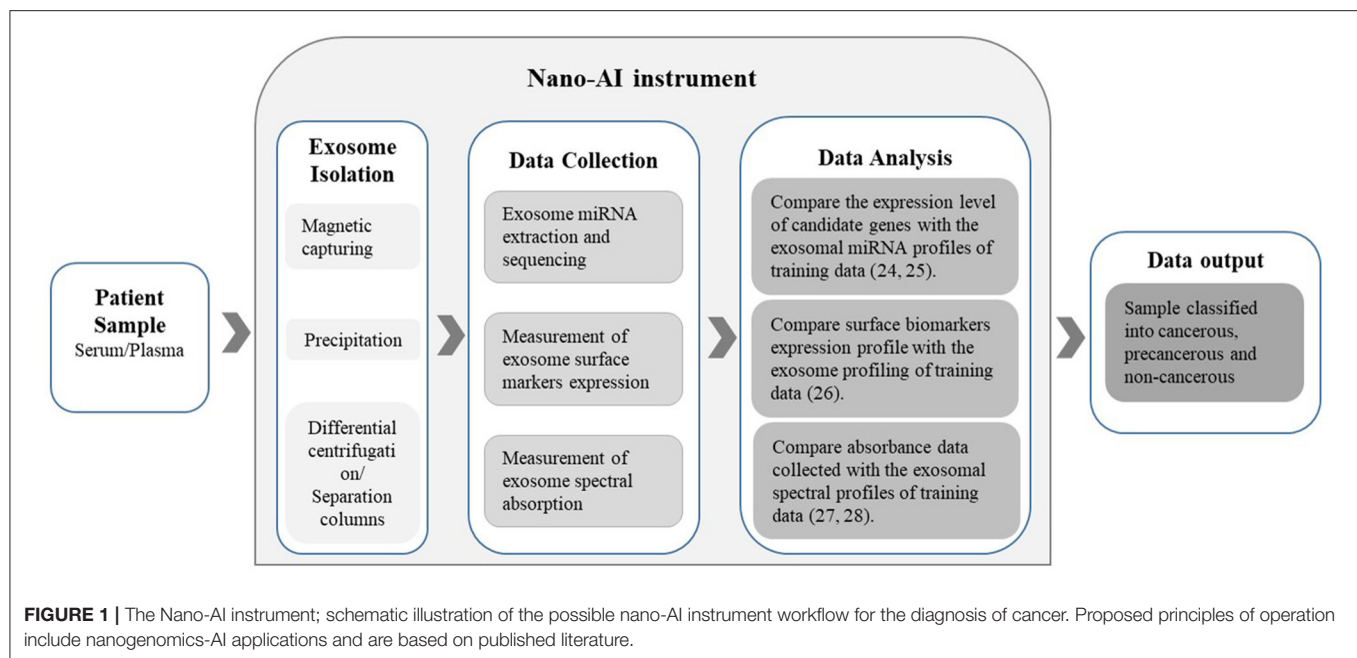
data (Tran et al., 2019). A branch of ML that is being employed in medical diagnosis is deep learning (DL). DL employs what are called “artificial neural networks” (ANNs). These networks have the structure of a biological nervous system, in that they are multilayer systems where different layers form synaptic connections with each other. Each layer of the network collects input from the lower layer and forms a more complex output. The more the number of layers, the more complex the final output will be (McBee et al., 2018).

For breast cancer diagnosis, DL models are mainly applied to perform analysis on the “gold standard” mammographic examinations. Digitalized mammogram images are interoperated through advanced ML. Most of the DL models used for the analysis are based on the convolutional neural network (CNN) algorithm, which is a class of ANN that is designed to work with data in the form of two-dimensional images (Geras et al., 2019). Several research groups have developed and validated different algorithms using images of breast cancer positive, false-positive, and negative mammogram examinations. Then, they compared the diagnostic performance of certified radiologists with and without the aid of AI. In all of these retrospective studies, the performance of the radiologists, including sensitivity and recall rate and time, was improved when supported by AI (Rodríguez-Ruiz et al., 2019a; Watanabe et al., 2019; Pacil et al., 2020). Until now, there is no single algorithm that could surpass the performance of human radiologists (Schaffter et al., 2020). Nevertheless, scientists continue to improve on previous work, and recent publications are reporting very promising outcomes for AI-based detection as a stand-alone mammography reading practice (Rodríguez-Ruiz et al., 2019b; Shen et al., 2019; Dembrower et al., 2020; Kim et al., 2020; Sasaki et al., 2020). DL models are also being developed for digitalized hematoxylin-eosin (H&E)-stained whole-slide images. This approach is crucial for the detection of malignancies in the breast and other body tissue biopsies including the prostate and the colon. It was reported that AI-based-histological evaluation enabled the fast evaluation of every single tissue patch on the slides of the patients, which led to a performance level comparable to that of a pathologist (Argov et al., 2002; Liu et al., 2019; Shamaï et al., 2019; Raciti et al., 2020; Ström et al., 2020). Other groups are attempting to create DL models that rely on non-image data sets. For example, one group used spectral data that reflect changes in collagen, lipids, and nucleic acid content of malignant breast tissues (Dulay et al., 2019). Meanwhile, another group analyzed tissue microarray data in conjunction with DL. This allowed for algorithm-automated gene selection and tumor classification with better performance than when compared to the standard methods (Dashtban and Balafar, 2017).

In addition to the conventional breast cancer screening and diagnostic tests, DL models are also being developed for novel cancer detection. Cancer cell-exomes, which are extracellular vesicles released by cancer cells into the bloodstream, are arising as vital cancer diagnostic and prognostic biomarkers (Al-Sowayan et al., 2019). Exosomes isolated from blood samples of patients with breast cancer were revealed to have characteristic content, mainly proteins and miRNA, which are indicative of the tumor sub-type, possible metastasis, and prognosis (Lee

et al., 2019; Tutanov et al., 2020; Wu et al., 2020). However, the utilization of exosomal genetic and proteomic data as a diagnostic tool is confined by the difficulties related to vast data analysis of the multiple molecular biomarkers. Therefore, it is only logical that DL models be developed also for the automated analysis of exosome-related data. One study developed an algorithm for clustering and candidate motif detection in exosomal miRNAs. This study used miRNA sequences downloaded from the “miRBase” database as training and testing data. The results revealed that the algorithm successfully completed the desired function with no human intervention (Gaur and Chaturvedi, 2019). In another study, a DL model that combines the measurement of eight exosomal miRNA biomarkers was used. Then, the model was applied on real-time PCR data of miRNA extracted from exosomes in the plasma of the patients. The algorithm successfully created predictive panels and classified patients with pancreatic cancer from healthy controls (Ko et al., 2017). Meanwhile, in another study, an algorithm that combines the measurement of four exosomal surface biomarkers to detect pancreatic cancer and breast cancer from plasma samples was used. The surface biomarkers were detected with quantitative super-resolution imaging. Then, the model was used to analyze the output of these multiple markers, which led to an accuracy level of 100% (Chen et al., 2019). In addition to exosomal miRNA and surface markers, researchers have also implemented ML in exosomal spectral data, where algorithms were used to build distinctive spectral profiles for cancer exosomes derived from the plasma of patients with lung cancer (Shin et al., 2020) and the saliva of patients with oral cancer (Zlotogorski-Hurvitz et al., 2019); the algorithms yielded an accuracy level of more than 90%.

This nano-AI-based approach could prove to be an advantageous screening and diagnostic method compared to the radiology- or histology-AI-based approaches. Exosomes hold enormous potential in cancer diagnostics as they contain a wealth of proteomic and genetic information. This not only presents a new insight into cancer biology but also enables an elevated level of personalized medicine and thus superior treatment outcomes. Moreover, examining the exosome-encapsulated content as biomarkers is reported to be more reliable compared to examining free-circulating miRNA and other biomolecules. This is due to the fact that exosomal content is highly sensitive to the status of the releasing cancer cell and its microenvironment. In addition, exosome-encapsulated miRNAs are highly stable when compared with free-circulating miRNAs since they are well-protected by the membrane bilayer of the exosome (Yuan et al., 2019). Moreover, the process of blood sample collection is less painful, less dangerous, less time-consuming, less expensive, and does not require a high level of proficiency to obtain when compared to the standard mammograms or biopsies. Theoretically, as proven in principle by the above mentioned studies, exosomes can be isolated from blood samples of patients with breast cancer with known diagnoses and prognoses. Then, the isolated exosome content can be investigated using arrays and analyzed by the computer-aided applications of bioinformatics. When sufficient data are collected, an “exosome-content-signature” for each tumor subtype, the estrogen-receptor statuses, and the



possible metastases can be constructed into distinct data sets. These data will serve as the training data for the algorithm to create the DL model. The blood samples are collected from females at any convenient location such as hospitals, family clinics, pharmacies, homes, prisons, and mobile clinics in rural areas. Then, the collected samples are brought back to the designated laboratory with the instrument fit with nano-AI. The instrument will isolate the exomes from the plasma/serum and then extract the data to allow the algorithms to analyze the exosomal composition and match it with the available profiles (Figure 1).

Of course, to realize such applications, there are few obstacles that need to be overcome. One main obstacle is the quality control issues related to the isolation and purification of the exosomes from patient samples. Obtaining a consistently pure and measurable yield of exosomes from the sera/plasma of patients is still a work in progress. However, recent advancements in nanotechnology are expected to circumvent all technical issues. Another major obstacle is algorithm creation; it is no doubt that the vast intratumor and interpatient heterogeneities will make it very challenging to assemble distinct molecular profiles for each diagnostic and prognostic variant. Moreover, a major concern in AI applications in general is the algorithm bias that results from using biased training data sets. In medical applications, biased data sets are a consequence of the underrepresentation of certain segments of the population in the evidence base e.g., ethnic minorities (Carter et al., 2020). Therefore, it is crucial to make sure that the clinically adopted algorithm

is designed to perform at the highest level with all the patients. This can only be done by using an all-inclusive training data set to create the model, then further validating it using patients with diverse demographic characteristics. In addition to the challenges of exosome isolation and training data creation, there are also general concerns about the utilization of AI in medical applications. These include the ethical and legal matters related to data acquisition such as consenting, confidentiality, ownership, etc. Also, the absence of infrastructure required to adopt the new technology and the fear of increased unemployment among medical professionals are also notable challenges. Nevertheless, the increasing amount of capital invested in the medical applications of AI (investments in start-ups implementing AI in healthcare reached \$4 billion in 2019, compared to \$2.7 billion in 2018) (Pifer, 2020) is expected to expedite the overcoming of these challenges and encourage a rapid regulation of the ethical, legal, operational, and social issues related to implementation. The clinical value of AI is projected to be fully realized in the very near future. When such nanogenomics-AI-based breast cancer screening approaches are applicable, a widespread, early detection of breast cancer will be achieved. This will positively impact the life of every female regardless of her social, economic, or geographical limitations.

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BA-S conceptualize the idea. All authors contributed to writing and editing the manuscript.

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Integrating Cycled Enzymatic DNA Amplification and Surface-Enhanced Raman Scattering for Sensitive Detection of Circulating Tumor DNA

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Circulating tumor DNA (ctDNA) represents an emerging biomarker of liquid biopsies for the development of precision cancer diagnostics and therapeutics. However, sensitive detection of ctDNA remains challenging, due to their short half-life and low concentrations in blood samples. In this study, we report a new method to address this challenge by integrating cycled enzymatic DNA amplification technique and Au nanoparticle@silicon-assisted surface-enhanced Raman scattering (SERS) technique. We have demonstrated a reproducible identification of a single-base-mutated ctDNA sequence of diffuse intrinsic pontine gliomas (DIPGs), with the limit of detection (LOD) as low as 9.1 fM in the spiked blood samples. This approach can be used to analyze trace amounts of ctDNA in translational medicine for early diagnosis, therapeutic effect monitoring, and prognosis of patients with cancer.

Keywords: surface-enhanced raman scattering (SERS), enzymatic amplification, circulating tumor DNA (ctDNA), DNA nanotechnology, biomarker

INTRODUCTION

Circulating tumor DNA (ctDNA) carries genetic information, such as point mutations, methylation, and copy number variations of sequences, of the tumor cells in patients with cancer, thus simultaneously serving as a diagnostic as well as a prognostic cancer biomarker based on liquid biopsies (Dawson et al., 2013; Weiss et al., 2013; Alix-Panabieres and Pantel, 2016; Wan et al., 2017; Reinert et al., 2018). Previous studies suggest that ctDNA is mainly derived from the processes of cell apoptosis, necrosis, and secretion (Diaz and Bardelli, 2014). The typical concentration of ctDNA in the clinical samples is in the range of 0.01–0.1 ng ml⁻¹, accounting for only a small fraction (nearly 1%) of the total cell-free DNA (cfDNA; Diehl et al., 2008; Wan et al., 2017). In addition, ctDNA features a much shorter half-life (<2 h) compared with other protein biomarkers, which usually remain for several weeks (Dawson et al., 2013; Bettegowda et al., 2014), and susceptibility to the variants in the liquid biopsy handling procedure. Technically, it is challenging to distinguish ctDNA with the fewest point mutations from wild-type DNA (W) and quantify the concentrations of ctDNA from the complicated background interference of biological samples.

The most widely used technologies for ctDNA detection include varieties of methods based on PCR, such as digital PCR (dPCR; Vogelstein and Kinzler, 1999; Taly et al., 2013); amplification

refractory mutation system (ARMS; Newton et al., 1989; Li et al., 2006); beads, emulsion, amplification, magnetics (BEAMing; Li et al., 2006); next-generation gene sequencing (NGS) technology (Forshew et al., 2012; Lai et al., 2018); and Sanger sequencing (Janne et al., 2006). However, there are concerns of generating false-positive results due to the ultra-high amplification capacity enabled by polymerases (Kitchin et al., 1990; Mathai and Adhikari, 2013). Many researchers have been trying to develop non-PCR-based techniques to detect ctDNA, including electrochemistry (Drummond et al., 2003; Yang et al., 2016), colorimetry (Li et al., 2017), and fluorescence (Li et al., 2016). Among them, surface-enhanced Raman scattering (SERS) spectroscopy has been considered an effective solution, due to its several advantages, such as ultra-high sensitivity, identification with characteristic fingerprint peaks, and the compatibility of both solid-form and liquid-form samples (Liang et al., 2019; Szekeres and Kneipp, 2019; Chen et al., 2020; Du et al., 2020; Fan et al., 2020; Langer et al., 2020; Li et al., 2020). Lin et al. (2019) reported sensitive detection of ctDNA for a good diagnostic sensitivity of 83.3% and a specificity of 82.5% in distinguishing patients with nasopharyngeal cancer from normal control groups by using the SERS substrate of Ag nanoparticles. Wee et al. designed a new laser wrapped graphene–Ag array to sensitively detect the methylated DNA by using SERS technology and demonstrated a limit of detection (LOD) of as low as $0.2 \text{ pg } \mu\text{L}^{-1}$ (Wee et al., 2016). There is still a great need to improve the LOD for the translational research of ctDNA.

Herein, we report a new method for sensitive detection of the sequence of ctDNA (H3.3 mutation) in diffuse intrinsic pontine gliomas (DIPGs) by using a combination of cyclized enzymatic DNA amplification and Au nanoparticle@silicon (Au NP@Si)-assisted SERS technology. DIPGs are high-grade glial tumors located in the pons of the brain and are the leading cause of fatal brain tumors in children. The traditional diagnostic methods of DIPGs heavily rely on *in vivo* imaging, which can become difficult for identification because of the deep location of the tumor sites in the brain. In this study, we have designed a SERS-tag-labeled probe DNA (P), which complements the H3.3 mutant DNA (T) to form a blunt end. The cleavage assisted by EXO III can generate a large amount of residual DNA in a cyclized manner to greatly improve the LOD of the SERS technique. We have demonstrated an ultra-high sensitivity (with the LOD of 7.9 fM) and specificity (being able to distinguish a single-base mutation) using this strategy. This is a promising approach for the sensitive detection of nucleic acids as a translational tool of ctDNA research.

EXPERIMENTAL SECTION

Materials and Reagents

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), sodium chloride (NaCl), acetone, and phosphate buffer saline (PBS) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Exonuclease III was provided by Thermo Fisher Scientific Co., Ltd. (United States). Tris borate EDTA (TBE) buffer (5×), tris EDTA (TE) buffer (1×), sodium dodecyl sulfate (SDS, 10%, w/v)

buffer, loading dye buffer solutions (6×), agarose, and saline-sodium citrate (SSC, 20×) buffer solutions were obtained from Solarbio Science & Technology Co., Ltd. (China). GelRed Nucleic Acid Gel Stain was purchased from Biotium, Inc. (United States). DNA ladder was provided by Sigma-Aldrich Co., Ltd. (China). QIAamp DNA Blood Mini Kit was purchased from Qiagen Co., Ltd. (Germany). All chemicals in our experiments were of analytical grade and used without further purification. Aqueous solutions were prepared using deionized water ($\geq 18 \text{ M}\Omega$, Milli-Q, Millipore). The SERS substrates were provided by Nanova Biomaterials Inc. (United States). High-performance liquid chromatography (HPLC)-purified oligonucleotides were provided by Sangon Biotechnology Co., Ltd. (China).

Instruments

The Raman microscope equipped with a He–Ne laser (633 nm, 20 mW) and a $100\times$ objective (NA: 0.9) was used to detect the Au NP@Si substrates (HR800, Horiba Jobin Yvon, France). SERS data were collected under a $100\times$ visible objective with a 633 nm laser radiation, with an acquisition time of 10 s and an accumulation count of one time. The scan of the wave number typically ranged from 1,000 to $1,800 \text{ cm}^{-1}$. The Raman spectral data were analyzed by the LabSpec5.6 Software. For each sample, 50 random spots on the substrate were tested for SERS signal. All SERS spectra have been elaborated by removing the baseline, with an example shown in **Supplementary Figure 1**. The correlation of the major Raman bands to the chemical bonds of Cy5 is summarized in **Table 1**. A DNA thermostat hybridization oven (HL-2000 HybriLLinker, UVP, United States) was used for the incubation experiments involving DNA. The substrate surface was characterized by contact angle measurement instrument (DataPhysics OCA). The gel electrophoresis of various sample mixtures was performed on a horizontal electrophoresis tank instrument (VE-186), followed by image acquisition with a Gel Imaging System (Tanon-2500).

Electrophoresis Experiments

The hybridization and the Exo III-assisted cleavage of different DNA samples were evaluated by agarose gel electrophoresis. Agarose (2%, w/w) was prepared in the buffer of $1\times$ TBE and mixed with $3 \mu\text{L}$ of GelRed stain. Specifically, $10 \mu\text{L}$ of the DNA sample was mixed with $2 \mu\text{L}$ of $6\times$ loading dye buffer solution. Different DNA samples were tested by gel electrophoresis under a voltage of 110 V for 25 min. The DNA sample mixtures included P, T, the hybridization of P and T (P + T), the mixture of the P, T, and Exo III (P + T + Exo III), and the residual DNA (R).

TABLE 1 | List of the oligonucleotide sequences in the experiments.

Oligonucleotides	Sequence (5' → 3')
Probe DNA (P)	Cy5-AAAATGAGTGCGTAGTTAGGGTTAGATA AGGGCGCACTCATGCCA
Mutant DNA (T)	TGCGATGAGTGCGCCCTCTACT
Wildtype DNA (W)	TGCGAAGAGTGCGCCCTCTACT
Capture DNA (C)	GCACTCATTTTAAATTTAA
Residual DNA (R)	Cy5-AAAATGAGTGCGTAGTTAGGGTTAGATA

ctDNA Detection on the Au NP@Si Substrate

The SERS signals of ctDNA testing experiments were harvested from the Au NP@Si substrate. In the first step, the Au NP@Si substrate was modified with the capture DNA (C) sequence. The substrate was precleaned with acetone, rinsed with DI water for three times, and dried in an oven at 200°C for 10 min. The pretreated substrate was incubated overnight with 10 μ L of C (10 μ M) and 400 μ L of the PBS solution at 37°C for bioconjugation through Au–S bonds. The sodium chloride (1 M) solution was used in the aging step for immobilization of C (15 μ L, three times with an interval of 20 min). The substrate was incubated overnight in the DNA thermostat hybridization oven at 37°C, followed by sequential washing with PBS and DI water three times. The reaction of cyclic enzymatic DNA cleavage/amplification was carried out by mixing 10 μ L of P (10 μ M), 10 μ L of ctDNA in the testing concentrations, 3 μ L of Exo III (60U), and 17 μ L of the enzyme-reaction buffer at 37°C for 2 h. Afterward, the mixture was incubated at 75°C for 10 min in order to inactivate the Exo III enzyme. The product mixture was obtained after the reaction was diluted with 360 μ L of PBS buffer and then incubated with the as-prepared substrate at 37°C for 2 h. The substrate was washed with 2 $\times$ SSC solution containing 0.1% (w/v) SDS for 5 min, washed with DI water several times, and dried before the Raman signal measurement.

Control Experiments in SERS Measurements

The feasibility of our assays was verified by a series of control groups in the tests, including (A) C; (B) C + P; (C) C + P + M; (D) C + M + Exo III; (E) C + P + W + Exo III; and (F) C + P + M + Exo III. All control groups were performed in the identical conditions.

Limit of Detection of the Assay

A series of ctDNA with gradient concentrations were prepared to determine the LOD of our assay. About 10 μ L of P (10 μ M) and 10 μ L of ctDNA with different concentrations were mixed for incubation at 37°C for 2 h. The final concentrations of mutant ctDNA were in the range of 10–100 nM.

Treatment of Blood Samples

The blood samples from healthy human donors were collected and treated according to the standard protocol. All human care and experimental procedures were conducted in compliance with relevant laws and the guidelines approved by the institutional committees for Human/Animal Experiments of the School of Basic Medical Sciences of Soochow University. An informed consent was acquired from all human subjects. The serum was obtained from the whole blood and mixed with H3.3-mutated ctDNA of various concentrations. Then, H3.3-mutated ctDNA was extracted from the serum according to the extraction procedure of QIAamp DNA Blood Mini Kit. Briefly, 100 μ L of serum mixed with H3.3-mutated ctDNA was taken into a 1.5 mL centrifuge tube, then 10 μ L of protease K and 100 μ L of AL buffer were added, and later incubated at 56°C for 10 min. After adding 50 μ L of ethanol, the solution was

centrifuged for 1 min at 8,000 rpm. About 500 μ L of AW1 and 500 μ L of AW2 were added to the separation tube and centrifuged for 1 min at 8,000 rpm. After 100 μ L of AE eluent was added, the solution was balanced for 1 min at room temperature and centrifuged for 1 min at 14,000 rpm to collect the eluent.

Statistical Analysis

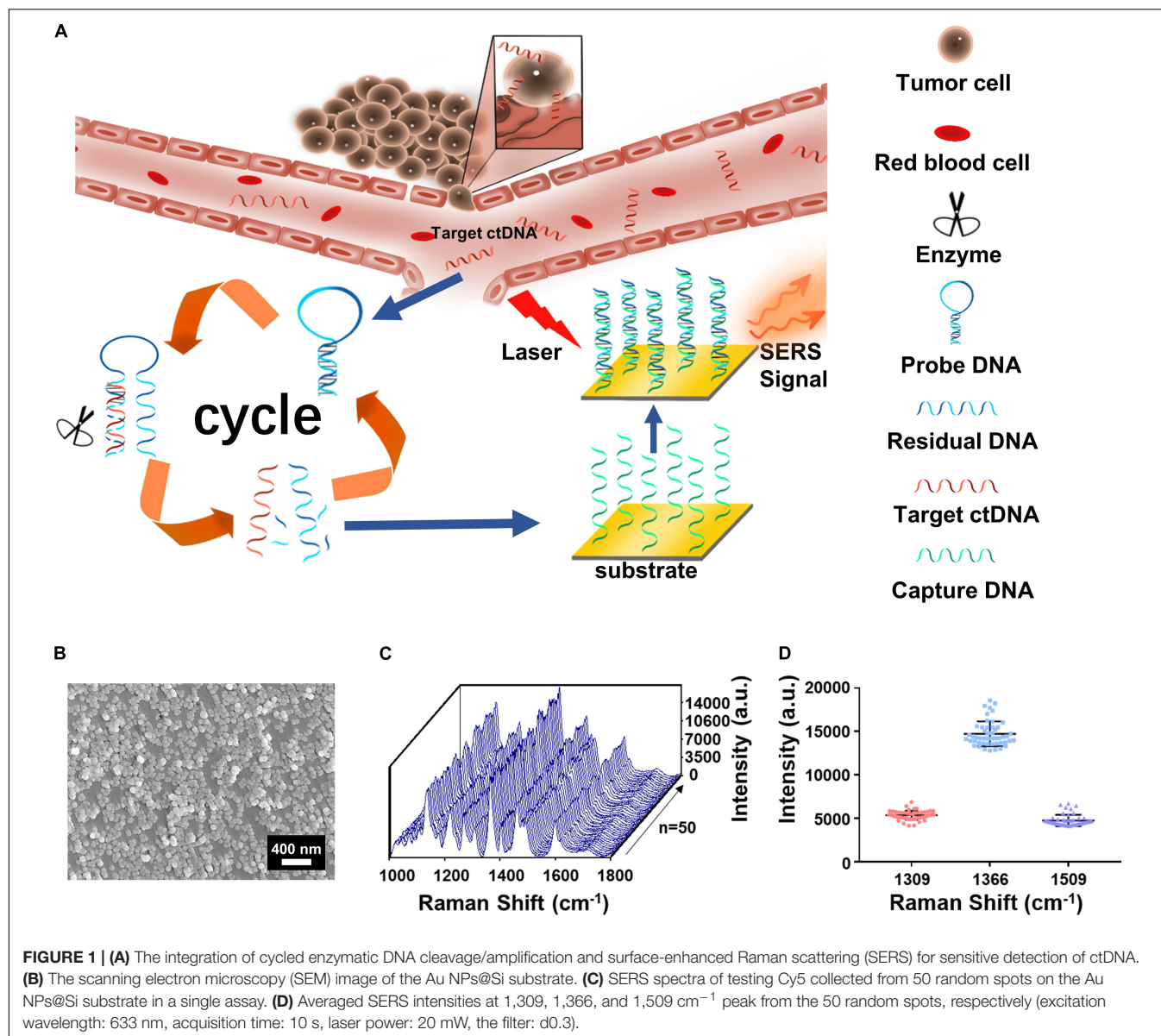
Statistical analyses were assessed with one-way ANOVA by using Prism 5.0 software (GraphPad Prism, United States). All intensities at the specified SERS peaks are presented as mean $\pm$ SD.

RESULTS AND DISCUSSION

The Assay Principle of ctDNA Detection and Characterization of the Substrate

The assay is featured with integration of the cyclic enzymatic DNA cleavage/amplification and SERS for the sensitive detection of ctDNA (**Figure 1A**). An oligonucleotide probe is designed to be folded in a stem-loop hairpin structure, tagged with cyanine dye Cy5 at the 5' end. The hairpin structure is stable, minimizing the undesired side hybridization with W sequence. In contrast, the stem-loop structure of the oligo probe can undergo changes to form a new double helix by hybridizing its 3' end with the target sequence of the mutant ctDNA. Consequently, the protruding 3' end in the new double helix can specifically be recognized by Exo III enzyme for the cleavage into nucleotides in a stepwise manner. Importantly, after completion of the cleavage process, the target sequence of ctDNA can be released into the solution and recycled for the next round of enzymatic DNA cleavage of the oligo probe. In this way, the residual DNA sequence generated by the digestion of the oligo probe can be accumulated to a great number by this cyclic reaction. Hybridization of the amplified R and C preimmobilized on Au NPs@Si will bring the Cy5 tag close to the substrate, producing intensive SERS signals with a high efficiency. Therefore, a trace amount of ctDNAs can initiate the cyclic generation of residual DNA sequences for amplified SERS detection.

Scanning electronic microscopy (SEM) (**Figure 1B**) suggested that the gold nanoparticles were uniform in size (60 nm) on the Au NP@Si substrate with a good distribution. The reproducibility in generating SERS signals was evaluated by scanning the substrate for spectral acquisition of up to 50 randomly selected spots. As shown in **Figures 1C,D**, there were minimal spot-to-spot variations of the intensities in the SERS spectra, with the coefficient variation values at the characteristic peaks of Cy5 at 1,309 cm^{-1} (CV < 9.88%), 1,366 cm^{-1} (CV < 9.73%), and 1,509 cm^{-1} (CV < 13.70%) and enhancement factor (EF) > 1.9×10^6 . The details of calculating the EF are listed in the **Supplementary Information**. We tested the SERS spectra at different laser powers by triturating the laser filters (**Supplementary Figure 2A**) and demonstrated the robustness of spectral data acquisition by switching laser power grades (high or medium) in five cycles (**Supplementary Figure 2B**). Therefore,



the Au NP@Si substrate can serve as a reliable platform for SERS measurements of a trace amount of molecules.

Oligo Sequence Optimization for ctDNA Detection

The DNA sequences, including P, T, C, R, and W, are summarized in **Table 1**. Based on the literature reports (Schwartzentruber et al., 2012), the H3.3-mutated ctDNA from DIPG is different from W in the healthy samples by a single nucleotide mutation (A to T), as specified in **Table 1**. A theoretic analysis using the online software (NUPACK) was performed to determine the minimum free energy (MFE) secondary structures of these oligos and hybridization efficiencies between each other (**Figure 2**). P tended to keep hairpin structure in the solution with a relatively favorable free energy ($-40.35 \text{ kJ mol}^{-1}$) for the secondary

structure (**Figure 2A**). The addition of H3.3-mutated ctDNA (T) to the solution would hybridize a segment of P competitively, thus changing its initial hairpin structure to form a partly hybridized helix with a 3' blunt end. This hybridization was driven by the decrease of free energy ($\Delta G = -61.20 \text{ kJ mol}^{-1}$). In addition, we compared the MFE changes for hybridization between W and P ($\Delta G = -47.09 \text{ kJ mol}^{-1}$), which suggested that P would prefer to hybridize H3.3-mutated ctDNA, even if W had only one base change. We also calculated the occupancy rates of the various oligos by NUPACK, in order to investigate their hybridization efficiency in silica at 37 °C. The calculation indicated that P was inclined to be hybridized with H3.3-mutated ctDNA with a high efficiency (nearly 47%); besides hybridization of 3% of W with P, the remaining W maintains its own structure (47%), leaving only 3% of the target DNA unhybridized in the solution (**Figure 2J** above, pie chart). We further compared the

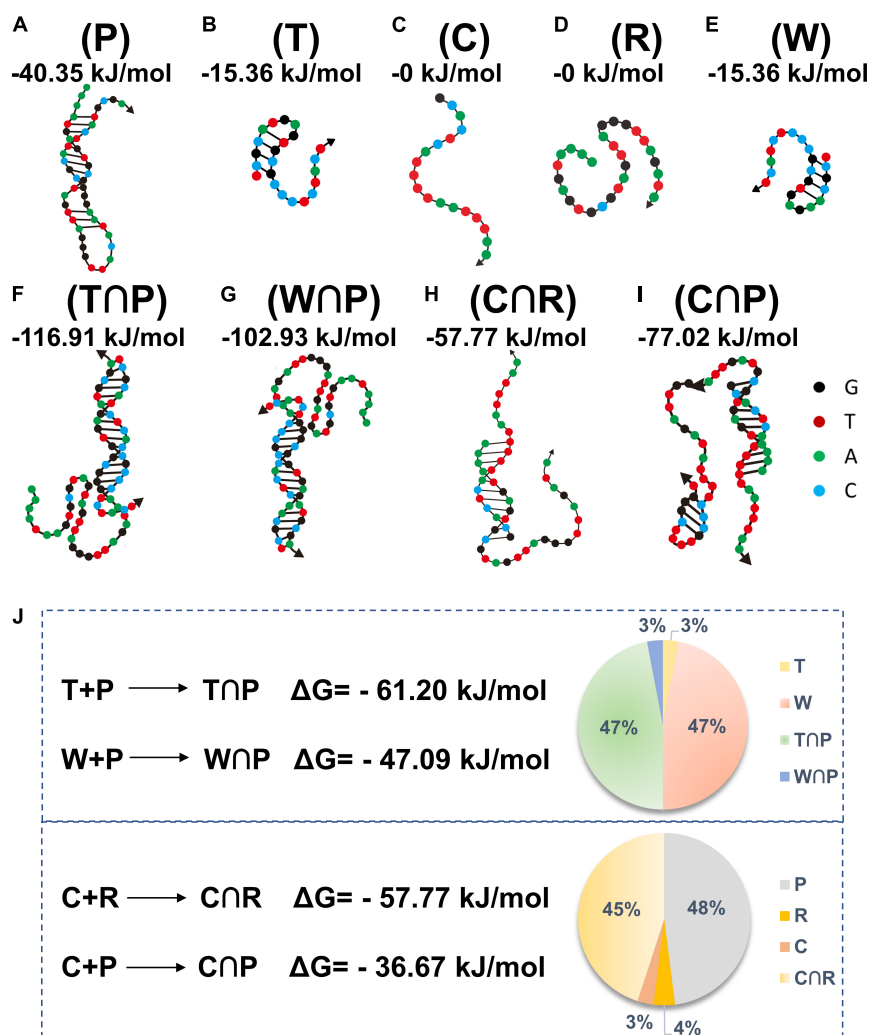


FIGURE 2 | The structures of different oligos (A–E) (oligo P, T, C, R, and W) and oligo hybridization with annotation of free energy calculated by NUPACK (F–I) (TnP, WnP, CnR, CnP). Theoretical calculations of the energy changes for the competitive hybridization between DNA oligos at 37°C (J).

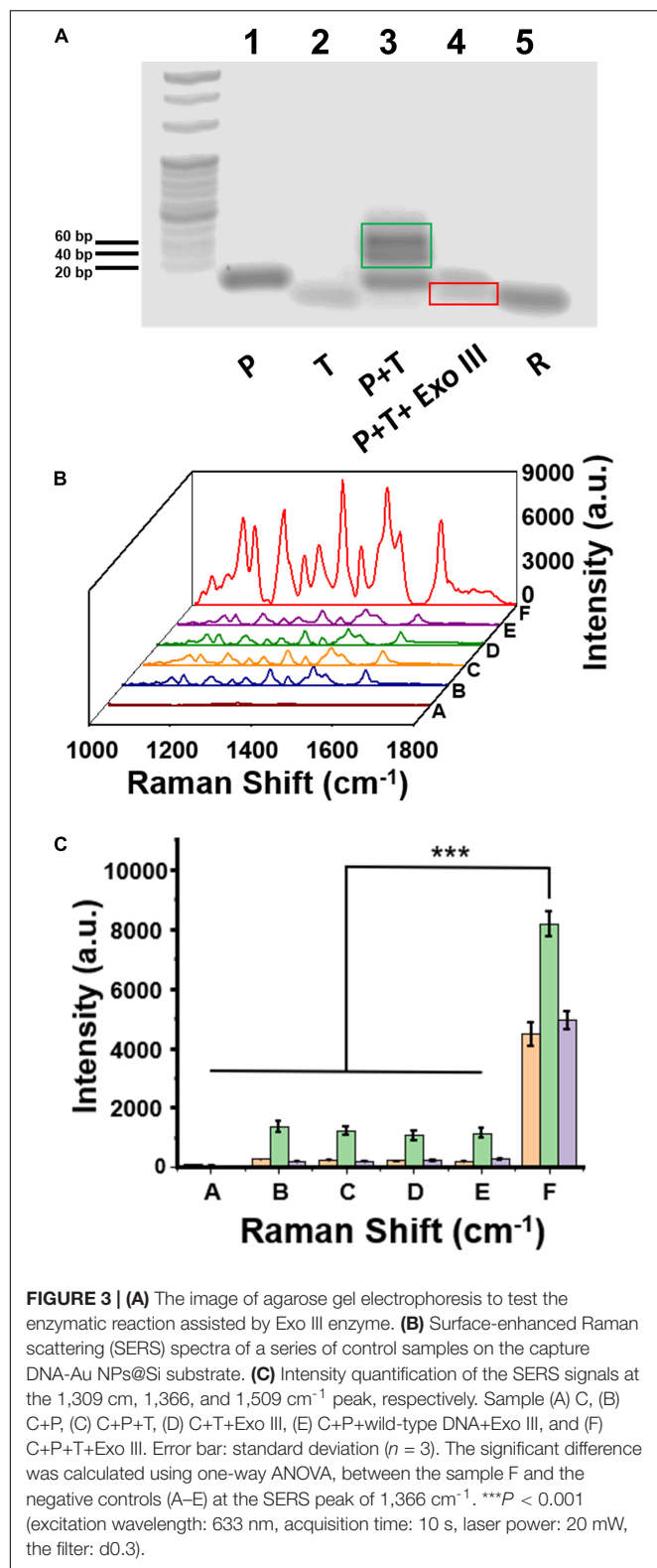
MFE changes for the hybridization scenarios between C and R (CnR , $\Delta G = -57.77 \text{ kJ mol}^{-1}$) or between C and P (CnP , $\Delta G = -36.67 \text{ kJ mol}^{-1}$). The results suggested that C would hybridize R, even when unreacted P was present in the solution. When the three kinds of DNA were mixed equally in mass, P would maintain its stable hairpin structure (48%), and most of the hybridization happened between C and R (45%, **Figure 2J** down, pie chart). This suggested that the presence of P (leftover after the cleavage reaction) would not influence the hybridization between R and C. Therefore, the oligo sequence design would promote the performance of our assay by minimizing the side reactions/hybridization of WnP or CnP as the sources of false-positive signals.

Feasibility Tests of the Assay

Agarose gel electrophoresis was employed to test DNA hybridization and Exo III enzyme-assisted DNA cleavage. As shown in **Figure 3A**, the bands in Lane 1, Lane 2, and Lane 5

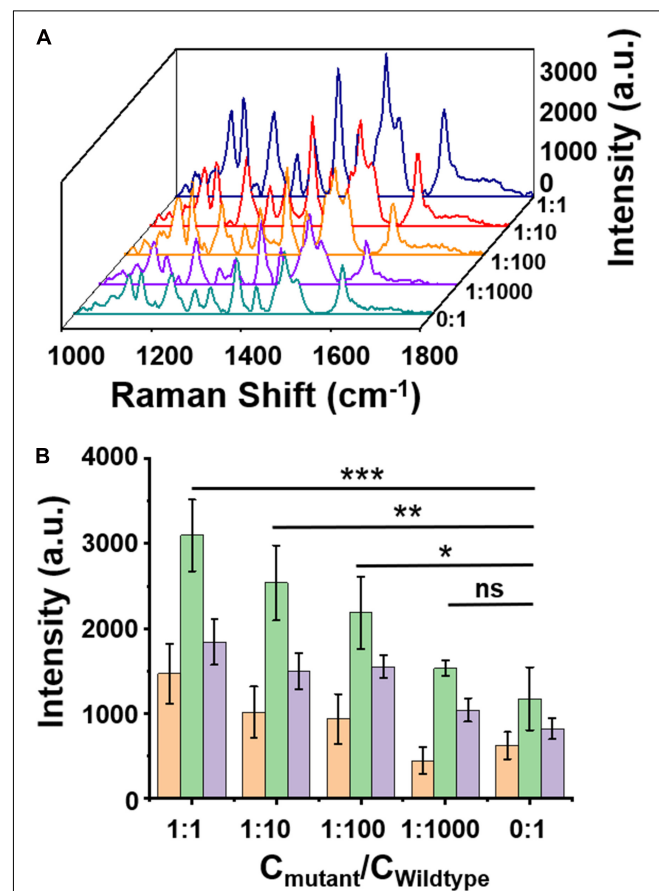
were corresponding to P, H3.3-mutated ctDNA (T), and R in the gel electrophoresis, respectively. The hybridized product of TnP was present as the dark band in Lane 3, with the light bands attributed to a small amount of the free oligos of P. After the addition of Exo III in Lane 4, the hybridized product of TnP disappeared, suggesting an efficient DNA cleavage assisted by the Exo III enzyme. The residual DNA was observed after the enzymatic digestion, which highly contributed to the degradation of the hybridized product of TnP by Exo III. GelRed usually stains double-stranded nucleic acids than single-stranded nucleic acids. It was interpreted that the band of T or R was weak because T tended to form only a short segment of double helix structure, while R was mostly single-stranded.

The feasibility of our assay was also validated with SERS measurements of a series of DNA oligo mixtures with or without Exo III enzyme, including five negative control groups and one positive experimental group (**Figure 3B**). There were no detectable SERS signals when only C was present on the



substrate (curve A in Figure 3B); the group of C+P (curve B) and C+P+T (curve C) observed the same trend. These experiments suggested that C would not hybridize to P, which

was consistent to our design and the theoretical calculation. We did not observe SERS signals for C+T+Exo III (curve D) or C+P+W+Exo III (curve E). In contrast, there was a significantly intensive SERS signal on the Au NPs@Si substrate in the positive experiment containing C, P, T, and Exo III (Curve F in Figure 3B). The signal intensity of the positive group (F) was nearly six to seven times higher than the other negative groups of B, C, D, and E at the characteristic peaks of Cy5 tag, such as 1,309, 1,366 cm, or 1,509 cm⁻¹ (Figure 3C). The SERS signal comparison between E and F verified that the assay was highly selective to distinguish a single-nucleotide difference between W and M sequences. In addition, we performed the reaction (C+P+target DNA+Exo III) in two separate substrates in parallel to evaluate the substrate-to-substrate variation, which suggested reproducible SERS signal acquisition (Supplementary Figure 3).



Specificity and Limit of Detection

The specificity of the assay was further examined by mixing the W (1 pM) and H3.3-mutated ctDNA oligos in gradient concentrations at different ratios (1 pM, 0.1 pM, 0.01 pM, 1 fM, and 0 fM). As shown in **Figure 4A**, when the concentrations of H3.3-mutated ctDNA were reduced from 1 pM to 0 fM, the SERS signal intensities gradually decreased with the gradient changes, while maintaining the spectral patterns of Cy5 tag. Quantitative analysis of the characteristic peak intensities at 1,306 cm⁻¹, 1,366 cm⁻¹, and 1,509 cm⁻¹ also demonstrated this trend (**Figure 4B**), suggesting that the assay can specifically distinguish the samples when the H3.3-mutated ctDNA oligos were diluted by 2 or 3 orders of magnitude with W.

The LOD of the assay was evaluated by titrating the H3.3-mutated ctDNA oligo concentrations in the PBS buffer and the human serum, separately. As shown in **Figures 5A,B**, the SERS signal intensities of the characteristic peak at 1,366 cm⁻¹ decreased when the ctDNA oligo concentration was diluted from 10 to 100 nM in PBS buffer. There was a good linear relationship ($R^2 = 0.980$) between the SERS signal intensities and ctDNA oligo concentrations, with an LOD of 7.9 fM (1.0 pg mL⁻¹) (signal-to-noise ratio ≥ 3).

Furthermore, we performed a pilot experiment to test the assay in human serum samples. H3.3-mutated ctDNA oligos were spiked into the human serum samples from healthy

donors with a concentration gradient from 10 to 100 nM. The DNA samples were then extracted from the mixture by referring to a standard DNA extraction procedure, in order to remove serum proteins or other contamination sources. As shown in **Figures 5C,D**, we observed a strong correlation (linearity $R^2 = 0.991$) between the SERS signal intensities and the spiked concentrations of the H3.3-mutated DNA oligos. The LOD was estimated to be 9.1 fM (1.2 pg mL⁻¹) according the criterion of the signal-to-noise ratio ≥ 3 . The experiments suggested that the SERS/enzyme amplification technique allowed for sensitive detection of M in complicated samples, thus offering a useful tool for potential clinical translation.

Diffuse intrinsic pontine gliomas is a brainstem tumor of malignancy in childhood for which median survival is below 1 year (Hoffman et al., 2018). More than 90% of patients with DIPG are estimated to have the point mutation in H3.3 (65% of tumors) or H3.1 (25% of tumors), while the rest are estimated to have histone 3 wild-type tumors (Vanan and Eisenstat, 2015). Among those mutations, the averaged survival span of H3.3 mutation is the shortest (10.4 months) (Cordero et al., 2017), thus being in an urgent need of developing sensitive diagnostic tools. By using the spiked blood samples, we have demonstrated an ultra-sensitive detection of the target DNA sequence for H3.3 mutation of DIPG with an LOD of 9.1 fM (1.2 pg mL⁻¹), nearly 10–100

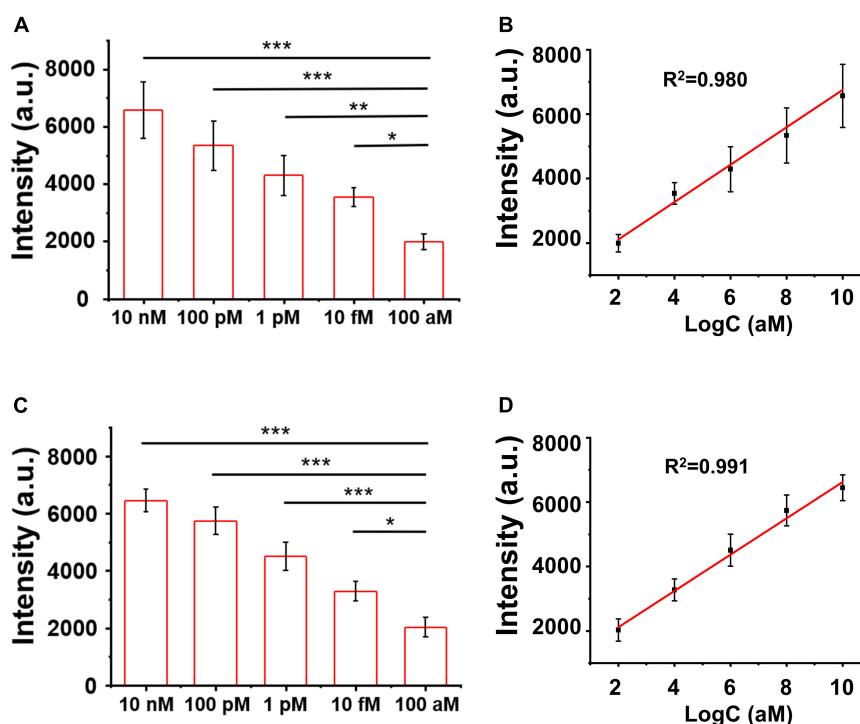


FIGURE 5 | (A,B) Surface-enhanced Raman scattering (SERS) intensity and the linear fitting of signal-to-noise ratios at the SERS peak of 1,366 cm⁻¹ with circulating tumor DNA in phosphate buffer solution (PBS). **(C,D)** SERS spectra and the linear fitting of signal-to-noise ratios at the SERS peak of 1,366 cm⁻¹ with ctDNA in the blood. Error bar: standard deviation ($n = 3$). The significant difference was calculated using one-way ANOVA, between the individual titrated samples against the most diluted one (100 nM) at the SERS peak of 1366 cm⁻¹. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (excitation wavelength: 633 nm, acquisition time: 10 s, laser power: 20 mW, the filter: d0.3).

folds lower than the typical ctDNA concentration range (0.01–0.1 ng ml⁻¹) of the disease. This study has not tested the blood samples from patients with DIPG yet due to a limitation of the clinical resources. It is necessary to further validate this technique in carefully grouped patient blood samples with references to the clinical outcomes in the future.

In our technique, the design of P is very flexible for the introduction of any target DNA sequence, thus allowing for an easy translation to sensitive detection of the ctDNA of other types of cancers without changing the enzyme. For instance, researchers have identified a variety of clinically useful mutations in the ctDNA samples, including G protein subunit alpha Q (GNAQ) Q209L mutation (626A > T) in metastatic uveal melanoma (Madic et al., 2012), p110a catalytic subunit of the class 1A PI3K (PIK3CA) E545K mutation (1633G > A) in breast cancer (Board et al., 2010), or epidermal growth factor receptor (EGFR) T790M mutation (2369C > T) in non-small-cell (Normanno et al., 2017). The applications of the technique on the sensitive detection of these mutations are promising in facilitating the early diagnosis of the diseases and treatment with personalized medicine. The key for successful development of a new assay by the technique is careful optimization of the competitive hybridization segment of P for minimal side reactions of hybridizing W or C.

CONCLUSION

In summary, we have developed a method that integrates cycled enzymatic DNA amplification and SERS spectroscopy for sensitive detection of ctDNA, by combining the advantages of highly efficient amplification by the Exo III enzyme and the SERS technique. It brings the LOD to as low as 7.9 fM in the mixture solution or 9.1 fM in the spiked serum samples. Our approach can differentiate a single-base mismatch between wide-type DNAs and mutated DNAs, which has better applications in medical laboratory research and translation for early clinical diagnosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional committees for Human/Animal Experiments of the School of Basic Medical Sciences of Soochow University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL and XM conceived and designed the experiments. XM and QF performed the experiments. XX and SL provided help with data analysis. RW assisted the characterization of the Au NP@Si substrates. JY provided help in the gel electrophoresis experiments. XM, BN, and JL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2021.676065/full#supplementary-material>

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RNA Interference and Nanotechnology: A Promising Alliance for Next Generation Cancer Therapeutics

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Cancer is a significant health hazard of the 21st century, and GLOBOCAN predicts increasing cancer incidence in the coming decades. Though several conventional treatment modalities exist, most of them end up causing off-target and debilitating effects, and drug resistance acquisition. Advances in our understanding of tumor molecular biology offer alternative strategies for precise, robust, and potentially less toxic treatment paradigms for circumventing the disease at the cellular and molecular level. Several deregulated molecules associated with tumorigenesis have been developed as targets in RNA interference (RNAi) based cancer therapeutics. RNAi, a post-transcriptional gene regulation mechanism, has significantly gained attention because of its precise multi-targeted gene silencing. Although the RNAi approach is favorable, the direct administration of small oligonucleotides has not been fruitful because of their inherent lower half-lives and instability in the biological systems. Moreover, the lack of an appropriate delivery system to the primary site of the tumor that helps determine the potency of the drug and its reach, has limited the effective medical utilization of these bio-drugs. Nanotechnology, with its unique characteristics of enhanced permeation and better tumor-targeting efficiency, offers promising solutions owing to the various possibilities and amenability for modifications of the nanoparticles to augment cancer therapeutics. Nanoparticles could be made multimodal, by designing and synthesizing multiple desired functionalities, often resulting in unique and potentially applicable biological structures. A small number of Phase I clinical trials with systemically administered siRNA molecules conjugated with nanoparticles have been completed and the results are promising, indicating that, these new combinatorial therapies can successfully and safely be used to inhibit target genes in cancer patients to alleviate some of the disease burden. In this review, we highlight different types of nano-based delivery strategies for engineering Nano-RNAi-based bio drugs. Furthermore, we have highlighted the insights gained from current research that are entering the preclinical evaluation and information about initial clinical developments, shaping the future for next generation cancer therapeutics.

Keywords: cancer, combinatorial therapy, nanotechnology, non-coding RNAs, RNA interference, targeted drug delivery

INTRODUCTION

With the growing global burden and economic impact, cancer has become the most significant public health challenge of this century. Cancer, an exemplar of deregulated genetic function, arises from a complex interplay of genes and their products. Conventional therapeutic regimens include surgical resection, chemotherapy, radiotherapy, and anticancer drugs or a combination of all these. Despite the fact that these treatment approaches have increased the overall patient survival for many cancers in their early stages, the lack of specificity, off-target effects, and acquisition of drug resistance stand as a hindrance in the path of considering these treatment methods as effective and patient-friendly. The significant distress caused by these side effects in patients is often a leading impediment in the course of adopting these as mainstream treatments. With the identification of several novel molecules, their mechanisms and pathways involved in cancer, it has become even more imperative to further distinguish and isolate the molecules which could help in the management of the disease.

Recently, much attention has been paid to the development of cancer therapeutics based on RNA molecules. RNA interference (RNAi), a process that involves sequence-specific gene silencing, is an endogenous post-transcriptional regulation process that consists of non-coding RNAs like microRNAs (miRNAs), small interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), which target and silence messenger RNAs (mRNAs) in a sequence-specific manner (Chalbatani et al., 2017). With the added benefit of simultaneously targeting multiple genes that share homology, RNAi is fast, economical, and has the potential for site-specific targeting. The primary role of RNAi in cells would be to downregulate the expression of their target gene and thereby the encoded proteins thus bringing about the desired results (Mahmoodi Chalbatani et al., 2019). Through careful sequence selection and synthesis of tailored non-coding RNAs (ncRNAs), this powerful approach could be used to circumvent conventional cancer therapy limitations, thus paving the way for next generation therapeutics (Lorenzer et al., 2015).

The *in vitro* experimental data obtained using RNA interference has shown promising results, demonstrating its feasibility to be taken from bench to bedside. Though advantageous in *in vitro* scenario, several impediments lie on its path in achieving gene silencing *in vivo* and making gene therapy a reality. The major hurdles being instability of RNA molecules, its low transfection efficiency and half-life, lack of site-specific targeting and distribution in the target tissue, heterogeneity of the tumor, etc. add up to the challenges of onsite delivery of drugs using RNAi. The unique characteristics of nanoparticles have enabled researchers to successfully demonstrate them as efficient chaperones for the delivery of RNAi molecules to the primary site of the tumor. Nanoparticles usually exist as particulate dispersions or solid particles with sizes ranging between 10–1,000 nm (Nagal and Singlab, 2013). Their enhanced permeability and retention (EPR) effect, capacity to prevent RNAi molecules from undergoing enzymatic degradation, and transportation efficiency across the

cell membrane make them a perfect carrier of RNAi molecules for targeted therapy (Xin et al., 2017). The conjugation of nanocarriers with the RNAi molecules widely opens the door to targeting mediators in cancer progression, identifying molecular targets, and engineering delivery vehicles conjugated with DNA/RNA as therapeutic devices, thus representing an ideal approach. Currently, a small number of Phase I clinical trials with systemically administered ncRNA molecules conjugated with different nanoparticles as delivery vehicles are already complete, indicating that these new therapeutics can safely inhibit targeted gene products in patients with cancer. In spite of elucidation of the immense potential of RNAi in cancer therapy in many explorative research trials, there is still a long way to go for successfully translating this exciting result from bench to bedside.

In this review, we discuss the importance of harnessing ncRNAs as next generation cancer therapeutics. We also discuss critical delivery strategies that are based on nanoparticles and the parameters to be considered for RNAi-based drug development. Furthermore, we have highlighted the insights gained from current research that are entering the preclinical evaluation and information about initial clinical developments, shaping the future for RNAi-based therapeutics in cancer.

DEBILITATING EFFECTS OF CHEMOTHERAPY AND THE NEED FOR NOVEL STRATEGIES

The practice of using chemicals to reduce the burden of cancer saw its beginning in the early decades of 20th century and has continued to treat this disease over the years. While surgery and radiation therapy were leading the cancer treatment, their failure to target inoperable metastatic lesions placed chemotherapy at an advantageous position. Therefore, most of the times an evidence driven choice was made to use a combination of these three modalities to induce remission of cancer (DeVita and Chu, 2008). While chemotherapy had become the standard care in numerous cancers, the ill effects of the chemicals on multiple organ systems of the body were also well known. The debilitating after effects and overall decreased quality of life that patients endure, directed research interest towards innovating novel strategies that are equally, if not more, effective as chemotherapy while being better tolerable by the human body (Gegechkori et al., 2017; Pearce et al., 2017).

Chemotherapy is harmful due to the lack of specificity and patients placed under any chemotherapeutic regimen report dissatisfaction due to undesired symptoms generated. It is also often an added factor for depression and anxiety in patients diagnosed with cancer (Niedzwiedz et al., 2019). Subjects placed on chemotherapy experience side effects that can range from mild (nausea, vomiting and diarrhea) to life threatening (cardiomyopathy with decreased ejection fraction, development of secondary malignancies such as leukemias) after effects. These can arise during the treatment phase (long term effects) or years later (latent effects) (Stein et al., 2010).

Chemotherapy regimen places patients in a loop wherein for minimizing adverse effects of one drug, they will be forced to take additional drugs and the cycle continues, often affecting their mental health and emotional state (Pearce et al., 2017). Pregnancy is another physiological state where chemotherapy is deemed unsafe and the detrimental effects include intrauterine growth restriction, limb anomalies and stillbirth etc. (Koren et al., 2013).

Chemotherapy affects rapidly dividing cells without differentiating the neoplastic and non-neoplastic cell. Gastrointestinal tract, germ cells, hematopoietic cells and hair follicles are prime targets, considering their replicative potential (Agarwal, 2016). Nonspecific targeting of gastric mucosal cells presents as diarrhea (immediate onset and late onset) is common with drugs like topoisomerase II inhibitors (irinotecan, topotecan), 5-Fluorouracil, taxanes (docetaxel/paclitaxel), antibodies against epidermal growth factor receptors and calcineurin inhibitors. Though there are treatment options like opioid based formulae and somatostatin analogs that are used to alleviate the pain and discomfort caused due to chemotherapy, these drugs too present with their very own set of side effects (Rock and Kono, 2008).

Meiotic cells are also affected by chemotherapy and this raises concerns about fecundability in female (often resulting in premature ovarian failure) and male subjects alike. While ovarian follicles and the endocrine axis are disrupted in females, males have diminished semen quality with spermatozoa that are low in number and/or of poor quality. Secondary sexual characteristics are affected in pubertal and prepubescent children undergoing chemotherapy which has an impact on their self-confidence (Vakalopoulos et al., 2015; Waimey et al., 2015; Poorvu et al., 2019). Chemotherapy induced alopecia is reported in numerous clinical and standard of practice trials which are considered a distressing effect as it affects the emotional state and body image of subjects (Saraswat et al., 2019). Hair loss is observed on the scalp, axillae, groin and all over non-glabrous skin surfaces which indicates the effects chemotherapy has on vellus and terminal hairs in a non-specific manner (Muth, 2017). Chemotherapy has an impact on childhood cancer subjects to an extent of impacting self-esteem in over two thirds of them and inducing significant stress among their parents. Chemotherapy in children in certain cases invalidates their germ cells and gonads resulting in decreased fertility and the psychological impact this creates often needs anticipatory counseling in their adolescence. Understanding the pathophysiology of chemotherapy induced alopecia to a certain extent has led to the use of techniques and medications such as hypothermia, YHO618 to minimize hair loss and improve hair growth but substantial research still needs to be done as no prevention and treatment guidelines are currently in place (You et al., 2019; Rossi et al., 2020).

Nervous system impact in subjects undergoing chemotherapy is well studied and includes impact on the central and peripheral nervous system manifesting as cognitive dysfunction, neuropathy and mental health concerns (Stone and DeAngelis, 2016). Chemotherapy results in a variety of neurological complications that include mood disorders, seizures, memory impairment ocular toxicity, ototoxicity and uncertain effects

when there is unspecified neuro-degeneration (Yang and Moon, 2013). Peripheral neuropathy is well noted with a variety of chemotherapeutic drugs like paclitaxel, vincristine, bortezomib and cisplatin. As the peripheral nervous system includes nerves constituting the autonomic and enteric nervous systems, effects such as gastroparesis and orthostatic hypotension can be observed post chemotherapy. These neurological effects have been understudied and the mechanisms and their prevention or treatment is limited (Zajackowska et al., 2019; Loprinzi et al., 2020).

Secondary malignancies are encountered years after chemotherapeutic regimens and drugs that are known to have such effects include alkylating agents (cyclophosphamide, ifosfamide, nitrogen mustards, melphalan), daunorubicin and doxorubicin. A life-long follow up is recommended to screen for the development of leukemias and lymphomas which are the most common secondary malignancies (Vega-Stromberg, 2003). The risk of secondary malignancies is higher in subjects placed on chemotherapy independent of irradiation status indicating the impact chemotherapy has on cells and their replicative potential (Natalapati and Jain, 2018). Chemotherapy impacts essential organ systems, overall quality of life, causes functionally impairing fatigue, distorts body image and self-esteem, compromises fecundability and with the risk of unpredictable secondary malignancies.

Therefore, there is an imminent need to replace chemotherapy with biological therapies. With the advent of various next generation technologies in genomics, transcriptomics, proteomics and high throughput data analysis, the molecular path to next generation drugs has been set in. One of the promising technologies is in the area of non-coding RNA based drugs that are considered to be next generation molecules in the field of molecular medicine. These molecules are classified as miRNAs, lncRNAs and circRNAs etc. They can act independently or in co-ordination with circular endogenous RNAs (ceRNAs) to bring about the regulation of genes. MicroRNAs (miRNAs) are short 22–25 nt non-coding RNAs that regulate a wide array of biological process including carcinogenesis. These molecules have revolutionized the field of cancer ever since their discovery as they have the capability of silencing multiple targets involved in one or different pathways thus bringing about a co-ordination among large oncogene regulatory networks. These miRNAs (miRs) are known to be deregulated in different stages of cancer development as they are known to regulate the expression of genes involved in onset and development of tumors, and such miRNAs are called oncomiRs. Thus these molecules along with numerous agents that can efficiently be part of molecular targeted therapy are being developed that can target cellular processes, such as angiogenesis, signal transduction, cell cycle regulation, apoptosis induction, protein translation, and metastases (Rishabh et al., 2021). To enhance the likelihood of achieving a positive response to this targeted therapy, further studies have to be done to understand the precise molecular mechanisms of cancer in each patient, then carefully designing bio-drugs to deal with the situation paving the way for personalized cancer therapeutics.

RNA INTERFERENCE AND ITS APPLICATIONS IN VARIOUS CANCERS

RNA Interference: An Introduction

RNAi is an evolutionary, endogenous, conserved, post-transcriptional, gene silencing pathway that involves double-stranded RNA-mediated degradation of mRNA. Usually mediated by small RNA molecules like siRNAs, lncRNAs, miRNAs, ceRNAs; RNAi in eukaryotes initiated by the RNase III enzyme called DICER that cleaves long double-stranded RNAs (dsRNAs) into mature small interfering RNAs (siRNAs) with an overhang at the 3' end. Constituting a passenger strand and a guide strand, siRNAs are incorporated into the RNA Induced Silencing Complex (RISC), where the guide strand complement pairs with the target mRNA sequences and initiates endonucleolytic cleavage through the action of induced Argonaute protein (Borges and Martienssen, 2015). Among these, miRNAs are well studied and known regulators of gene expression in various cellular processes like cell development, differentiation, apoptosis, and proliferation (Hutvagner and Zamore, 2002). The disruption of miRNAs and their cellular functions has been reported to be responsible for the initiation and progression of various cancers and other fatal human ailments. Though their functions are elucidated as oncogenes and tumour suppressors, the complete function of miRNAs, especially in humans, is not fully understood. Thus the function of RNAi and its biological significance within the organism's body in which it is present is also a mystery to the scientific world. The accurate and expeditious nature of RNA interference technology makes it a popular strategy to learn the gene expression of various organisms. Loss of function studies and systemic RNAi-based genetic screens performed on organisms like plants, *C. elegans*, and *Drosophila* highlighted the role of this technique in functional genomics. The fact that transfecting mammalian cells with long dsRNAs induced interferon response leading to global gene silencing and, consequently apoptosis, hindered the use of RNAi in mammalian cells in the earlier stages. This was at a time when the structures of these molecules were not elucidated. However, chemical synthesis of siRNA molecules specific to mRNA targets in later years has enabled efficient targeting and silencing of a particular gene, leading to the widespread use of the same in mammalian cells (Karagiannis and El-Osta, 2005).

RNAi and Cancer

With a complex etiology, cancer cells with their characteristic lesion often resemble their normal counterparts except for the difference in a series of genetic alterations (Wilda et al., 2002). It is well known that the mutations in the gate keeping genes are the ones that give rise to tumorigenesis in the first place. Subsequent mutations in different genes that belong to cellular oncogenes (c-onc), viral oncogenes (v-onc), tumor suppressor genes (TSG) classes also cause tumor initiation and progression. Different experimental approaches have been used to compare tumor cells with normal cells and have shown that deregulation of a set of non-coding RNAs (ncRNAs) that might be acting as a master switch, might be vital in their differences. The earliest example of

a non-coding RNA involved in cancer was the H19 gene (Hao et al., 1993). Cellular oncogenes usually encode various proteins like growth factors and their receptors, signal transducers, and transcription factors associated with tumor initiation and progression. The downregulation of the K-Ras gene through the RNAi pathway is among the first RNAi-mediated deregulations discovered. The silencing of the K-Ras gene, was found to trigger anchorage-independent growth and thus tumorigenesis (Brummelkamp et al., 2002). Various other cellular oncogenes that code for Bcl-2, CDK-2, Mdm-2, PKC- α , TGF- β 1, H-Ras, VEGF, and GFP proteins are cognate targets for siRNAs, effectively leading to suppression of cancer cell proliferation. Viral oncogenes, integrated into the host genome through DNA and RNA viruses, are also found to be regulated by RNAi. In cervical cancers and hepatocellular carcinomas (HCC) that are caused by HPV and HBV viruses, respectively, these viral oncogenes turn out to be the target for cancer treatment (McCaffrey et al., 2003; Yoshinouchi et al., 2003). Belonging to the tumor suppressor gene prototype, Retinoblastoma protein (RbP) is found to undergo somatic inactivation in various cancers. In order to study RbP in cancer, components of the dE2F/dDP/RBF pathway were silenced by RNAi, and a remarkable division of labor between family members of this pathway was analyzed through examination of gene expression changes (Agami, 2002). DNA damage-based biological response is usually governed by the effectiveness of DNA repair processes called the checkpoint cell cycle responses and induction of processes that favor or inhibit pathways leading to apoptosis of the cell. The viability of the cell may not be affected by the defects in DNA repair enzymes, but it can lead to genomic instability, and thereby enhancing the rate of genetic changes and thus the rate of tumor formation. Suppression of Rad51 gene, a DNA damage repair gene, by RNAi method, elucidated the role of the same after both endogenous and exogenous double-strand break formation. This was complemented by mutating SPO11 and MRE11 proteins (Takanami et al., 2003). RNAi-based functional genomic approaches have also enabled the identification of chromosomal DNA degradation mediating nucleases like CPS6 and NUC1. siRNAs developed against the ATR gene have also been in use to analyze its role in checkpoint responses, along with p53 binding protein. These have turned out to be a significant target in clinical application since then (Cortez et al., 2001; Morales et al., 2003).

During invasion and metastasis, tumor cells can undertake either single-cell locomotive or cohort migration strategies. The membrane-anchored glycoprotein RECK inhibits tumor metastasis and angiogenesis by negatively regulating matrix metalloproteinases (MMPs). RNAi-mediated inhibition of RECK in CL-1 human lung cancer cells exterminated the inhibitory effect of trichostatin A (TSA), and HDAC inhibitor, on MMP-2 activation (Liu et al., 2003). RNAi studies done on MDA-MB-231 human breast cancer cells showed the overexpression of CXC chemokine receptor-4 (CXCR4) could be controlled by knockdown of the gene and thereby metastasis. Matrix metalloproteinases MMP-9 and cathepsin B promote invasion and metastasis of gliomas through matrix degradation. Studies showed that the suppression of gene

expression of these proteins is caused due to short hairpin RNAs in SNB19 cells, leading to a significant reduction in cell-cell interaction of human microvascular endothelial cells (Lakka et al., 2004).

Angiogenesis is a major contributor to the development and growth of cancer. Various proteins involved in angiogenesis have been targeted by RNAi technology. The angiogenic factor VEGF is regulated by the interaction of its GC-rich motif with that of Sp protein. RNAi-based studies have been done on Sp proteins to analyze their role in the regulation of VEGF. Sequential knockdown of the Sp1, Sp3, or Sp4 gene with the help of siRNA showed that all three proteins regulated transactivation in pancreatic cancer cells transfected with the pVEGF1, pVEGF2, and pVEGF3 constructs containing VEGF promoter inserts. This indicates that Sp1, Sp3, and Sp4 cooperatively regulate VEGF expression in tumor cells (Abdelrahim et al., 2004). Another molecule that regulates the function of VEGF is HIF. RNAi approach has been used successfully to determine functional differences between HIF-1 α and HIF-2 α in different human cell lines (Warnecke et al., 2004). HIF-1 α knockdown in cervical cancer cell lines reduced hypoxia-induced mRNA stimulation of glucose transporter 1 GLUT1, lactate dehydrogenase A (LDH-A), VEGF, carbonic anhydrase IX (CA IX), and HIF prolyl hydroxylase 2 (PHD2) by 40–60%. In contrast, HIF-2 α knockdown had no effect on these HIF target genes. Tie-2 is a small molecule inhibitor of endothelial cell-specific tyrosine kinases, which, when interrupted by RNAi, make endothelial cells lose their viability. This was further investigated and found to be due to AKT signaling inhibition, leading to increased thrombospondin expression (Niu et al., 2004). The tumor growth occurs due to the imbalance between cell proliferation and apoptosis. The livin gene expressed in numerous cancers is found to have an anti-apoptotic function. A study based on vector-based livin-siRNAs plasmid found that it silenced endogenous livin gene expression through association with caspase-3 activation, thus, in turn, enhancing apoptotic rate (Crnkovic-Mertens et al., 2003). Bcl-2 and xIAP are other anti-apoptotic factors. Studies showed that the sensitivity of MCF-7 breast cancer cells to treatment with the drugs etoposide and doxorubicin is increased after silencing of Bcl-2 or xIAP by siRNA (Lima et al., 2004). RNAi is developing from a powerful tool utilized to elucidate the function of novel genes to a potential therapeutic modality in cancer therapy. Further research is needed to understand and unravel the precise mechanism, by which RNA interference modulates gene expression inside the body, especially during cancer, and to exploit this powerful tool and use it to its full potential.

RNA MOLECULES AS NEXT-GENERATION THERAPEUTICS IN CANCER

With the development of novel therapeutic strategies like gene silencing and genome editing, diverse RNA molecules have come to the forefront as potential therapeutic molecules (Fire et al., 1998; Cong et al., 2013; Mali et al., 2013). The fact that they can interact with all the major biological molecules like DNA, RNA,

and protein led to the idea of RNA therapeutics, which is now the primary reason for the expansion in the range of druggable targets, including conventional proteins and previously undruggable transcripts and genes. Basically, RNA can take up three different roles in therapy. Firstly, RNAs can form oligonucleotide molecules called aptamers that can bind to the extracellular, cell surface, or intracellular proteins targeted by small molecule drugs thus bringing about silencing (Gragoudas, 2004). Secondly, siRNAs, miRNAs, and their mimics/knockdown molecules can target specific mRNAs leading to gene silencing or control of gene expression for treatment of diseases. Thirdly, mRNA molecules can be transfected in the cells where they can get translated into protein and thus be a part of vaccination or protein replacement therapy (Sahin et al., 2014; Lieberman, 2018). Though scientists knew that the transcription of many genes in eukaryotic cells are repressed, genes are transcribed into mRNA that never gets translated, post-transcriptional mechanisms are in place to add another level of control over this already existing complex system, identification of the function of these endogenous and exogenous molecules revolutionized the way gene expression can be manipulated (Christopher et al., 2016).

MicroRNA

MicroRNA plays a significant role in gene regulation transcriptionally and translationally. The fact that one miRNA can compromise the expression of several different target genes simultaneously indicates that it has a very different role in pharmaceutical targeting when compared to siRNAs. Ambros and colleagues discovered the first miRNA lin-4 in 1994 (Lee et al., 1993). As an extension to this discovery, various other miRNA molecules like let-7 were identified and were observed to perform sequence-specific RNA-RNA interaction with mRNA, leading to inhibition of gene expression (Rawoof et al., 2020). These non-coding RNA molecules were found to possess highly conserved nature suggesting that these molecules are essential gene regulatory factors. Hsa-miR-15a and miR-16-1 were the first cancer-related miRNAs discovered. These were tumor-suppressing miRNAs found in chronic lymphocytic leukemia that induced apoptosis through repression of the Bcl-2 gene (Calin et al., 2002). Further studies on the deletion of miR-15 and miR-16-1 cluster in mice recapitulated chronic lymphocytic leukemia-associated phenotypes observed in humans. This convincingly demonstrated that these two miRNAs are crucial for tumor suppression (Klein et al., 2010). Hsa-miR-143 and miR-145 are often found deregulated in lung cancer, resulting in decreased expression of both miRNAs. The abnormal expression of certain miRNAs can also be due to the deregulation of various transcription factors like c-Myc and p53. c-Myc is found to repress the transcriptional activity of tumor-suppressive miRNAs like miR-15a, miR-26, miR-29, miR-30, and let-7, thus setting in proliferation (Chang et al., 2008). Tumor suppressor gene p53, on the other hand, positively regulates the expression of various miRNAs like miR-34, miR-605, miR-1246, and miR-107.

The failure of chemotherapy in cancer has paved the way for development of miRNA molecules as therapeutic targets, especially for patients with drug resistance issues. The fact that

there exists an alteration in the expression of these miRNAs in cancer makes it possible to manipulate miRNA expression in such tumor tissues. These manipulations are often facilitated by injecting miRNAs similar to the use of antisense mRNAs. By identifying signature miRNAs, their mechanism of action in cancer, and strategies to attain this miRNA molecule delivered at the target, miRNA therapeutics can be applied more efficiently in cancer therapy and thus would emerge as a novel therapeutic tool (Kumar et al., 2020).

Long Non-coding RNA

After the discovery of the first long non-coding RNA (H19) associated with a tumor, several ncRNAs have been identified, implicated to have functions in different cancers. These ncRNAs usually constitute small and long ncRNA, and some of these long ncRNAs can even give rise to miRNAs (Metzler et al., 2004; Eis et al., 2005). Recent studies have revealed the role of lncRNA in cancer development and in maintaining the hallmarks of cancer. This is mainly by virtue of their ability to interact with the major biological molecules like DNA, RNA, and protein, or even a combination of them. LINE-1, HERV, Satellites, and TERRA are specific lncRNAs that can contribute to disease progression through immune response activation. Other than this, LINE-1 is also found to support chromatin formation. TERRA, on the other hand, supports alternative lengthening of telomeres (Parasramka et al., 2016). Highly upregulated lncRNA in liver cancer (HULC) is a molecule that has a prominent role in the early stages of cancer progression. The binding of the promoter region of HULC to that of CREB protein is found to activate an autoregulatory loop involving HULC and miR-372, which further leads to phosphorylation of CREB and thus the chain of similar events (Wang et al., 2010). HULC also promotes HCC proliferation by targeting and suppressing p18 and IGF2BP1 (Hämmerle et al., 2013). Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is another lncRNA upregulated in HCC and non small cell lung carcinoma (NSCLC) cells that is associated with the alternative splicing of various precursor mRNAs (Ji et al., 2003; Schalken et al., 2003; Lee et al., 2011; Lai et al., 2012). Detection of certain lncRNAs in body fluids and serums has propounded the idea of using these molecules as cancer biomarkers. Plasma levels of HULC are higher in patients with HCC when compared to healthy humans. PCA3 is a prostate-specific lncRNA found usually upregulated in patients and thus is used as a biomarker (Lee et al., 2011). Prostate-Specific Gene 1 (PCGEM1), small dendritic non-translatable RNA BC1, and DD3 ncRNA genes are also over expressed in prostate tumors compared to normal and primary tumor specimens. DD3 is an ideal molecule for cancer therapy as it has not been detected in any of the healthy tissues like breast, bladder, testis, gastrointestinal organ, and musculoskeletal tissue (Schalken et al., 2003). Similarly, BC200 ncRNA, a brain-specific small cytoplasmic RNA, is found to be expressed in cancers like breast, cervix, esophagus, lung, ovary, parotid, and tongue though absent in healthy individuals (Chen et al., 1997).

Circular RNA

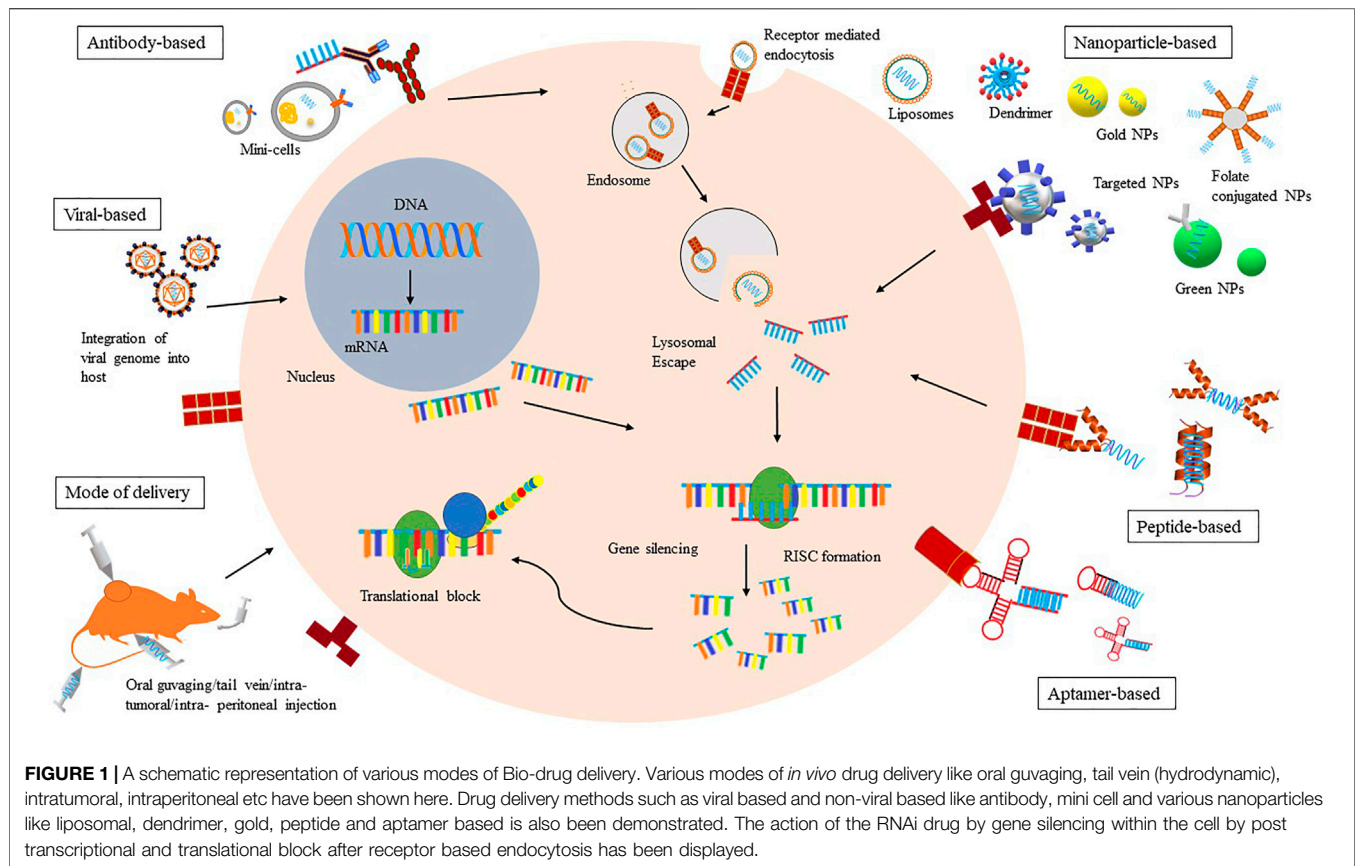
The circRNAs are functional non-coding RNAs that lack 3' and 5' ends, existing as circular transcripts. Expressed tissue specifically,

these transcripts, identified to be coded by thousands of genes, were till recently considered aberrant spliced by-products. Nigro and colleagues in 1991 identified the first circRNA in mammalian cells (Nigro et al., 1991). They observed the transcript of the DCC tumor suppressor gene to lack 3' and 5' end and existed as circular molecules. They termed it as scrambled exons. With the development of high-throughput sequencing and bioinformatics pipelines, the identification of more and more circRNAs became accessible in the past decade (Santer et al., 2019). The most frequently described function of circRNAs is that of sponging. With one circRNA having the ability to target more than one miRNA, these non-coding RNAs bind to miRNA molecules and prevent them from binding onto its canonical targets. The circRNA CDR1as have more than 70 conserved binding sites for miRNA-7 (miR-7), inhibiting its activity (Memczak et al., 2013). Studies in liver cancer showed that circHIPK3 was upregulated and could sponge at least nine different tumor suppressor miRNAs with a total of 18 potential miRNA-binding sites (Zheng et al., 2016). Some of the circRNAs were found to have the protein-interacting ability. Circular RNA, circ-FOXO3 is one of them, with expression levels negatively correlating with cell proliferation, inhibition of circ-FOXO3 function promoted cell proliferation, whereas overexpression repressed cell cycle progression (Du et al., 2016). Though numerous challenges are there on the path of knocking down this circular transcript, various gain and loss of function experiments are being conducted that can elucidate the function of circRNAs. The success of such research will decide the potential of using circRNAs as diagnostic and therapeutic tool for clinical application.

Eventhough we celebrate the potentiality of RNA interference, there are immense challenges to be overcome to take this from bench to bedside. The RNAi molecules being negatively charged, their capability to get infused through a cell membrane and to stay stable in the cellular matrix is questionable. Though studies that anchor on increasing the half-life of these molecules in the serum through chemical modification exist, the chance of them becoming immunotolerant inside an organism's body is meager. Thus, the development of carrier molecules that is in nanoscale, which can stay undetected or are pH-sensitive, is required for the development of RNAi to a potent therapeutic molecule. With the advancement in nanotechnology and its application in medicine and pharmaceutical companies, more and more molecules are being experimented for stable and safe delivery into the target tissue.

NANOTECHNOLOGY BASED DELIVERY SYSTEMS FOR RNA INTERFERENCE

The advent of gene therapy in clinical realm has facilitated the use of nucleic acids or oligonucleotides like plasmid DNAs, small interfering RNAs, short hairpin RNA etc. in therapeutic approaches, opening a new paradigm for treating wide range of genetic, innate and acquired diseases including cancer. A comprehensive approach in the oligonucleotide delivery deals with two major aspects. Firstly, to assimilate the oligos into a type of carrier that helps determine their precise morphology, tissue



distribution, half-lives and other biological interactions, and secondly, to attempt the possible modifications in the nucleotide itself, say with a targeting ligand without altering the nature of the conjugate (Juliano, 2016). In order to address the complications associated with therapeutic intervention, efforts are being made to comply homeostasis within the therapeutic findings and shell out the prospects of desired outcomes. Drug delivery is a crucial part of treatment having its own attributes and advantages. However, regardless of the significant potential of bio-drugs in gene silencing, a key limitation in transition to bedside is the lack of appropriate delivery systems that do not reduce the drug efficacy. Even though the phenomenon of RNAi is promising, direct administration of small oligos has not been fruitful because of the lower half-lives of the oligos and presence of nucleases in biological fluids. Furthermore, its low molecular weight hinders oligos to effectively penetrate the endothelial lining and diffuse into the extra-cellular matrix. In addition to these biological perturbations, nucleic acid itself possesses negative charge which lowers its penetration efficiency through the negatively charged cellular membrane. Also, the endosomal pathway offers harsh environment, resulting in degradation of these bio-molecules. The pursuit to make a viable novel antisense drug has given birth to various strategies, delivery approaches and modifications that address limitations associated with direct assimilation of bio-molecules. These strategies facilitate the targeted carriage of bio-molecules, evading biological barriers

of the body and they confer better *in vivo* stability, tissue bioavailability, and targeted cellular delivery to the bio-drugs (Figure 1). Both viral and non-viral vectors have been developed for improving target cell penetration as well as minimizing toxicity by off-target hybridization.

Nanoparticle-Based Delivery Systems

The multifaceted nature of science has paved the way for novel combinatorial and synergistic approaches in clinical terms from bench to bedside. Nanoparticles are versatile vehicles that can be used as drug carriers because they can be tweaked to address the limitations of delivery systems by permitting concurrent transport of numerous therapeutic agents for active therapy. Nanotechnology enables the design and assemblage of intended drugs with unprecedented control over their size and shape. Nanoscale methods have focused on developing adaptable bio-composites by conjugating therapeutic agents of interest into them, offering prominent advantages in drug delivery and imaging. Intracellular targeting describes the site where the drug has to be delivered; whether a tissue, an organelle or a compartment. Nanoparticles being sub-cellular and sub-micron in size, have the ability to penetrate deep into tissues or any other desired region by crossing the epithelial lining, ultimately getting permeated into the cell for achieving their therapeutic effects (Vinogradov et al., 2002). The enhanced permeation and retention effect (EPR effect) in cancerous tissues is essentially exploited by adopting various drug delivery methods, particularly

with nano-based techniques. The defective tissue morphology and leaky vasculature of cancers increase the EPR effect, making them accurate targets for effective drug delivery (Brannon-peppas and Blanchette, 2004). The nanoparticle-based delivery systems can further be segregated into solid, lipid, and polymer based and in the next section we deal with each of these three systems taking appropriate examples.

Solid based Delivery Systems

Recently biocompatible magnetic nanoparticles have been used to deliver small oligos with high gradient external magnets attracting the drug complex, to the target site within the body providing great transfection efficiency (Nagal and Singlab, 2013), (Xin et al., 2017). Halloysite nanotube (HNT) based carrier was recently developed to deliver antisense oligonucleotides to target anti-apoptotic genes in cancer. In order to study the functionality of the carrier as well as the target gene, HNT carriers were surface modified with γ -aminopropyltriethoxysilane and the oligonucleotides were labelled with fluorescein. Confocal and transmission electron microscopic images recorded efficient cellular uptake and the variable gene expression (Shi et al., 2011). Conducive to construct efficient delivery systems, efforts were directed to preserve the bioactivity of oligonucleotides throughout the administration, from start to target site. Two different strategies have been devised which could be used to bind siRNA on gold nanoparticles. A covalent approach, based on thiolated siRNA bound to nanoparticle surface, and an ionic approach based on the electrostatic interaction between the negatively charged siRNA backbones. Both methodologies proved to be efficient for siRNA delivery, attaining precise gene silencing in both *in vitro* and *in vivo* biological systems (Tortiglione and de la Fuente, 2019). In leukemia mouse models, administration of cancer cell-specific mRNA drug conjugated with gold nanoparticles resulted in efficient drug delivery, appropriate release, and competent cellular uptake in both *in vitro* and *in vivo* experiments (Gossai et al., 2019). Also, gold nanoparticles have shown to act as a functional scaffold for various medicinal applications such as drug delivery, imaging etc. A gold nanoparticle (AuNP) based non-viral delivery system was created to direct siRNA into prostate cancer cells. Consequently, polyethylenimine (PEI) capped AuNPs were synthesized and combined with folic acid, a targeting ligand for folate receptors (AuNPs-PEI-FA) which could effectively integrate siRNA through electrostatic interactions. Flow cytometric analysis showed that, AuNPs-PEI-FA could exactly deliver siRNA into LNCaP cells, a prostate cancer cell line overexpressing prostate specific membrane antigen (PSMA). To inspect and counter-validate the complex, internalization of siRNA into PC-3 cells, a prostate cancer cell line not expressing PSMA or folate receptors, remained unattained via AuNPs-PEI-FA, signifying the potential of AuNPs-PEI-FA for targeted delivery in treatment of prostate cancer (Rahme et al., 2019).

Lipid based Delivery Systems

Lipid nanoparticles (LNPs) are the most progressive nanocarriers for siRNA delivery due to their high encapsulation efficacy and potent gene knockdown ability. For precisely targeting the subsets of lymphocytes and to offer RNAi payloads in a cell-specific

fashion, targeted lipid nanoparticles (tLNPs) were used as a unique favorable approach holding abundant potential for the manipulation of gene expression. This method exploits particular antibodies that adorn the lipid nanoparticle that is aimed at targeting lymphocyte subsets (Hazan-Halevy et al., 2019). Antisense oligonucleotides were designed and conjugated with Liposome-Polycation-DNA (LPD) nanoparticles linked with animoside, a targeting ligand to study the down-regulation of an anti-apoptotic protein called survivin in lung cancer (Hill and Carolina, 2006). 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) based nanoliposomes were used to deliver siRNA directly into the tumor cells *in vivo*, with greater efficiency. Xenograft and orthotopic tumor models treated with DOPC incorporated siRNA, resulted in substantial regression in the tumor size and target genes causing no distress or toxicity (Ozpolat et al., 2010). Multi-stage delivery systems demonstrated the advancement of nanovectors. Nanovectors with spherical, quasi-hemispherical and discoidal silicon microparticles have unique roles in cellular adhesion and internalization, and the fabrication of such nanovectors have considered size, shape, and design. *In vivo* studies report increased therapeutic efficiency of liposome coated siRNA when administered through multi-stage systems as compared to free nanoparticles (Serda et al., 2011). Cervical cancer being one of the most life threatening types of cancer among the women, is generally known to be resistant to chemotherapy. Formulations of different solid lipid nanoparticles (SLNs) having chemotherapeutic agent and genetic material (Paclitaxel and siRNA against Bcl-2 oncogene) were tailored respectively. The intent of the study was to understand efficacy with respect to release amount of siRNA, when administered through vaginal suppositories (Büyükköroğlu et al., 2019). Multifunctional envelope typed nano device (MEND) was designed to address the novelty of non-viral gene delivery system. It comprises a complex nucleic acid core, a ligand for specific targeting, fusogenic lipids to enhance endosomal escape and a cell-penetrating peptide to increase the intracellular availability of novel drugs (Hatakeyama et al., 2011). Specific antisense oligonucleotides were incorporated into Cationic amphiphilic bolaamphiphiles (CABs), which were created and tested for their potential to form nano-sized vesicles based on various properties. Results demonstrated significant effect in treating *C. difficile* infection (CDI) by effective targeting and controlling the disease. For treatment options, antisense therapies promise to be a feasible alternative to the conventional antibiotic therapies (Sharma et al., 2018).

Polymer based Delivery Systems

Numerous carriers have exhibited promising results in terms of delivering antitumoral oligonucleotides like siRNAs to xenograft tumor models, where the formulation was administered through intravenous mode. Chitosan-coated poly (isobutylcyanoacrylate) nanoparticles were used as transporter molecules to deliver siRNAs in xenograft tumor mice models. siRNAs coupled with the nanoparticles by adsorption made the polyelectrolyte complexes formed between the chitosan and nucleic acids very stable and non-toxic (Vauthier, 2019). Treatment of mammary

tumors with intratumoral delivery of c-Myc shRNA complexed to rhodamine labelled nanoparticles in Brca2/p53 conditional knockout mouse models, resulted in regression of tumors, prolonging the survival, indicating the suppression of tumors treated with c-Myc shRNA linked-nanoparticles compared to control (Tangudu et al., 2015). Some malignancies like pancreatic cancers, being refractory for regular treatment options (chemotherapy), have the potential to play an important role in precision medicine for cancers by silencing the expression of genes when small-interfering RNAs (siRNAs) are used. For targeted carriage, star-shaped polymeric nanoparticles were developed which self-assembles siRNA into them, for delivery into pancreatic cancer cells in orthotopic mouse pancreatic tumor models. Results indicated substantial reduction in the tumor size and slow recovery in health of the mice after successful administration (McCarroll et al., 2019). Spherical Nucleic Acids (SNAs) are nanostructures that typically consist of a core where functional repositories such as siRNA, miRNA mimics or antagonists could be housed and used as gene regulators. It is known to confer heightened resistance towards degradation, facilitate ease of cell entry in the absence of transfection vehicles. In murine models of glioblastoma, precisely administered siRNA or miRNA conjugated with SNAs, penetrate blood-brain and blood-tumor barriers, regressing tumor progression (Tommasini-Ghelfi et al., 2019).

Aptamer-Based Delivery Systems

For tumor-targeted drug delivery, currently aptamers are being used when addressing cancer treatment with a therapeutic approach. In a recent study, a 64Cu-labeled modified A10 aptamer was designed to target prostate cancer by conjugating with the drug (p-SCN-Bn-NOTA), labeled with 64 Cu radioisotope. This extends the hope for diagnostic potential, non-invasive imaging, and to trace their bio-distribution *in vivo* paving the way for clinical applications (Kang et al., 2019). Tumor-specific carrier systems for siRNA delivery have extended the scope for reaching targets in treatment of cancer. For targeted combinatorial approach, Folate-conjugated siRNA polyplexes were developed based on sequence-defined oligomer platforms. *In vivo* and *in vitro* experiments validated the efficiency of these polyplexes in gene silencing, receptor-directed killing of cancer cells (Lee and Wagner, 2019). Synergistic combinations of oligonucleotides and drugs are the new edges in finding effective therapeutic approaches. To expound active combinations in triple negative breast cancer cell models, resourceful protocols to screen siRNA libraries were developed. For validation of effective transfection reagent for intracellular delivery of siRNA, lipid-grafted low molecular weight (1200Da) Polyethylenimines (PEIs) were used owing to its advantages (Thapa et al., 2019). Optimized DNA nanosuitcases was constructed as tractable DNA prisms that can encapsulate and selectively release siRNA upon recognizing an oligonucleotide trigger. It was designed with much adaptability of oligonucleotides, where it could be made to respond to an oligonucleotide trigger of interest like shRNA or miRNA, thus aiding dual therapeutic approaches (Bujold

et al., 2016). Dendrimers being nano sized and radially symmetric molecules have been used as a carrier in gene delivery systems. For specific gene delivery in head and neck squamous cell carcinomas (HNSCC), a fluorescently labeled folic acid-decorated polyamidoamine (PAMAM) dendrimer was conjugated with folic acid (FA) as targeting moiety. When linked with a plasmid or siRNA, this complex is known to significantly enhance the gene transfection or knockdown efficiency in mouse xenograft models (Xu and Yang, 2019). Trastuzumab (TZ) is a monoclonal antibody that binds explicitly to human epidermal growth factor 2 (HER2) receptors and is presently being used for the treatment of HER2-positive breast cancer. A targeted carrier system made up of trastuzumab-conjugated poly (amido) amine dendrimers demonstrated a strategy for site-specific carriage of siRNA to HER2-positive breast cancer cells, subsequently resulting in definite targeting without off-target effects (Kulhari et al., 2019). Higher levels of miRNAs in cancer cells are frequently related with oncogenic effects. Targeted delivery of synthetically modified antagomiR molecules to malignant myeloid cells and B cells by linking with single stranded phosphorothioated oligodeoxynucleotides (PSO), reported to persuasively reduce the levels of target miRNA. Therefore, the PSO-antagomiR has demonstrated its role, by virtue of regulating the expression of downstream protein targets, both *in vitro* and *in vivo* (Su et al., 2019). Synergistic approach by delivery of small interfering RNA (siRNA) and chemotherapeutic agent (cisplatin) for Human antigen R (HuR) mRNA in cancer cells by means of a polyamidoamine (PAMAM) dendrimer, was developed. *In vitro* results established that this strategy was effective in lung cancer cell lines H1299 and A549, along with reduced toxicity in normal lung fibroblast MRC9 cells (Shivdasani, 2006). During invasion and metastasis of cancer from a primary organ, patients have to undergo treatment with anti-tumor drugs by means of systemic administration, instead of local injection. Unfortunately, when oligonucleotides are administered, they tend to be degraded by ribonucleases in the blood leading to poor accumulation of the effector molecules in target sites. To overcome this, double-stranded RNA/DNA chimera (dsRDC) was used as a strategy instead of small interfering RNAs in *in vivo* breast cancer models. This kind of chimera sidesteps off-target properties leading to the formation of RISC complex by the sense strand, thereby conferring more durability and stability in bloodstream (Taniguchi and Imai, 2019). DNA nanostructures offer a simple yet powerful technique for the self-assembly of designed oligonucleotides into them. Multivalent DNA nanostructures consisting of unmethylated CpG motifs were prepared and tested for their ability to withstand nuclease degradation, stability, and transfection efficiency. Studies demonstrated that CpG motifs are recognized by toll-like receptors of the cell, proving their specificity and high potential in drug targeting (Li et al., 2011). Engineered gold nano-shell based oligonucleotides were therapeutically designed to discharge its cargo on mandate upon illumination with near-infrared laser irradiation at 800 nm. A fluorescently labeled Green fluorescent protein (GFP) expressing human lung cancer

H1299 cell line *in vitro*, was used to determine the cellular uptake, controlled release, and gene silencing induced by the desired siRNA (Huschka et al., 2012).

Peptide and Cell-Based Delivery Systems

Peptide-based nanoparticles form a stable complex of proteins and nucleic acids. These nanoparticles facilitate efficient delivery of cargo into cells highlighting the importance of cellular uptake, independent of the endosomal pathway. Cell-penetrating peptides initiate electrostatic interactions with proteoglycans, allowing modification of actin network in the extracellular matrix, thereby facilitating the uptake of nanoparticles (Crombez et al., 2008). In one study, a morpholino based oligonucleotide was constructed and linked to a tumor-targeting RGD peptide to form oligoconjugates. These were further bound to a single molecule of human serum albumin. The resultant nanoconjugate showed a greater enhancement in receptor-specific intracellular delivery of oligonucleotides and markedly boosting the functional activity at low nanomolar concentrations, overcoming the limitations of cytotoxicity (Ming et al., 2013). The delivery of siRNA through modular L1, a peptide carrier bearing CXCR4 targeting ligand was evaluated for its ability to condense siRNA, mediating endosomal escape. This resulted in forming complexes with siRNA to provide efficient VEGFA gene knockdown by substantial reduction of VEGFA gene expression in EA.hy 926 endothelial cells and in A172 glioblastoma cells (Egorova et al., 2019). Biodegradable nanoparticles developed from poly D, L-lactide-co-glycolide (PLGA) were studied for their localization, release, and delivery of therapeutic proteins, plasmid DNA, and small molecules suggesting that they are efficient in overcoming the endo-lysosomal compartment, thus moving into the cytosol after administration (Panyam and Labhasetwar, 2012). An innovative biomolecule compound Atelocollagen was developed to deliver tumor-specific siRNAs in nude mice. Atelocollagen being positively charged forms a complex with siRNA by electrostatic interactions. In orthotopic tumor models of prostate cancer in nude mice, this complex overcame degradation by nucleases and was proficiently transported in the bloodstream to the target tumor tissues, where the siRNA/atelocollagen complex integrated through endocytosis, promoting anti-metastasis and tumor regression (Takei, 2019). The usage of cationic polymer Polyethylenimine-Polycaprolactone-Polyethylene glycol (PEI-PCL-PEG) showed increased efficiency as it formed a superior complex with nucleic acids and thus proved better for gene delivery. Moreover, this polymer permits targeting moieties to be coupled to the micelleplex, the overexpressed receptors found within tumors. With this approach, indium labeled siRNA encapsulated with PEI-PCL-PEG nanoparticles and folic acid targeting ligand was developed for their evaluation in *in vivo* tumor targeting in orthotopic ovarian cancer models (Feldmann et al., 2019). Bacterial minicells, nanosized enucleated cells, have been exploited as a productive delivery method for nucleotide based therapeutics. Minicells retain all of the molecular components of the parent cell, except chromosome, lacking the ability to divide. Due to numerous advantages like convenience, efficient packaging of si/shRNA, stability, and easy surface modification with antibodies/ligand for active targeting, they can be manifested selectively to

tumor cell-surface receptors with any targeting moieties conjugated on minicells. The functionality of minicells was observed *in vivo* in xenograft mice tumor models and in *in vitro* selected cancer cell lines (Jivrajani and Nivsarkar, 2019).

RNA INTERFERENCE AND NANOTECHNOLOGY IN PRE-CLINICAL STUDIES: AN INSIGHT INTO BOTH *IN VITRO* AND *IN VIVO* METHODS IN DIFFERENT CANCERS

The detailed understanding of molecular targets underlying cancer has paved way for personalized therapy, overcoming the intrinsic complexity and asymptomatic nature of various cancers. With an ability to interfere with the function of specific molecular targets, targeted therapy controverts the conventional and empirical approach of developing cytotoxic chemotherapeutics. The past decades have witnessed the manipulation of diverse molecules like tyrosine inhibitors, serine/threonine inhibitors, small molecule drug conjugates, and monoclonal antibodies into developing targeted therapeutics with anti-cancer activity. Although these molecules were effective compared to the conventional methods, researchers found it challenging to circumvent the challenges related to protein durability. This is where RNA interference interceded and revolutionized targeted therapies. With an uncanny ability to target cancer-related genes, the RNAi pathway employs non-coding RNAs to bind messenger RNAs and silence gene expression in eukaryotic cells. Although profuse alternatives for gene silencing exist like CRISPR/Cas and TALENs, RNA interference remains the most favored option due to its precise functional mechanism, high potentiality, high specificity of gene silencing, and scanty side effects (Mansoori et al., 2014). The emergence of RNAi-based cancer therapies has materialized the usage of nanoscale particles as delivery molecules. With an enhanced ability to accumulate in tumor cells compared to normal cells due to the EPR effect, nanoparticles protect the RNAi molecules from undergoing enzymatic degradation and prevent them from being recognized by the immune system, thereby showing higher transportation efficiency compared to other carriers (Xin et al., 2017). However, a detailed preclinical characterization of the nano molecule and studies on the mode of offloading the loaded non-coding RNAs by these carrier particles have to be analyzed both *in vitro* and *in vivo* before taking these molecules to clinical trials. Evaluation of rate, extent, and perpetuation of silencing and the time-frame of delivery of the nanocarriers to the cancer niche is crucial in preclinical studies and the information generated enhances the chance of success in clinical trials (Wu et al., 2014). Almost every cancer tissue with a prominent biomarker and a potential to regress after gene silencing has been targeted for RNAi-based anti-cancer therapy. We are summarizing the results of pre-clinical obtained with nanoparticle-RNAi system in each type of cancer.

Breast cancer

Advancement in gene therapy has accentuated the potentiality of RNAi molecules in breast cancer therapy over recent years. Lu

Han *et al.* targeted HOX transcript antisense intergenic RNA (HOTAIR) by using specific siRNAs and by transfecting it into three human breast cancer cell lines (MCF-7, MDA-MB-231, and SKBR-3) resulting in repressed proliferation, invasion, and migration of cancer cells (Han *et al.*, 2018). Studies have analyzed the interaction between miR-145 and Hepatitis B virus X-Interacting Protein (HBXIP), an oncoprotein promoting breast cancer progression and metastasis. Anti-miR-145 and si-HBXIP upon transfection revealed that miR-145 directly targets HBXIP and is a useful therapeutic target (Jiang *et al.*, 2019). In order to knock down the expression of Ubiquitin-associated protein 2-like (UBAP2L), an oncogene associated with various cancers like prostate cancer, colorectal cancer, breast cancer, and glioma, shRNAs targeting the same has been transfected into breast cancer cell lines like ZR-75-30 and T-47D, successfully regressing the tumor (He *et al.*, 2018). Other than these, Ca^{+2} -dependent phospholipid and membrane-binding proteins, Annexin A3 (ANXA3), underwent gene knockdown in MDA-MB-231 cells with the help of sh-ANXA3 containing lentiviral vectors leading to inhibition of cell proliferation, migration, and invasion (Zhou *et al.*, 2017).

A large intergenic non-coding RNA-regulator of reprogramming (lincRNA-ROR) is a newly identified lncRNA associated with initiation, development, and metastasis of multiple tumors, including breast cancer. This lincRNA-ROR has been shown to enhance the resistance of radio- and chemotherapy of cancer cells along with the ability to inhibit the gemcitabine (Gem)-induced apoptosis and autophagy of breast cancer cells (Takahashi *et al.*, 2014; Chen *et al.*, 2016a; Yang *et al.*, 2017a). Even after tamoxifen (TAM) has been clinically proven to reduce the risk of development of breast cancer, its resistance towards therapy is still a drawback. Thus, shRNA targeting lincRNA-ROR has been transfected into MDA-MB-321 BC cells. This showed decreased cell proliferation and increased TAM sensitivity by reducing its resistance (Lu *et al.*, 2019). TAM is also found to promote apoptosis by suppressing the activation of the P13K/Akt/mTOR signaling pathway. The PRDI-BF1 and RIZ (PR) domain zinc finger protein 14 (PRDM14) is found to be overexpressed mostly in three out of five breast cancer patients, out of which some exhibit gene amplification. siRNA targeted against PRDM14 in nude mice successfully reduced size of breast tumors and lung metastases (Taniguchi and Imai, 2019).

Colon cancer

Previous studies have revealed the hindrance of colon cancer proliferation and invasion *in vitro* via the down-regulation of TSPAN1 expression (Chen *et al.*, 2010). Enacting the role of a potential prognostic factor in colorectal carcinoma, TSPAN1, through its inhibition by miRNA mediated RNAi, paved the way to the deduction of miR-638 as a tumor suppressor miRNA (Zhang *et al.*, 2014). Similarly, a knock-down study on *s100p* gene in colon cancer cells using lentivirus-mediated RNAi, indicated significant reduction of tumor growth and liver metastasis *in vivo* (Jiang *et al.*, 2011). Via in-silico screening, forced overexpression of miR-124 in colon cancer cells reduced Collagen prolyl-4-hydroxylase α subunit 1 (P4HA1) expression and subsided its malignant phenotype. The malignant phenotype was also

diminished by treating the cancer cells with diethyl-pythiDC, a small molecule inhibitor of P4HA1 (Agarwal *et al.*, 2019). Colo-320 cells were transfected with two complementary pairs of short-hairpin siRNA oligomers specific to human BRCP/ABCG2 cDNA sequence which led to a significant reduction in protein expression and chemoresistance (Hu *et al.*, 2017).

Colon cancer-associated transcript 2 (CCAT2) is an lncRNA which is found to be highly overexpressed in microsatellite-stable colorectal cancer. A meta-analysis on the same revealed that the overexpression of CCAT2 gene can be used as a novel prognostic factor in several cancers (Fan *et al.*, 2017). Thus, shRNAi plasmids targeting CCAT2 have been transfected into human gastric cancer BGC-823 cell lines. The corresponding silencing of the CCAT2 gene reduced the tumor cell proliferation and promoted apoptosis and autophagy in BGC-823 cells (Yu *et al.*, 2018). The regulatory interlink between HOTAIR and insulin like growth factor 2 mRNA-binding protein 2 (IGF2BP2) has been shown to influence the invasion and migration of colon cancer LoVo cells (Wu *et al.*, 2019). The lncRNA, upon targeting and silencing HOTAIR, could significantly lower the proliferation of tumor cells as well as promote apoptosis through suppression of IGF2BP2 and Epithelial to Mesenchymal Transition (EMT). LINC01234, a highly abundant mammalian noncoding RNA has been proven to be a novel molecule in the field of tumor biology with associations with almost all types of cancers like breast, gastric, ovarian and colon cancers (Guo *et al.*, 2016; Gu *et al.*, 2017; Guo *et al.*, 2017). Serine hydroxymethyltransferase 2 (SHMT2) is an enzyme regulating the serine/glycine metabolism pathway, a pathway identified to be critical in tumor metabolism since they have high energy and anabolic consumption capability. shRNA interference vectors targeting LINC01234 and SHMT2 were injected into BALB/c nude mice which had already been transplanted with tumor through subcutaneous injections of LoVo and HCT119 cell lines. Knockdown of LINC01234 arrested serine/glycine metabolism pathway thereby suppressing the cell proliferation and invasion (Lin *et al.*, 2019a). Furthermore, LINC01234 was identified to be functioning as a competitive endogenous RNA (ceRNA) for miR-642a-5p, thus implying that LINC01234-miR642a-5p-SHMT2 axis plays a critical role in colon cancer proliferation.

Nuclear paraspeckle assembly transcript 1 (NEAT1), one of the nuclear lncRNAs is found to be associated with the deteriorated prognosis of colorectal cancer (CRC). siRNA and antisense oligonucleotides targeting NEAT1 as well as knockdown of miR-193a-3p modulated KRAS proteins, thus providing a therapeutic and diagnostic marker for CRC (Zhu *et al.*, 2019). Similarly, the function of Tripartite Motif Containing 25 (TRIM29) was explored in colorectal cancer cells by transfecting HT-29 and SW1116 cells with si-TRIM29. This knockdown reduced tumor cell proliferation, migration and invasion followed by reducing the phosphorylation levels of JAK2 and STAT3, which are the regulators of JAK/STAT signaling pathway (Xu *et al.*, 2016).

A novel oncogenic lncRNA, Sprouty4-Intron 1 (SPRY4-IT1) is found to be upregulated in multiple cancers regulating cell growth, invasion and apoptosis (Li *et al.*, 2017a). To knockdown its expression, CRC (LoVo and SW480) cell lines

were transfected with siRNA sequences specific to SPRY4-IT1 (Shen et al., 2017). Thus, cell proliferation, migration and invasion of CRC cells were suppressed via EMT-related gene modulation by negatively regulating the expression of miR-101-3p (target of SPRY4-IT1).

Cervical cancer and Prostate cancer

Cervical cancer is one among the most common types of cancer found in women worldwide. Several non-coding RNAs have been associated with cervical cancer and a few of them were taken forward for preclinical analysis. In 2012, Gibb et al. found the abnormal expression of growth arrest specific 5 (GAS5) lncRNA by performing serial analysis of gene expression (SAGE) in cervical cancer (Gibb et al., 2012). In order to downregulate GAS5, RNAi sequence targeting this lncRNA was transfected into cervical cancer HeLa cell lines using liposome INTERFERin and its regulatory role was unraveled (Li et al., 2018a). Mitogen-activated protein kinase 1 (MAPK1), identified as a direct target of miR-329-3p, is upregulated in cervical cancer tissues and found to depict an inverse correlation with miR-329-3p expression. The knockdown of MAPK1 by transfecting human cervical cancer cell lines with si-MAPK1 significantly inhibited tumor cell growth, migration and invasion (Li et al., 2017b). These results were similar to the results obtained by overexpression of miR-329-3p inferring its therapeutic ability in cervical cancer.

Diverse cell-penetrating peptides (CPPs) have been in use to deliver anti-VEGF siRNA into cancer cells (Choi et al., 2010; Kanazawa et al., 2012; Egorova et al., 2016; Chung et al., 2017). With inadequate cell-type specificity being a major drawback, CPPs like BR2 specific to cancer cells have been reported (Lim et al., 2013). An amalgamation of siVEGF and BR2, when delivered intracellularly in HeLa cell lines, down regulated VEGF levels notably reducing toxicity, increasing antitumor efficacy indicated a specific and efficient delivery of siRNA by BR2 CPP (Lee et al., 2018).

Prostate cancer (PCa) is the most prevalent visceral cancer in men worldwide (Chen et al., 2015). Special AT-rich sequence-binding protein 1 (SATB1), a transcriptional factor is found to be overexpressed in various types of malignant cancers like nasopharyngeal carcinoma, cutaneous malignant melanoma, osteosarcoma and small cell lung cancer (Chen et al., 2014a; Hou et al., 2016; Lee and Pelletier, 2016; Xiong et al., 2017a). Three siRNAs targeting SATB1 region containing nucleotides 2,147–2,185 of complementary DNA were synthesized and were transfected into prostate cancer cell line DU145 using siPORT lipid transfection reagent (Mi et al., 2016). Tumor cell growth, invasion and migration capabilities were significantly inhibited *in vitro* implying that SATB1 siRNA could be a potential agent for human PCa therapy. Playing a major role in Ca^{+2} homeostasis, transient receptor potential cation channel subfamily M member 8 (TRPM8) has also emerged as a potential therapeutic target in PCa (Fidaleo et al., 2015), (Cao et al., 2017). The specific siRNA complementary to TRPM8 complexed with Lipofectamine 2000 was transfected into prostate cancer (LNCaP) cells thus, inhibiting cell proliferation and enhancing epirubicin chemosensitivity of cancer cells via promoting apoptosis (Chen et al., 2012). Prostate stem cell antigen (PSCA) is a

glycosylphosphatidylinositol (GPI)-anchored cell membrane glycoprotein which is highly expressed in prostate cancer (Gu et al., 2000). Prior study by Zhigang Zhao and team produced *in vitro* validation that 21-nt long siRNA targeting PSCA could significantly inhibit cell proliferation and invasion of human PCa (PC-3M) cells (Zhao et al., 2016). The same group of researchers in the pursuit of validating *in vivo*, injected si-PSCA subcutaneously into male SCID mice bearing PCa xenografts (Leconet et al., 2018). The expression of PSCA was completely shut down as shown by immunohistochemical analysis as well as the reduced tumor growth volumes and metastasis onset. As ZEB1 was overexpressed in malignant cancers like prostate cancer (Heinrich et al., 2018), when the antisense lncRNA ZEB1-AS1 was expressed it positively regulated ZEB1 expression promoting tumor growth and metastasis via functioning as an oncogene in primary liver cancer (Huang et al., 2020). Based on these observations when two different siRNA vectors targeting ZEB1-AS1 were transfected into prostate cancer cell lines they inhibited ZEB1 overexpression and thereby the cell proliferation (Su et al., 2017).

Lung cancer

In 2019, Fei Cao et al., reported that targeting epidermal growth factor receptor (EGFR) mutations by RNAi can possibly treat residual lung cancer. PEG-PEI-EGFR siRNA nanocomposite was created by conjugating PEG-PEI polymer with siRNA targeting EGFR. This when injected intratumorally significantly reduced cell proliferation and invasion of tumor cells in BALB/c nude mice which had been subcutaneously injected with HCC827 lung cancer cells, subsequently rejecting the human lung xenograft model in mice (Cao et al., 2019). Growth arrest-specific 5 (GAS5) is known to be a vital tumor suppressor lncRNA in some types of cancers. NSCLC cells were transfected with plasmid vectors of GAS5 siRNA sequences. This study determined that miR-205 was a direct target of GAS5 in lung cancer and the Phosphatase and Tensin homologue (PTEN) was a direct target of miR-205. Upregulation of GAS5 aided in the suppression of tumor growth and invasion via miR-205/PTEN axis (Dong et al., 2019). On the contrary to this, X inactive-specific transcript (XIST) is known to function as an oncogenic lncRNA in bladder cancer, CRC and NSCLC (Fang et al., 2016; Xiong et al., 2017b; Chen et al., 2017). In order to unzip the correlation between XIST and TGF- β induced EMT, siRNA sequences targeting XIST and zinc finger E-box-binding protein (ZEB2) were transfected into human NSCLC (A549) cells using Lipofectamine 2000 transfection reagent. The knockdown of XIST repressed ZEB2 expression thereby inhibiting TGF- β induced EMT, cell proliferation and invasion. In addition to *in vitro* validation, the sh-XIST sequences were intravenously injected into female nude mice along with stable A549 cells. The decreased pulmonary metastasis observed from metastatic nodules on the lung surface revealed the role of XIST and ZEB2 in promoting EMT (Li et al., 2018b).

Programmed death receptor 1 (PD-1), being a specific programmed death receptor ligand 1 (PD-L1), plays a major role in immune escape by malignant tumor cells and is found to be overexpressed in most of the cancer types. The antitumor

effects of dendritic cell (DC) immunization have been observed to be inhibited by the signaling functions of PD-1/PD-L1. The shutdown of the PD-L1 expression by lentivirus-mediated RNAi in SCID-hu mice injected with human pancreatic cancer PaTu8988 cells (Wang et al., 2019), reduced the tumor growth and lung metastasis thereby increasing the survival time of SCID-hu mice. Staphylococcal nuclease domain-containing protein 1 (SND1), also termed as P100 is a major component of RISC complex which regulates gene expression at both transcriptional and translational levels. Various studies have revealed the interaction of P100 with c-Myc, STAT5 and STAT6 (Dash et al., 1996; Yang et al., 2002; Välineva et al., 2005). Anna et al (year) reported the upregulation and chemoresistant characteristics of P100 in lung cancer (Zagryazhskaya et al., 2015). So, when miR-320a targeting P100 was transfected into human lung cancer cell lines, it inhibited cell migration, thus potentiating the biomarker effects in prognosis of lung cancer (Xing et al., 2018). Cancer cells require enhanced lipid biosynthesis since the fatty acids and phospholipids supplied by the gut and the liver alone are insufficient when compared to the consequent increase in the expression and activity of crucial enzymes like fatty acid synthase (FASN). Ning Zhan and his team constructed a lentiviral vector containing shRNA complementary to FASN and then transfected it into human non-small cell lung cancer (A549) cells making use of Lipofectamine 2000 as transfection reagent (Zhan et al., 2018). They observed that the FASN knockdown increased the radiosensitivity of NSCLC cells by effectively promoting apoptosis.

Aquaporins (AQP) which are integral membrane proteins facilitating water transport between cells are known to be prominently abundant in diverse malignant tumors, promoting cell proliferation and angiogenesis (Hu et al., 2006), (Verkman et al., 2008). In particular, AQP3 has been reported to be regulating cancer cell growth, invasion and migration (Lieberman, 2018), (Christopher et al., 2016) and hence inhibition of it can decrease the proliferation of NSCLC cells (Chen et al., 2015). So, two siRNA oligonucleotides targeting AQP3 were transfected into lung cancer (XWLC-05) cells and nude mice were subcutaneously inoculated with lung cancer cells transfected with si-AQP3. Cellular experiments revealed the inhibition of cell proliferation and invasion promoting apoptosis whereas reduced tumor growth was observed in *in vivo* studies (Xiong et al., 2017a). Lung cancer (A549) cell lines were transfected with four siRNA oligonucleotides cloned into lentiviral expression vectors to silence RNA helicase (DHX9), a protein overexpressed in a wide variety of cancer (Fidaleo et al., 2015; Lee and Pelletier, 2016; Mi et al., 2016). Subsequently using enoxacin to inhibit cell proliferation, this particular knockdown of DHX9 completely suppressed lung cancer cell growth (Cao et al., 2017). The combined effect of RNAi and tyrosine kinase inhibitors (TKIs) by targeting EGFR in NSCLC cells and subsequently silencing the gene in the cancer has also been reported (Chen et al., 2012).

Gastric cancer

Being rarely diagnosed at early stages, gastric cancer (GC), though could be treated using surgery, radiation, and chemotherapy at its

earlier stages is very unresponsive to the same at its advanced and metastatic stage. The use of RNA interference has been thus emphasized in gastric cancer treatment to enhance both diagnosis and prognosis. The MYCL1 being a proto-oncogene, has been found to be upregulated in most of the gastric cancer cells. The usage of lentiviral MYCL1 shRNA expression vectors to knockdown the overexpression of this gene reduced migration and invasion of MGC-803 cell line *in vitro* (Qin et al., 2019). This effect of MYCL1 gene on migration phenotype can be exploited to enhance the prognosis rate of gastric cancer patients. Plasmacytoma variant translocation 1 (PVT1), has been reported to be a potential lncRNA biomarker for Gastric cancer patients. The shRNA targeting lncRNA PVT1 when transfected into BGC823 and AGS cells using lipofectamine, the expression of c-Myc was reduced which in turn decreased migration and invasion ability of tumor cells (Ren et al., 2019).

Methyltransferase-like 3 (METTL3) is known not only for N6-methyladenosine (m6A) modification of mRNA but also for regulating the translation of oncogenes. Human gastric cancer cell lines, AGS and MKN45 when transfected by shRNA targeting METTL3, it significantly decreased Bcl2 and increased Bax and active Caspase-3 implying activation on the apoptotic pathway. Thus, downregulation of METTL3 suppressed the proliferation and invasion of human gastric cancer cells (Lin et al., 2019b). Many researchers have reported the role of lncRNA LINC00978 in lung cancer, breast cancer and gastric cancer (Yang et al., 2015; Ke et al., 2017; Fu et al., 2018). In order to understand the biological function of this lncRNA in gastric cancer, Min Fu and his colleagues targeted the lncRNA using its specific shRNA in human gastric cancer (MGC-803 and SGC-7901) cell lines using LipoFiter as transfection reagent. The knockdown of this overexpressed lncRNA induced apoptosis by suppressing cell cycle progression. Also, since the migration and invasion of GC cells were found to be inhibited, LINC00978 could serve as a prognostic biomarker for gastric cancers (Fu et al., 2018).

Another lncRNA involved in cell proliferation, apoptosis and tumor development in liver and gastric cancer is small nucleolar RNA host gene 15 (SNHG15) (Zhang et al., 2016). The knockdown of this gene, in human NSCLC (A549) cells using Lipofectamine 2000, using specific siRNA oligonucleotides significantly suppressed cell proliferation, invasion and metastatic abilities of cancer cells thereby initiating apoptosis (Chen et al., 2016b). In order to investigate the role of TRIM25 in gastric cancer, two different siRNA oligonucleotides specific to TRIM25 were transfected into GC cell lines (MGC-803 and AGS). This silencing of TRIM25 expression decreased cell migration and invasion by regulating TGF- β signaling pathway (Dong et al., 2018). Being a transmembrane protein, the IQ motif containing GTPase-activating protein three gene (IQGAP3) is known to be upregulated in gastric cancer cells and could serve as therapeutic target (Yasui et al., 2011). So, the siRNA sequences targeting IQGAP3 were conjugated with Lipofectamine RNAi-MAX and then transfected into human GC (MKN-1) cells. Performing spheroid colony formation assay elucidated that both the number and size of the spheres formed by GC cells were remarkably reduced (Oue et al., 2018). Another transmembrane glycoprotein shown to be overexpressed in GC

is liver-intestine cadherin (CDH17). Lentiviral-mediated RNAi performed by transfecting human GC (MKN28) cells with shRNA oligonucleotides specific to CDH17, promoted apoptosis by notably inhibiting cell proliferation of GC cells. Furthermore, subcutaneously injected GC mice xenografts showed significant lessening of tumor growth and volume thereby restricting tumorigenicity (Su et al., 2008).

Other Cancers

First ever trials of RNAi in humans were focused on assessing the potentiality and activity of lipid nanoparticles (LNPs) formulated with siRNAs targeting VEGF and kinesin spindle protein (KSP) in liver (Tabernero et al., 2013). With this, not only intravenous administration of ALN-VSP liver was shown to be effective and safe but also complete regression of liver metastases was observed in endometrial cancer. Endometrial cancer (EC) being the most common malignant female genital cancer in the world, one of the candidate biomarkers of EC, chitinase-3-like protein 1 (YKL-40) is overexpressed in patients diagnosed (Fan et al., 2013) has a pivotal role in cancer cell proliferation (Bröchner et al., 2012), angiogenesis (Francescone et al., 2011) and anti-apoptosis (Lee et al., 2009). In order to silence the expression of YKL-40, siRNA oligomers targeting it were first cloned into retroviral vectors and then transfected into EC (HEC-1A) cells using Lipofectamine 2000 followed by lentiviral-mediated transduction (Li et al., 2018c). Tumor cell attributes like cell proliferation, migration and invasion were found to be inhibited consequently increasing the mean cellular apoptotic rate. One of the oncogenic lncRNAs, HOTAIR, has been reported to be mediating chemoresistance to cancer cells (Liu et al., 2013). In order to ascertain the role of this lncRNA in endometrial cancer, the Ishikawa human EC cell line along with cisplatin-resistant clones were transfected with three si-HOTAIRs with the help of Lipofectamine 2000 as transfection reagent (Sun et al., 2017). This knockdown of HOTAIR inhibited the cell proliferation of EC cells thereby decreasing their cisplatin-induced chemoresistance.

The oncogenic transmembrane glycoprotein MUC1, being overexpressed in several tumors, upon silencing *via* RNAi inhibited the growth of Hepatocellular carcinoma cells indicating its crucial role in tumorigenicity (Li et al., 2014). The following study performed by the same group of researchers indicated that MUC1 initiates the autocrine TGF- β signaling in HCC cells by activating JNK/AP-1 pathway (Li et al., 2015). JNK, being a member of the MAPK family, has been displaying its role in HCC development and progression (Ozpolat et al., 2010). In order to collectively target both MUC1 and JNK, this team (Wang et al., 2017) firstly transfected HCC (SMMC-7721) cells with si-MUC1 and si-JNK1/2 or SP600125 (inhibitor of JNK) separately complexed with X-fect transfection reagent. Cell viability of MUC1-transfected cells was decreased and showed similar results with si-JNK and its inhibitor. Since unmodified siRNA is not stable in bloodstream and does not get transported through cell membranes (Whitehead et al., 2009), both the si-MUC1 and si-JNK were chemically modified by conjugating with 5'-cholesterol and 2'-O-methyl groups and then intratumorally injected into tumor bearing mice which in fact did not show variable results compared with negative control.

Thus, a more effective delivery method has to be innovated or developed to inhibit JNK/AP-1 pathway.

Most of the human malignancies which have epithelial origin of cells are associated with overexpressed EGFR. So, shRNA targeted against EPS8 (EGFR pathway substrate 8) conjugated with polyamidoamine dendrimers through receptor-mediated delivery has been utilized to assess the phenotype of head and neck squamous carcinoma cells (Yuan et al., 2019). This showed that it inhibited cell growth, proliferation, migration and malignancy of tumor cells. Signal peptidase complex 18 (SPC18), encoded by SEC11A gene has been reported to induce the secretion of transforming growth factor (TGF- α), which phosphorylates EGFR and stimulate pathways involved in cell proliferation and anti-apoptosis. So, siRNA targeting SEC11A was transfected into human bladder cancer (T24 and KMBC-2) cell lines using Lipofectamine RNAi-MAX. The knockdown of SEC11A prominently inhibited cell growth and invasiveness contrary to the forced expression of SEC11A (Shigematsu et al., 2019). Small Nucleolar RNA Host gene 7 (SNHG7), an lncRNA has been found to be overexpressed in bladder cancer tissues and cell lines. Thus, such overexpression has been downregulated by siRNA-mediated RNA interference. This downregulation not only had regulatory effect on Epithelial-Mesenchymal Transition (EMT) related markers but also inhibited cell proliferation and invasion thereby promoting apoptosis (Zhong et al., 2018; Katoch et al., 2021).

Bone cancer pain (BCP) has both the components: nociceptive and neuropathic. The nociceptive component is associated with the release of algogenic substances by tumor and thus plays a pivotal role in inducing cancer-related deaths. The neuromedin U receptor 2 (NMUR2) has been shown to be upregulated in rat models which were injected with walker 256 cells (Peng et al., 2019). Thus, siRNA targeted to silence NMUR2 successfully relieved pain by assessing paw withdrawal threshold (PWT) value.

Recent studies have shown that the hypoxia pathway is dysregulated in pancreatic cancer. Also, glucose transporter-1 being a transmembrane transporter is upregulated in many types of cancers and is responsible for transformation into malignant tumor. Folic acid receptor is overexpressed on almost all the malignant tumor cell surfaces since it is the essential vitamin for proliferative cells. The siRNA complementary to HIF-1 α which has been transfected with graphene oxide nanoparticles targeting folate receptor was injected intravenously into nude mouse tumor models with pancreatic cancer PaTu8988 cells (Wan et al., 2019). This shutdown of the expression of HIF-1 α and GLUT1 under hypoxic conditions significantly inhibited the proliferation and metastasis of tumor cells and thereby speeding up the apoptosis of tumor cells. In addition, hypoxic state of tumor tissues was reduced, which is usually higher in malignant tumor cells.

Tumor-derived exosomes (TEXs) are not only widely distributed in body fluids of cancer models but also have significant molecular level effects on immune cells like NK cells (Andre et al., 2002; Kim et al., 2007; Dai et al., 2008; Reiniers et al., 2014). In order to determine the mechanistic role of oral cancer-derived exosomes (OCEXs), Yang and his colleagues performed *in vitro* knockdown of NF- κ B-activating kinase-associated protein 1 (NAP1) using siRNA in human oral cancer (OC) cell lines using

Lipofectamine RNAi-MAX transfection reagent, and observed that OCEX derived NAP1 induces activation of interferon regulatory factor 3 (IRF-3). Thus, exosomal NAP1 enhances the tumor-suppressing functions of NK cells specifically cytotoxicity towards tumor cells and could be used as immunotherapy for treating oral cancer (Wang et al., 2018).

Research has shown that, Human antigen R (HuR) being the most ubiquitously expressed RNA-binding protein in eukaryotes is one of the key regulators in post-transcriptional gene expression mechanisms (Schwanhüsser et al., 2011) consequently causing several types of cancers like breast cancer, ovarian cancer and colon cancer (Govindaraju and Lee, 2013; Wang et al., 2013). Balden et al. demonstrated that HuR is overexpressed also in thyroid cancer (Balden et al., 2016) and to determine its biological activity, the siRNA targeting HuR were transfected into anaplastic thyroid cancer cell lines (SW1736 and 8505C) using Dharma-FECT 1 as transfection reagent and this, reduced colony forming ability (Allegrì et al., 2018). Apart from using the RNAi mechanism to silence HuR, SAHA an FDA approved drug was also used in mouse epidermal JB6 C141 cells.

Urinary bladder cancer (UBC) is another most common genitourinary malignancy worldwide. ASAP1-Intron 1 (ASAP1-IT1), is an intronic transcript of ASAP1 whose overexpression has been implied in cancer development (Guo et al., 2018). Human UBC cell lines were transfected with si-ASAP1-IT1 cloned into a lentiviral vector using Lipofectamine 3,000 transfection reagent thereby reducing stemness of UBC (Yang et al., 2017b). Also, Snail-1 is one of the crucial transcription factors in the process of regulation of EMT. Musavi Shenash et al. transfected EJ-138 bladder cancer cells with three different siRNA sequences targeting Snail-1 (Musavi Shenash et al., 2017). The expression of a few miRNAs involved in EMT such as miR-29b, -21 and -203 was shown to be reduced significantly by the knockdown of Snail-1. In addition, Snail-1 siRNA aided in induction of apoptosis as measured by the TUNEL test.

The human trophoblast cell surface antigen 2 (TROP2), is a cell surface glycoprotein and its overexpression in several cancers has been linked to tumorigenicity, poor survival and metastasis (Cubas et al., 2009). Though Chen et al. reported the association of TROP2 overexpression with poor prognosis in gall bladder carcinoma (GBC), mechanisms underlying proliferation and metastasis are still unknown (Chen et al., 2014b). To determine this, human GBC cell lines were transfected with shRNA targeting TROP2 conjugated with Lipofectamine 2000 reagent. In addition to this, BALB/c mice were subcutaneously injected with GBC cells transfected with sh-TROP2. The *in vitro* results confirmed the inhibition of cell proliferation, invasion and migration of GBC cells whereas, *in vivo* studies showed the postponed EMT of cancer cells analyzed by immunohistochemistry (Li et al., 2017c).

RNAi AND NANOTECHNOLOGY-BASED CLINICAL TRIALS: WHERE DO WE STAND?

In a world where the development of new drugs is painfully slow and the pipeline of new therapeutics frightfully thin, the discovery of RNA interference has paved way for the development of

relatively rapid approaches towards cancer drug design. The transition from preclinical work to clinical trials requires a novel therapeutic that shows sufficient promise to be effective and safe in treating human diseases. The fact that the number of nanocarriers that have reached the phase of clinical approval is meager and the high diversity of the nano platform makes concluding a particular nanoparticle to be “promising” is complicated (Mirkin et al., 2015). However, with the success and validation in *in vivo* models, various RNAi and nanoparticle-based molecules were taken for clinical trials in the past decade.

Approximately 25 different siRNA/shRNA molecules have been approved for clinical trials till now for anti-cancer therapy (Ozcan et al., 2015). Unlike other antisense therapies that have a stoichiometric effect on mRNA, siRNA's ability to reduce off-target toxicity along with its ability to act with increased potency, makes it a better contender than any other therapeutic molecules. These RNAi molecules can be transported to the patient's body *via* either natural carriers like bacteria or virus and using synthetic nanocarriers, the strategic use of these depends on the application, the required duration of the therapeutic, and the targeted tissue (Burnett et al., 2011; Chakraborty et al., 2019). Usually in clinical trials, the natural carriers are used to deliver to target cells *ex vivo*, which are then reinfused back inside the patient's body. These bacteria and viruses often possess shRNAs that will undergo intracellular modifications to become functional siRNAs. Synthetic carriers like nanoparticles are often delivered systemically through IV injections (Whitehead et al., 2009).

Being that most predominant molecules coming under the category of ‘FDA approved small RNA drugs’ to enter the paradigm of clinical application, nano-conjugated miRNA molecules have not still entered the United States government's website recording clinical trials. Several pharmaceutical and biotech companies have launched miRNA projects based on miRNA mimics and antagomiRs. Several companies like Dharmacon, BioSyn and GenScript tried to produce natural and chemically modified RNA, to stabilize and reduce the high reactive nature of RNA molecules. While some companies have shown some success, most of them are struggling to impact patient outcomes. For example, FDA halted the Phase1 clinical trial by MiRNA Therapeutics on miRNA mimic, MRX34, as a consequence of adverse side effects in patients caused due to lack of immune tolerance (Ling et al., 2017). MiRagen Therapeutics is actively developing MRG-106, an LNA antagomiR that targets miR-155 and is now in phase1 and phase2 clinical trials. Several companies work in preclinical and large screening studies to identify potential biologic miRNA such as CURNA program by Opko, Alnylam Pharmaceuticals, Interna Technologies and Mello Biotech (Bonneau et al., 2019). The first siRNA-based delivery system that was approved for clinical trials was that of the cyclodextrin polymer (CDP). Denoted as CALAA-01, this CDP polymer attached to polyethylene glycol (PEG), steric stabilization agent, and human transferrin targeted transferrin receptors that are usually found upregulated in cancer cells (Davis, 2009). Calando Pharmaceuticals (Pasadena, CA, United States) in 2010 conjugated siRNA targeting M2 subunit of ribonucleotide reductase to CALAA-01 regressing the tumor

growth (Davis et al., 2010). Though there was an intratumoral downregulation of ribonucleotide reductase, the fact that the trial took place only in a few patients made these results preliminary.

Liposomes, being a potential agent in transferring siRNA to the target tissue, many liposome conjugated siRNAs have been sanctioned by the FDA for clinical trials. With an ability to improve siRNA entrapment efficiency, 1,2-dioleoyl sn-glycero-3-phosphatidylcholine (DOPC) when used to encapsulate EphA2 siRNA showed promising results in orthotopic ovarian carcinoma. This conjugate was taken for Phase I clinical trial and is currently in the recruiting phase (Landen et al., 2005). Atu027 is a lipoplexed siRNA that can target the protein kinase N3. Constituting a positively charged AtuFect01, a neutral, fusogenicDPHyPE helper lipid and the PEGylated lipid MPEG-2000-DSPE (Schultheis et al., 2014), Atu027, due to its success in treating lymph node metastases in mouse models of the prostate, pancreatic cancer, and lung metastasis, was approved for clinical trials (Aleku et al., 2008). Silence Therapeutics (London, United Kingdom) conducted Phase I clinical trials and found that it is tolerable and does not show dose-dependent toxicity (Strumberg et al., 2012). The efficacy of Atu027 in clinical trials is also being tested with gemcitabine in metastatic and advanced pancreatic cancer patients.

Stable Nucleic Acid Lipid Particles (SNALP), due to their high bioavailability and enhanced permeability and retention, has become a notable carrier of siRNA in clinical trials. Found to be well tolerated at high dose, SNALP encapsulated version of siRNA targeting PLK1 was taken for Phase I trials by Tekmira Pharmaceuticals Corporation (Burnaby, BC, Canada). Moreover, SNALP was used to create the first dual-targeted siRNA drug. ALN-VSP02, developed by Alnylam Pharmaceuticals (Cambridge, MA, United States), targeted both VEGF and KSP and in Phase I trial was well tolerated at high doses in hepatic and extra hepatic tumor patients (Morrissey et al., 2005; Shen et al., 2012). The first dual-target nanoparticle-based RNAi showed promising results as one of the patients exhibited complete response, and the rest were stable for eight to twelve months of treatment (Mahmoodi Chahbatani et al., 2019). DCR-PHXC-101 is another lipid formulation that has entered the Phase I trial. It targets the transcription factor Myc and is showing metabolic response in patients with solid tumors, multiple myeloma, and lymphoma (Tolcher et al., 2015). Besides these, miniature biodegradable polymeric matrices were loaded with KRASG12D-targeting siRNA to target the K-Ras gene in pancreatic cancer patients. Phase I and II clinical trials on the same showed no evidence of cancer progression (Strand et al., 2019). A summary of the nano-conjugated siRNAs in clinical trials has been presented in the table below (Table 1).

CHALLENGES FACED BY RNAi AND NANOTECH-BASED THERAPEUTIC STRATEGIES

Despite RNA interference being a promising therapeutic strategy in cancer therapy, it faces a lot of hurdles during the process of

being recruited from bench to bedside. Potent and effective gene silencing in the tumor cells is mandatory for determining the efficiency of siRNA-based drugs in combating cancer. To achieve such effective gene knockdown *in vivo* and in clinical trials, efficient delivery of RNAi molecules to the target tissues is required (Petrocca and Lieberman, 2011). The efficient uptake of siRNA molecules by the cell is usually intercepted due to its high negative charge. These molecules need to survive in the extracellular matrices that constitute the RNA degrading endonucleases. Although endocytosis of these molecules is an elegant strategy to enter the cells, the hassle of releasing the RNA efficiently from the endosome and preventing it from undergoing endosomal degradation is unavoidable. This, along with the fact that siRNAs undergo rapid excretion via the renal system after intravenous injection, is what reduces the potency of naked siRNA-based therapy (Bora et al., 2012).

Another major obstacle to RNAi-based therapeutic strategy is intracellular delivery. This is primarily in the case of treating disseminating cancer cells that cannot be removed through surgery. To combat this, many biotechnological and pharmaceutical companies have come up with multiple delivery strategies. Chemical conjugation of siRNAs to specific molecules, being one of them, has, to an extent, increased the half-life of these molecules *in vivo* and has thus enhanced the potency of the same in the serum (Braasch et al., 2003; Chiu and Rana, 2003; Layzer et al., 2004). Modification of these nucleic acid molecules so as to have extended cholesterol, glycan, and folate branches that bind to cell receptors and incorporating these within nanocarriers, liposomes, and aptamers to do cell-specific targeting have shown promising results when induced in mice and non-human primates (Elmén et al., 2008). Nevertheless, the fact that only a few siRNAs among these have entered clinical trials and various companies are still working to resolve this RNAi-based molecule delivery problem shows that none of these current solutions are ideal, and the payoff for the same could be enormous.

Besides accomplishing target specificity, developing therapeutic strategies that give minor off-target effects is another major challenge (Jackson et al., 2003). The ultimate answer to such a problem would be to develop an efficient delivery system. Site specific delivery of siRNA, if appropriate, would reduce the number of normal cells exposed, favoring the uptake by cancerous cells and thus reducing the potential off-target effects. This would also minimize the dose to be administered, lowering potential dose-related detrimental side effects. Some of the siRNA molecules can also induce immunostimulatory effects. RNAi-based molecules can induce the innate immune system to release inflammatory cytokines, such as interferon- α , tumor necrosis factor- α , and interleukin-6, which may lead to considerable toxicity (Elbashir et al., 2002; Karikó et al., 2004; Hornung et al., 2005).

However, despite all these drawbacks, RNAi-based cancer therapeutics is still the best option available in bringing about apoptosis of highly malignant cells in a specific manner especially for the tumor cells resistant to conventional therapy. The development of personalized cancer therapies that combine monoclonal antibodies with RNAi has come up due to their

TABLE 1 | A summary of the nano-conjugated siRNAs in clinical trials.

Sl. No	Name	Type	Target	Company	Status	Reference Identifier number (clinicaltrials.gov)
1	CALAA-01	Cyclodextrin Polymer based nanoparticle	M2 subunit of ribonucleotide reductase in solid tumors	Calando Pharmaceuticals (Pasadena, CA, United States)	Completed	NCT00689065
2	EphA2-targeting DOPC-encapsulated siRNA	DOPC-liposome	EphA2 in advanced malignant solid neoplasm	M D Anderson cancer center, Houston, Texas, United States	Recruiting	NCT01591356
3	Atu027	Liposome	Protein kinase N3 in solid cancers and metastatic pancreatic cancer	Silence therapeutics (London, United Kingdom)	Completed	NCT00938574
4	TKM-PLK1	SNALP	PLK1 in solid tumors, lymphomas, adrenocortical carcinoma, liver cancer, and neuroendocrine tumors	Tekmira pharmaceuticals corporation (burnaby, BC, Canada)	Completed	NCT01808638 NCT01262235
5	ALN-VSP02	Lipid based NP	VEGF and KSP in solid tumors	Alnylam pharmaceuticals (cambridge, MA, United States)	Completed	NCT00882180
6	DCR-PHXC-101	Lipid based NP	Myc in solid tumors, multiple myeloma, non-hodgkin's lymphoma	Dicerna pharmaceuticals, Inc.	Terminated	NCT02110563
7	siG12D LODER	LODER polymer	KRAS in pancreatic cancer	Silenseed Ltd.	Recruited	NCT01676259

ability to complement each other's vulnerabilities (Schultheis et al., 2016). This is also considered more effective than using either alone. RNAi being a revolutionary tool to treat several diseases, through rational strategies that take into consideration all the cautionary drawbacks, could be developed into a powerful weapon that can combat cancer.

CONCLUSION AND FUTURE PROSPECTS

Arising from a complex interplay of oncogenes and tumor suppressor genes, cancer is a collective manifestation of deregulated gene functions. In spite of the innovation of various approaches of therapeutic modalities like chemotherapy, radiotherapy, and immunotherapy to combat cancer, the fact that these strategies have encountered numerous limitations compelled the scientific world to go on a quest for a better treatment paradigm. This is where RNA interference entered basic and clinical research, and revolutionized it. Termed as 'next generation therapeutic molecules', alongside recombinant protein and monoclonal antibody, RNAi has shown propitious results *in vitro* due to its high efficacy, and specificity. Despite its potential, several hurdles arise on the path of using RNAi *in vivo* due to its instability and short half-life in the serum. To overcome these problems, use of non-viral vector delivery systems that protect the RNAi molecules from degradation have been extensively explored. Drug delivery systems at the nanoscale have a promising future with the demonstrated superiority (by virtue of targeted approach, minimal collateral damage and accumulation at pharmacological levels at the desired site) compared to the traditional chemotherapy modalities. The addition of active targeting molecules to nanoparticles is found to enhance the tumor-targeting and transfection efficiency towards tumor cells. However, the use of nanoparticles too has its own limitations due to cytotoxicity, hurdle of delivery, and immunogenicity that prevents the reach of RNAi from bench to bedside. As constant progress is being made to achieve improved tumor targeting accuracy, interaction of these

nanoparticles with the immune system of the host remains an interesting current and future prospect (Zolnik et al., 2010; Bolkestein et al., 2016). The increased uptake of liposomal preparations in therapeutics by tumor cells has been attributed to certain defining features of malignancy such as increased vascular permeability, the microenvironment that is created by the neighboring stroma, the presence or absence of sustentacular cells, the lymphatic composition and mononuclear cell presence etc (Bolkestein et al., 2016). The interaction of chemical mediators such as bradykinin, nitrous oxide and physical factors (such as the smooth muscle in the blood vessel wall) in controlling nanoparticle movement and reach to the tumor site have been demonstrated in certain studies as the real hindrances in large scale adoption of nanoparticles as therapeutic vehicles (Stylianopoulos et al., 2012). These factors are fundamental to the enhanced permeability and retention effect (EPR effect) which holds great importance in nanoparticle and tumor biology (Stylianopoulos et al., 2012; Bolkestein et al., 2016; Golombek et al., 2018).

The immune status of the preclinical model appears to play a role in dictating tumor targeting efficacy of RNAi-nanoparticle therapeutics. Some factors which have been demonstrated to have an effect are opsonization (which is the process of making a target 'ready' for an immune system interaction), structural and electrochemical features of the nanoparticle and the duration of nanoparticle presence in the host (retention effect) (Zahednezhad et al., 2019). Mice models with deficient adaptive immune system were demonstrated to have low levels of neoplastic growth inhibition when the kinase inhibitor sorafenib was used, indicating the probable prerequisite of an intact adaptive immune status for the nanoparticles to function (Zhao et al., 2018). The interaction of nanoparticles with immune system components like the complement system and mononuclear phagocyte system has led to clinically observed effects such as CARPA (complement activation-related pseudoallergy) and increased clearance of therapeutic nanoparticles. The variability in antitumor efficacy with the use of CTLA-4 and PD-1 inhibitors (implying the immune

status) has led to the question of validity of preclinical models being used (La-Beck and Gabizon, 2017).

The extent of interaction between the immune system and nanoparticles drives the need to test these drug delivery systems in immuno-competent models and humanized mice models (which are often achieved by reconstituting SCID mice with human bone marrow introduction). As the immune system is complex, interaction with other subgroups such as the Th1 and Th2 systems needs to be studied further to have better outcomes (La-Beck and Gabizon, 2017). The gap between the pre-clinical and clinical stages of nanoparticle therapeutics needs to be bridged and the progress might be seen when hurdles such as clearance and toxicity of nanoparticles are circumvented and the use of humanized models is adopted for large scale studies (Rosenblum et al., 2018). With backing of the advantages they offer, this combinatorial therapeutics have the potential to be game changers in attacking cancer cells and tissues and are expected to yield revolutionizing clinical outcomes. Such a future is possible through constant improvement in the materials used in the nanoparticle drug delivery system and by moving towards an individualized and patient centric approach (Golombek et al., 2018), (Navya et al., 2019).

In spite of the remarkable progress made till now in terms of increasing the efficiency of nanobased RNAi delivery system with novel complexation or conjugation approaches, there are still specific challenges posed by cell types in different target organs to be resolved. Therefore, as an important strategy, RNAi therapeutics must be validated for varied targets to obviate this challenge. It would then be conceivable to promptly advance RNAi therapeutics innovatively and empirically to target different tissues and organs by fine tuning nano-based delivery system through experimental studies. Furthermore, the detailed molecular mechanism behind the RNA interference and its off-target effects are to be deciphered as the multi-targeting network of their pathways are often very complicated and

challenging. Considering the immense influence our understanding of RNAi regulatory network in neoplasia has in the development of RNAi based anti-cancer therapeutics, there is a compelling need to decipher such networks to place RNAi based therapeutics on a firm footing. Even though novel clinical studies on the same have ushered hope, there is a necessity for the development of more multi-centered, randomized, and placebo-controlled treatment methods that would be effective irrespective of the racial or ethnic variations among the patients. With the development of safe, biocompatible, and biodegradable targeted delivery systems for the clinical application and with proper knowledge on the RNAi networks, this promising alliance of nanotechnology and RNA interference is sure to transform anti-cancer therapeutic methods and interventions in future.

AUTHOR CONTRIBUTIONS

LDK has conceptualized the topic, critically reviewed, edited and rewritten many parts of the MS. GS and AS have written the major aspects of the manuscript. AK contributed the figure, introduction, conclusion and editing of the manuscript. VB has written the clinical aspects of the review, and VD contributed to the preclinical section of the manuscript.

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Encapsulating Anti-Parasite Benzimidazole Drugs into Lipid-Coated Calcium Phosphate Nanoparticles to Efficiently Induce Skin Cancer Cell Apoptosis

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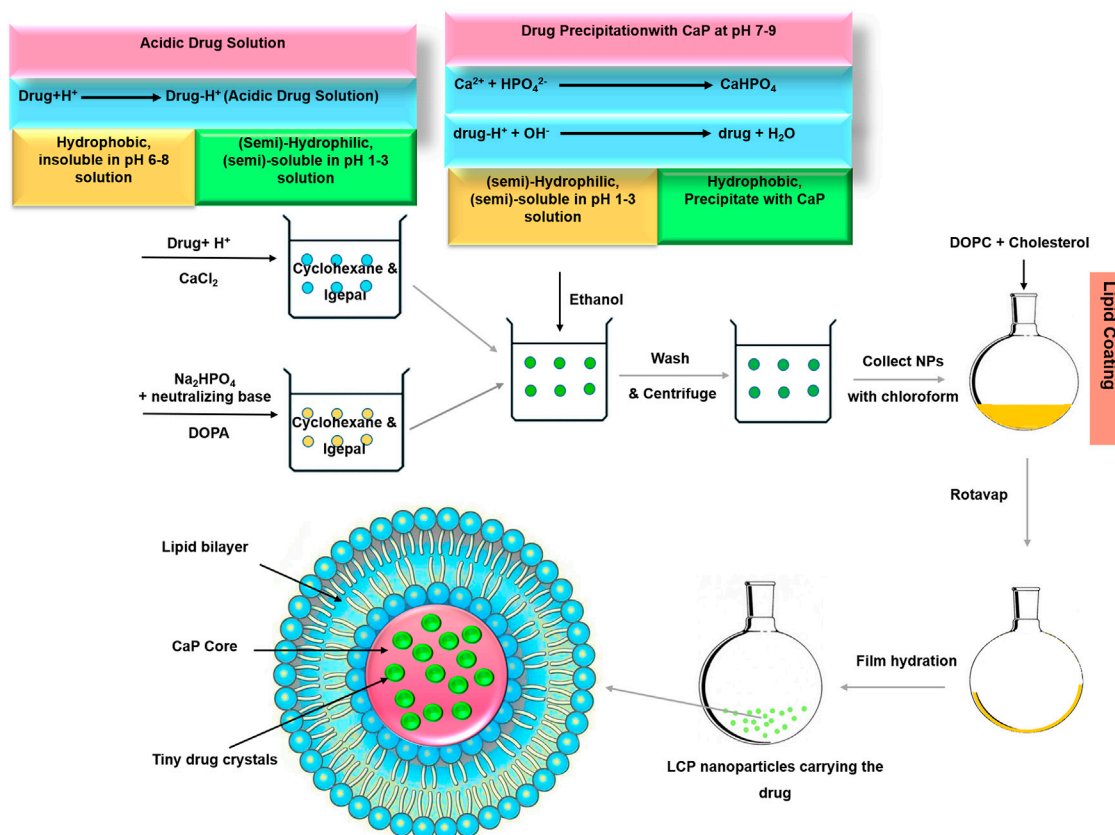
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Benzimidazole (BMZ) family of anti-worm drugs has been now repurposed as anti-cancer drugs. However, offering a general reformulation method for these drugs is essential due to their hydrophobicity and low aqueous solubility. In this work, we developed a general approach to load typical BMZ drugs as tiny nanocrystals within lipid-coated calcium phosphate (LCP) nanoparticles. BMZ drug-loaded LCP nanoparticles increased their solubility in PBS by 100–200% and significantly enhanced the anti-cancer efficacy in the treatment of B16F0 melanoma cells. These drug-LCP nanoparticles induced much more cancer cell apoptosis, generated much more reactive oxygen species (ROS) and inhibited Bcl-2 expression of cancer cells. Moreover, BMZ drug-loaded LCP nanoparticles caused morphological change and extension disruption of cancer cells, and significantly reduced migration activity, representing high possibility for inhibition of tumor dissemination and metastasis. Very advantageously, BMZ drug-loaded LCP nanoparticles did not show any obvious toxicity, Bcl-2 inhibition and morphological changes in HEK293T healthy cells. In conclusion, BMZ drug-incorporated LCP nanoformulations may be a valuable nanomedicine that is able to inhibit primary tumors and prevent tumor dissemination with minimum side effects on healthy cells and tissues.

Keywords: benzimidazole family of anti-worm drugs, lipid-coated calcium phosphate nanoparticles, enhanced solubility, enhanced anti-cancer toxicity, inhibition of cancer dissemination

INTRODUCTION

Benzimidazoles (BMZs) are a family of anti-helminth drugs widely used in humans and livestock since 1960s to treat parasitic infections (Stasiuk et al., 2019). Many members of this family including albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ) and thiabendazole (TBZ), are cost-effective FDA-approved drugs which are associated with very mild side effects (Carvalho and Gadelha, 2007). In recent years, many studies have reported the anti-cancer effect of these drugs on a broad range of cancers (Noorani et al., 2015; Castro et al., 2016). The new findings have a great importance not only because of offering cancer chemotherapeutics with minimum side effects but also due to the considerable reduction of Research and Development (R & D) and commercialising



SCHEME 1 | Schematic procedures for drug-loaded LCP nanoparticle preparation.

costs compared to the costs of developing new anti-cancer agents, a substantial challenge of global pharmaceutical industry at the moment (Pushpakom et al., 2018).

Because of various similarities between parasites and cancer cells (Dorosti et al., 2014), benzimidazole drugs are also potent to suppress cancer through anti-parasite mechanisms such as inhibiting tubulin polymerization, disrupting the formation of mitotic spindle, causing cell cycle arrest, and inducing apoptosis (Hasanpourghadi et al., 2016). However, the clinical anti-cancer application of these drugs is still limited due to low aqueous solubility and limited bioavailability (Daniel-Mwambete et al., 2004).

Providing a small size and a large surface area to volume ratio, nanoformulation has been considered as a promising approach for repositioning the poorly water-soluble drugs such as BMZs (Kumar et al., 2020) by improving their solubility, dissolution rate and bioavailability (Thorat and Dalvi, 2012). In addition, nanoparticles provide the possibility of drug penetration through the tight junctions of the skin and blood brain barrier as well as offering enhanced permeability and retention (EPR) effect, stimuli-responsive and targeted delivery (Kohane, 2007). Having the benefits of both calcium phosphate (CaP) nanoparticles and liposomes, lipid-coated calcium phosphate (LCP) nanoparticles are considered as ideal carriers for nanoformulation of BMZ drugs due to pH-responsiveness,

controlled size and the possibility for targeted delivery (Tang et al., 2015).

To demonstrate the capability of LCPs as benzimidazole drug carriers for the general cancer therapy, it is essential to examine apoptosis induction in tumor cells, inhibit resistance-mediating factors such as B-cell lymphoma 2 (Bcl-2), and reduce tumor cell dissemination. As schematically shown in Scheme 1, our new approach for preparing ABZ-loaded lipid-coated calcium phosphate (LCP) nanoparticles (Movahedi et al., 2020) is generalized. Since BMZ-drug is protonated and dissolved in acidic aqueous solution, BMZ drug is first dissolved in acidic CaCl_2 solution, which is then made into the first W/O emulsion. The second W/O emulsion is made to contain phosphate and base, which will neutralize and precipitate upon mixing with the first emulsion. During the mixing, protonated BMZ-drug molecules are neutralized and then precipitate as tiny nanocrystals within the simultaneously precipitated calcium phosphate matrices, which are then encapsulated with the lipid bilayer after hydration (Scheme 1). Such LCP nanoparticles are expected to retain the tiny drug crystals within CaP nanoparticles in physiological solution but release the drug in a pH-responsive way upon exposure to acidic organelles (endosome and lysosome) in tumor cells, leading to enhanced anti-cancer efficacy due to facilitated cellular uptake and improved solubility.

The objectives of this research were thus to: 1) generalize our approach for preparation of BMZ drug-loaded LCP nanoparticles (such as ABZ, FBZ, MBZ, and TBZ); 2) show enhanced solubility of these drugs in the LCP nanoparticle form; 3) confirm the cytotoxic effect against B16F0 melanoma cells but not healthy cell line HEK293T in the LCP form; and 4) demonstrate the apoptosis-induced mechanisms, such as inhibition of Bcl-2 expression and overproduction of reactive oxygen species (ROS), morphological changes and reduction of tumor cell migration. This research has specifically shown the potential of BMZ-LCP nanoparticle formulations as promising anti-cancer nanomedicines.

MATERIALS AND METHODS

Materials

Albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ), thiabendazole (TBZ), igepal-CO520, calcium chloride, sodium phosphate, DMSO and cholesterol were purchased from Sigma-Aldrich, cyclohexane from Merck (Germany), and 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Avanti polar lipids. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Invitrogen and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) from Promokine (Germany). Apoptosis kit was provided by Biolegend and Bcl-2 FITC Antibody was purchased from eBioscience. All other chemicals were obtained from Sigma-Aldrich unless indicated. Water was deionised Milli-Q water (18.2 MΩ cm at ambient temperature).

Lipid-Coated Calcium Phosphate Nanoparticle Preparation and Characterisation

In order to synthesise drug-loaded LCPs (ABZ/FBZ/MBZ/TBZ-LCP), drugs were incorporated into calcium phosphate core of LCPs as illustrated in Scheme 1. In brief, acidic drug solution (625–5,000 μg/ml) in CaCl₂ aqueous solution was dispersed in cyclohexane/Igepal CO-520 (7/3 v/v) to prepare a well-dispersed microemulsion. After stirring for 10 min, the microemulsion was mixed with the other well-dispersed DOPA (20 mM in chloroform)-containing W/O microemulsion consisting of aqueous solution of ammonia and Na₂HPO₄. After stirring for another 20 min, absolute ethanol was added to collect drug-loaded calcium phosphate (CaP) cores by centrifugation at 10,000 g for 20 min and washing with absolute ethanol thrice. CaP cores were then redispersed in chloroform and mixed with cholesterol and DOPC chloroform. Chloroform was evaporated under reduced pressure and the resultant film was hydrated with PBS (pH 7.4), followed by gentle ultrasonication to obtain the well dispersed LCP NP suspension.

As summarized in **Supplementary Table S1** ABZ/FBZ/MBZ/TBZ-LCP #1-4 were denoted for nanoparticles prepared at various drug concentrations in acidic solutions (630, 1,250, 2,500 and 5,000 μg/ml), respectively.

The hydrodynamic diameter, PDI and zeta potential of drug-loaded LCPs were determined by Zetasizer (DLS, Zetasizer Nano, Malvern, United Kingdom). The microscopic images of drug-loaded LCP NPs were taken in a transmission electron microscope (TEM, JEM-3010, ZEOL, Tokyo, Japan) operated at the voltage of 100 kV.

To determine the encapsulation efficiency, drug-loaded LCP NPs were incubated with Tris Buffer (2 mM EDTA and 0.05% Triton X-100 in pH 7.8) at 65°C for 10 min. The resultant samples were centrifuged for 15 min at 20,000 g, and the drug concentration in the supernatant was calculated by the absorbance measured at 290 nm for albendazole (ABZ), 305 nm for fenbendazole (FBZ), 235 nm for mebendazole (MBZ) and 300 nm for thiabendazole (TBZ), respectively.

Cell Growth Inhibition

The growth inhibition of B16F0 cells and HEK293T cells by BMZ family drugs in the free form and the LCP form was assessed by MTT assay. Briefly, 4,000 cells in 100 μl of DMEM per well were seeded in a 96-well plate after incubation overnight at 37°C and 5% CO₂. Then, culture medium was replaced with fresh one containing variable amounts of drugs in the free or LCP form (ABZ/FBZ/MBZ/TBZ-LCP) and cells were incubated for 24 h. After removal of culture medium, MTT in DMEM (5 mg/ml) was added and incubated for another 4 h at 37°C and 5% CO₂. Then, DMEM containing MTT was carefully removed and replaced with 50 μl DMSO to dissolve formazan crystals. After shaking the plate for 10 min, the absorbance was read at 570 nm, and cell viability was calculated based on the absorbance compared to that of untreated cells.

Apoptosis Analysis

B16F0 cells were treated with BMZ drugs in the free or LCP form for 24 h. The cells were then detached by trypsinisation (0.25% trypsin) and centrifuged at 2000 rpm for 5 min. Resultant cell pellets were washed with PBS and resuspended in 100 μl of Annexin V Binding Buffer and added with 5 μl of FITC Annexin V and 10 μl of propidium iodide solution. After incubation for 15 min in dark, 400 μl of Annexin V Binding Buffer was added to each tube and fluorescence of the cells was evaluated with a flow cytometer (CytoFLEX, Beckman, United States) and analysed by CytExpert software.

Reactive Oxygen Species Detection

B16F0 or HEK293T cells were treated with BMZ drugs in the free or LCP form for 6 h at 37°C and 5% CO₂, and then co-cultured with 2',7'-dichlorodihydrofluorescein diacetate (20 μM) for 30 min. The cells were collected and analysed in a flow cytometer (CytoFLEX, Beckman, United States).

Bcl-2 Detection

To determine the level of Bcl-2, cells were incubated in DMEM media with drug-loaded LCP NPs for 24 h at 37°C and 5% CO₂, then detached by trypsinisation (0.25% trypsin) and centrifuged at 2000 rpm for 5 min, followed by washing with PBS twice. Then, cold fixation buffer (2% PFA) was added dropwise while vortexing the cells. After incubating for at least 30 min at 4°C, cells were

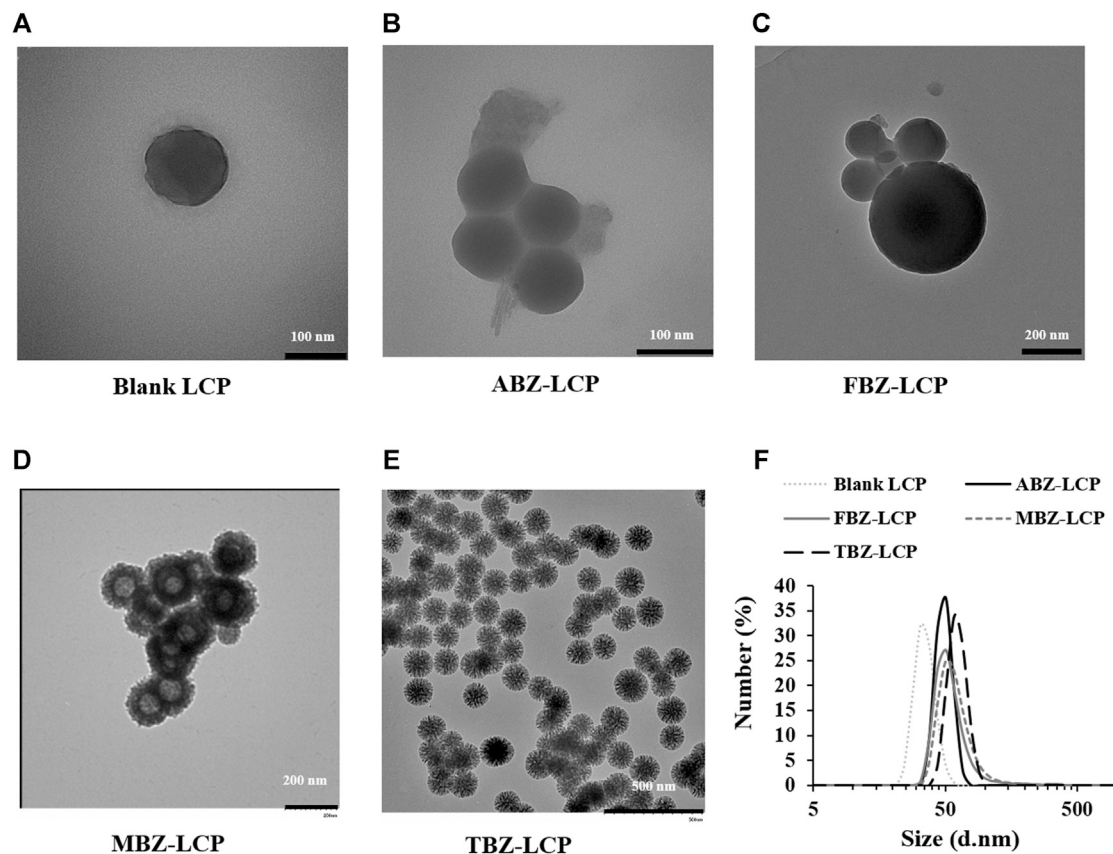


FIGURE 1 | Morphology and size of BMZ drug-loaded LCP NPs. (A–E) TEM images of BMZ drug-loaded LCP nanoparticles; and (F) the size distribution of BMZ drug-loaded LCP NPs.

centrifuged for 5 min at 300 g and washed twice with cold PBS. Cells were then resuspended in permeabilisation buffer (0.1% in triton-X in PBS) and incubated for 15 min at room temperature, followed by centrifugation for 5 min and washing twice with PBS. Afterward, 150 μ l of blocking buffer (0.5% BSA, 2% FBS in PBS) was added to cells. The cells were vortexed and incubated for 30 min, then centrifuged and resuspended in 100 μ l of antibody solution, followed by incubation for another 30 min on ice in dark. Samples were washed twice with FACS buffer (2% FBS in PBS) and analysed using a flow cytometer (CytoFLEX, Beckman, United States) or spectrophotometer.

Migration Assay

To evaluate the effect of the treatment on migration activity of B16F0 or HEK293T cell line, cells were incubated in a 12-well plate overnight to form a confluent monolayer. Then the scratch was artificially made using a 100 μ l pipette tip. The suspended cells and debris were washed away with PBS twice, and the cells in the plate were incubated in DMEM containing free drugs or drug-loaded LCP NPs for 4 h. Olympus IX81 microscope was used to take the images and the relative migration was calculated as the percentage of the number of control cells.

Statistical Analysis

Data presented as mean $\pm$ SD were analysed by t-test using GraphPad 7.03 software. The *p* value less than 0.05 was considered as statistically significant. *p* < 0.05: *; *p* < 0.01: **; *p* < 0.001: ***.

RESULTS AND DISCUSSION

General Physicochemical Characteristics of Benzimidazole Family-Loaded Lipid-Coated Calcium Phosphate Nanoparticles

As demonstrated in TEM images (Figure 1), all typical LCP NPs, i.e., loaded with about 15 wt% of drug molecules, were spherical in shape. The average size and the size distribution of these typical drug-loaded LCP NPs were very similar (Figure 1E; Table 1) with a similar distribution profile (polydispersity index (PDI) of 0.3–0.4). Relatively, FBZ-LCP and ABZ-LCP NPs were smaller with the number-mean diameter of 44.0 ± 5.1 and 49.5 ± 6.3 nm, respectively, while MBZ-LCP and TBZ-LCP NPs had the number-mean diameter of 58.8 ± 5.3 and 57.8 ± 4.2 nm

TABLE 1 | The average size, PDI and the zeta potential of typical LCP NPs loaded with about 15 wt% of BMZ drugs.

Sample	Size (d.nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Loading Capacity (wt%)
Blank LCP	35.7 ± 5.2	0.32 ± 0.06	-17.0 ± 3.1	–	–
ABZ-LCP	49.5 ± 6.3	0.35 ± 0.10	-16.5 ± 4.9	65.4	14.9
FBZ-LCP	44.0 ± 5.1	0.36 ± 0.08	-17.1 ± 2.5	80.0	15.6
MBZ-LCP	58.8 ± 5.3	0.33 ± 0.05	-19.1 ± 4.8	40.0	15.6
TBZ-LCP	57.8 ± 4.2	0.39 ± 0.03	-16.4 ± 4.7	39.1	15.3

(Table 1). In specific, MBZ-LCP NPs had a clear core with the diameter of ~40 nm and a thick layer (~30 nm) as well as a very rough surface (Figure 1D). The core size seems to be very similar to that of ABZ-LCP and FBZ-LCP NPs (Figures 1B,C). In contrast, the cores were not seen in TBZ-LCP NPs, which had a very hairy surface (Figure 1E). While factors such as calcium/phosphate molar ratio and mixing style affect the size of LCPs, the optimized condition was adopted from previous studies in our lab (Tang et al., 2015) to achieve the mean size of LCPs lying in the ideal range for cancer therapy to avoid recognition by the immune system (>200 nm) or clearance by kidneys (<5 nm) (Liu et al., 2013).

The surface charge of all drug-loaded LCP NPs was almost similar, varying from -19 to -16 mV. The negative surface charge of LCPs would reduce the adsorption of serum proteins, and benefit the prolonged half-life in blood circulation and enhanced tumor accumulation of NPs (Blanco et al., 2015).

Variable Loading and Enhanced Solubility of Benzimidazole Drugs in Lipid-Coated Calcium Phosphate NPs

Alteration in encapsulation efficiency and loading capacity was examined by varying the initial concentration of BMZ family drugs in the nanoemulsion, as illustrated in Figure 2; Supplementary Table S1. The loading capacity of these drugs was varied from 0–15.3% (TBZ) to 0–23.2% (FBZ) (Supplementary Table S1). In general, the encapsulation efficiency (EE) was relatively high (>50%). Especially, the EE was 60–85% and 59–93% for ABZ and FBZ, and 40–80% and 39–67% for MBZ and TBZ, respectively. The lower encapsulation efficiency for MBZ and TBZ may stem from their relatively higher aqueous solubility of these two drugs in PBS, particularly in the case with the high drug loading (Supplementary Table S1).

As represented in Figure 3, the commercial MBZ and TBZ were found to have a solubility of 30 and 45 µg/ml, while it was 10 and 6 µg/ml for commercial ABZ and FBZ, respectively, which are similar to that reported for these anti-parasite drugs elsewhere. For example, the solubility of TBZ was reported to be 50 µg/ml at 25°C (Thiabendazole-DrugBank), MBZ 1–70 µg/ml in physiological conditions (Swanepoel et al., 2003) and that of ABZ and FBZ 1–8 µg/ml (at pH 6–8) (Kang et al., 2015) and 6 µg/ml in physiological conditions (Ryu et al., 2013), respectively. Consequently, the higher aqueous solubility of MBZ and TBZ may lead to a lower precipitation rate during formation of CaP

cores, resulting in a smaller amount of the drug being encapsulated.

Furthermore, as demonstrated in Figure 3; Supplementary Table S2, the solubility of all drugs was almost doubled in the LCP form. As discussed in our previous study, the enhanced solubility arises from incorporation of tiny drug nanocrystals in the CaP matrix (a few nm in size) -with crystalline structure confirmed by XRD results-, which is significantly smaller than very large crystals in the free form of commercial drugs (>1,000 nm confirmed by TEM imaging) (Movahedi et al., 2020). After the lipid bilayer and the CaP matrix are dissolved partly (particularly in acidic environment of tumor), the tiny drug nanocrystals are exposed to the aqueous phase and dissolved much more than large crystals (commercial products) due to enhanced surface area, thinner diffusion layer and higher differential concentration (Kumar et al., 2020). Melian et al., also have reported the improved solubility of fenbendazole by nanocrystallisation (Melian et al., 2020). Additionally, at lower pH of tumor environment, the released drug becomes more hydrophilic due to protonation process of the aromatic amines (Chen et al., 2015).

We further hypothesise that some surface MBZ and TBZ tiny nanocrystals may be already dissolved partially during hydration, and the left LCP-BMZ cores may interact the lipid molecules and form some complex structures, such as a hairy/rough surface, as observed in TEM images (Figures 1D,E).

In summary, LCP-based BMZ drug-loaded NPs were controllably made in the number-mean diameter of 40–60 nm with the BMZ drug loading capacity varying from 0 to ~25 wt%. Once encapsulated in LCP NPs, these hydrophobic BMZ drugs showed a double solubility in PBS. Thus the preparation approach would be a general strategy for encapsulating acid-soluble hydrophobic drugs into the CaP matrix, a general way to enhance the bioavailability and activity of the drugs, as presented in the following sections, taking these MBZ-loaded LCP NPs as examples for the cellular anti-cancer therapy.

In Vitro Cell Growth Inhibition by Benzimidazole-Loaded Lipid-Coated Calcium Phosphates

The cytotoxic effect of BMZ family drugs in the free and LCP forms is shown in Figure 4. No B16F0 toxicity was observed for blank LCP NPs (Figure 4A). All drugs demonstrated a dose-dependent anti-cancer effect against B16F0 cells in both forms, while BMZ-LCP NPs were significantly more active

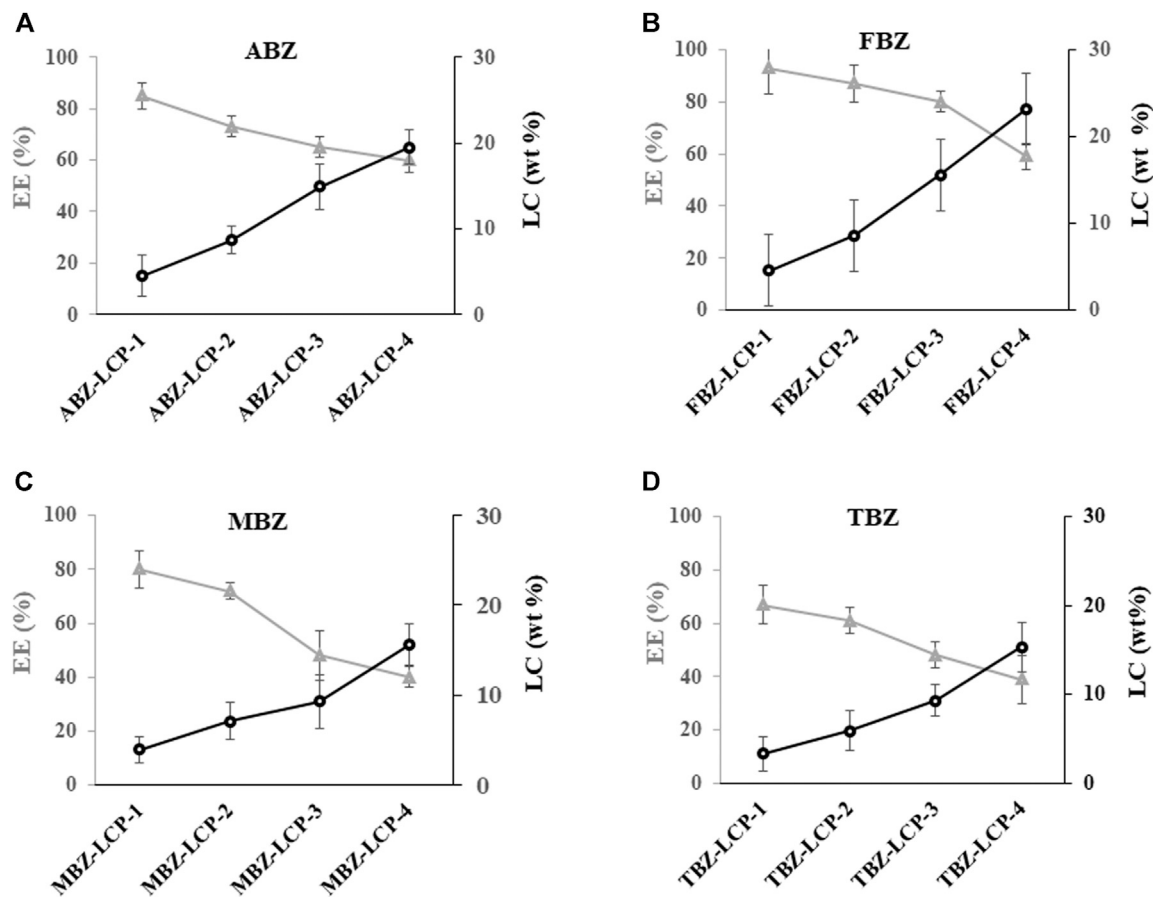


FIGURE 2 | Profiles of the encapsulation efficiency (%) and loading capacity (wt%) of BMZ family drugs loaded into LCP NPs at different initially added concentrations (**Supplementary Table S1**).

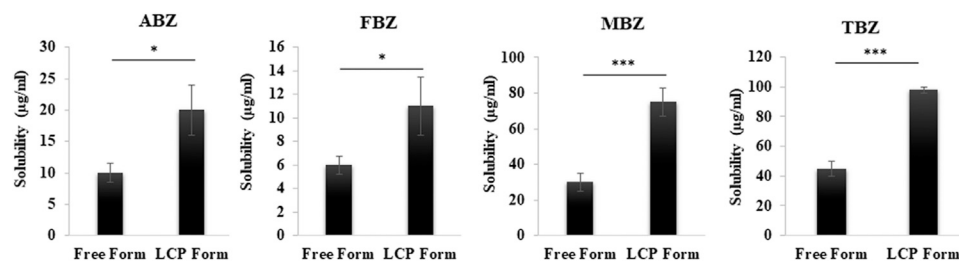
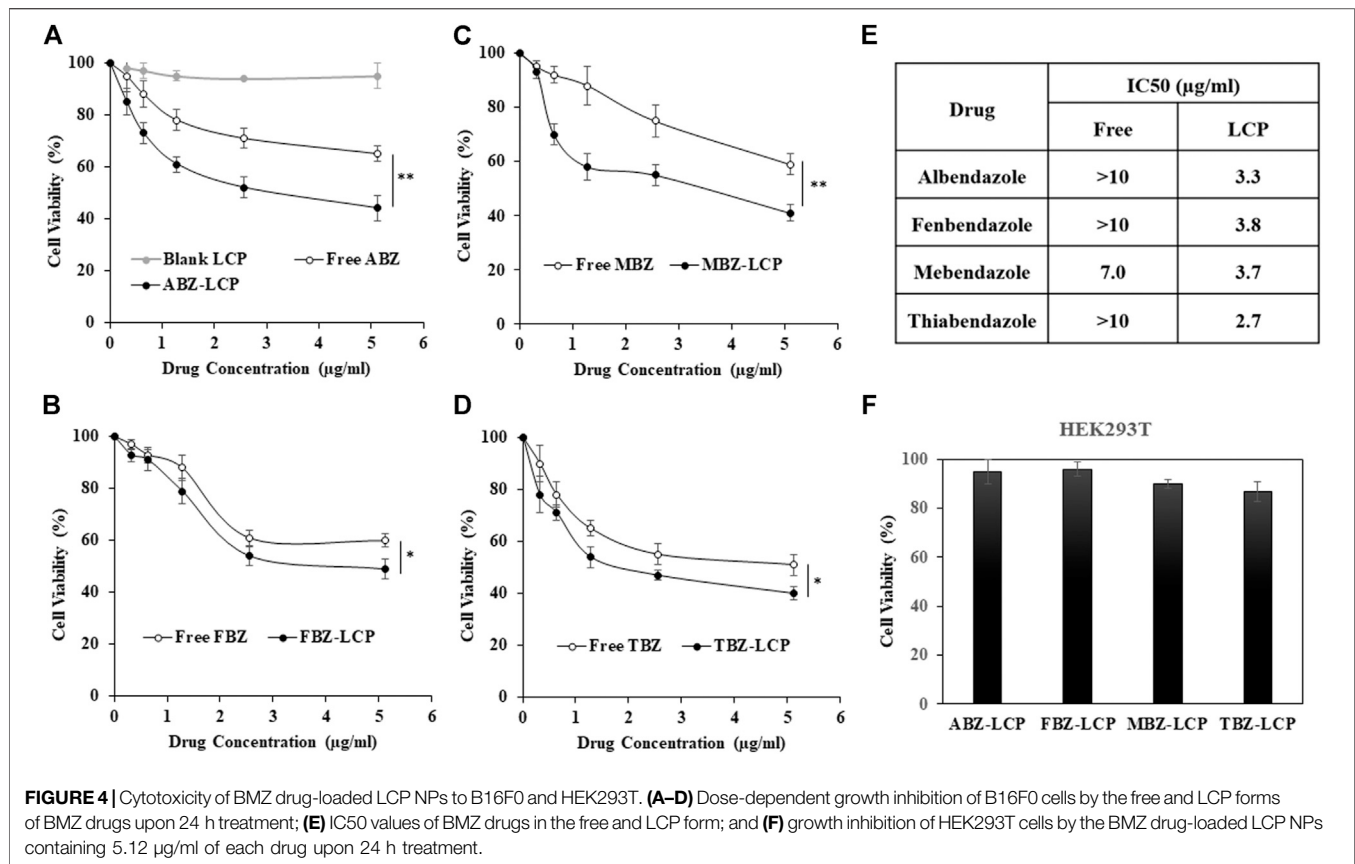


FIGURE 3 | Comparison of the solubility of BMZ family drugs in free form and LCP form after 24 h dissolution in the physiological buffer (pH 7.4) at room temperature.

than that of their corresponding free form (**Figures 4A–D**). Approximately, IC_{50} values (**Figure 4E**) were varied from 7 to >10 $\mu\text{g/ml}$ for the free drugs. In sharp contrast, the IC_{50} value was remarkably decreased to 2.7–3.8 $\mu\text{g/ml}$ for their corresponding LCP forms. Enhanced cytotoxicity of BMZ-LCP NPs may come from improved solubility, as discussed previously, and moreover, facilitated cellular uptake via LCP NP carriers (Wu et al., 2017).

The reported anti-cancer effect of these BMZ drugs in their free form is comparable to our observations. For example, 85% cell viability was observed after 24 h treatment of B16F0 melanoma cells at 0.32 $\mu\text{g/ml}$ of free ABZ, which is very similar to 90% cell viability for A375 and A2058 melanoma cell lines after 12 h treatment at 1 μM (0.265 $\mu\text{g/ml}$) of ABZ and $\sim 70\%$ after 72 h treatment (Patel et al., 2011). B16F0 cell viability was 54 and 49% at 2.56 and 5.12 $\mu\text{g/ml}$ of free FBZ in the



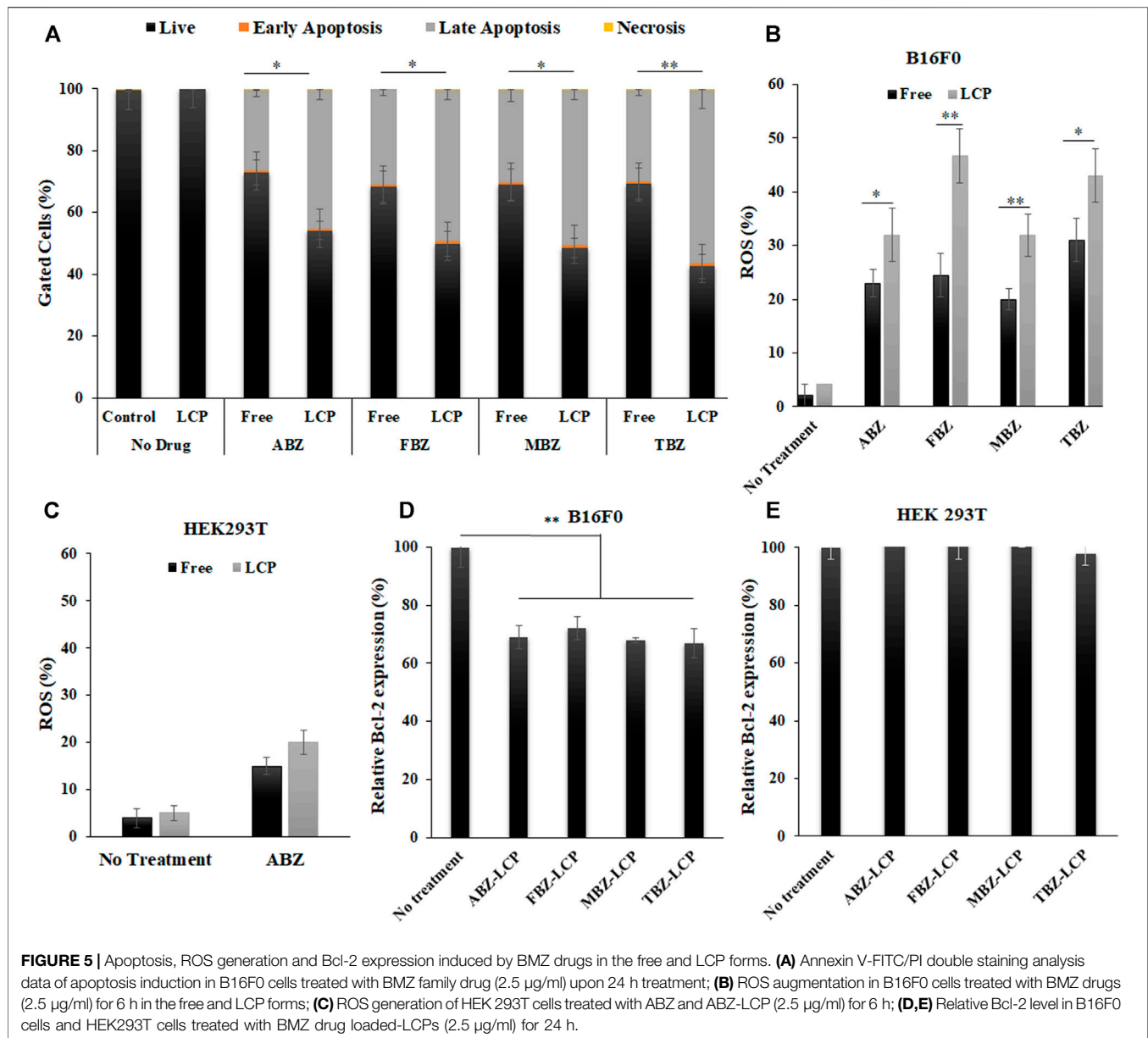
current test, which is similar to 40% viability of EMT6 cells upon treatment with 10 µM (4 µg/ml) of FBZ (Duan et al., 2013). Similarly, MBZ showed some anti-cancer effect on cell lines such as MCF7, T47D, and MDA-MB-231 breast cancer (Zhang et al., 2019) in the drug dosage range of 1–10 µM (0.3–3 µg/ml). In addition, daily intraperitoneal injections of 50 mg/ml TBZ led to almost 75% tumor reduction in the HT1080 fibrosarcoma xenograft mouse model after 27 days treatment, representing high anti-cancer efficacy of this drug (Cha et al., 2012). Clearly, LCP-based BMZ drug NPs developed in this research have demonstrated much higher anti-cancer activity than their free forms, which is the motivation of this research to repurpose these anti-parasite drugs for anti-cancer therapy.

Very interestingly, the highest drug dosage examined in the LCP form showed no obvious toxicity to healthy cell line HEK293T (Figure 4F). This is similar to that no obvious toxicity to HUVEC healthy cells was observed after treatment with MBZ at the dosage highly toxic to lung cancer cell lines (Mukhopadhyay et al., 2002). Similarly, ABZ did not show any toxicity to HOSE normal ovary cells, but to ovarian cancer cell lines in the similar dose range (Noorani et al., 2015). Thus, the safety of BMZ family drugs is a principal advantage over many chemotherapeutics as these drugs have the minimum side effects on healthy tissues/organs and are widely used orally by humans at the dose of up to 15–20 mg/kg every day for an adult (Despommier et al., 2017).

Apoptosis Induction through Reactive Oxygen Species Augmentation and Bcl-2 Inhibition

Apoptosis induction by BMZ family drugs in the free form and the LCP form was analyzed by Annexin V-FITC/PI double staining method (Supplementary Figure S1). As represented in Figure 5A, both forms of drugs induced major late apoptosis while the LCP form induced a much higher level of late apoptosis (45–56% compared to 26–30% for the free drugs). There was also a few percent of early apoptosis and necrosis in all treatments. The elevated later apoptosis may be the reason behind the higher cytotoxic effect of these drugs in the LCP form, which may be attributed to the enhanced solubility and facilitated cellular uptake of drugs via the LCP NP carriers (Tang et al., 2015).

Since the defect in apoptosis plays a fundamental role in cancer pathogenesis, providing time for genetic mutation, increasing angiogenesis and promoting invasion apoptosis induction is considered as the most promising non-surgical approach for cancer therapy (Pfeffer and Singh, 2018). Some studies have confirmed apoptosis induction in cancerous cells by BMZ family drugs. These reported data included 30% apoptosis of human NSCLC cells induced with 1 µM (0.3 µg/ml) of FBZ for 32 h and 50% of Ehrlich carcinoma cells in animals with 20 mg/kg of ABZ (Castro et al., 2016).



Both the free and LCP forms of BMZ drugs elevated the ROS level in B16F0 cells, as demonstrated in **Figure 5B**; **Supplementary Figure S2**. Similar to apoptosis induction, the ROS level was augmented much higher upon treatment with the LCP form of BMZ drugs (32–47%) than that with the free form of drugs (20–31%). As reported by Castro et al., ROS in MCF7 cells was overgenerated upon treatment with 25 μM (6.6 $\mu\text{g/ml}$) of ABZ for 1 h (Castro et al., 2016), and treating A549 cells with 10 μM (3 $\mu\text{g/ml}$) of FBZ for 4 h significantly enhanced ROS production (Dogra and Mukhopadhyay, 2012). Since ROS plays a key role in regulation of cell signalling and pathways of apoptosis, ROS augmentation may be responsible for enhanced apoptosis in drug-loaded LCP NP-treated cells.

ROS enhancement was much lower in the healthy cells, e.g., HEK293T cells treated with both free ABZ and ABZ-LCP NPs

(**Figure 5C**; **Supplementary Figure S3**). Considerably lower ROS induction by ABZ -as the representative of BMZ drugs- in HEK293T cells was demonstrated just to support the fact that these drugs are selectively toxic on cancer cells by unbalancing the ROS level. In comparison with cells treated without any drugs, ROS enhancement was 10–20 fold in drug-treated B16F0 cells (**Supplementary Figure S2**), but it was only 1.5–2.5 fold in drug-treated HEK293T (**Supplementary Figure S3**). This may be the reason that increased ROS generation by only 1.5–2.5 fold did not affect HEK293T cell viability (**Figure 4F**). Thus ROS generation enhanced by BMZ-LCP NPs can selectively kill cancer cells but not healthy cells, as a selective approach for cancer therapy (Perillo et al., 2020).

Consistently, the relative level of Bcl-2 (**Figure 5D**) was all significantly dropped in B16F0 cells after treatment with

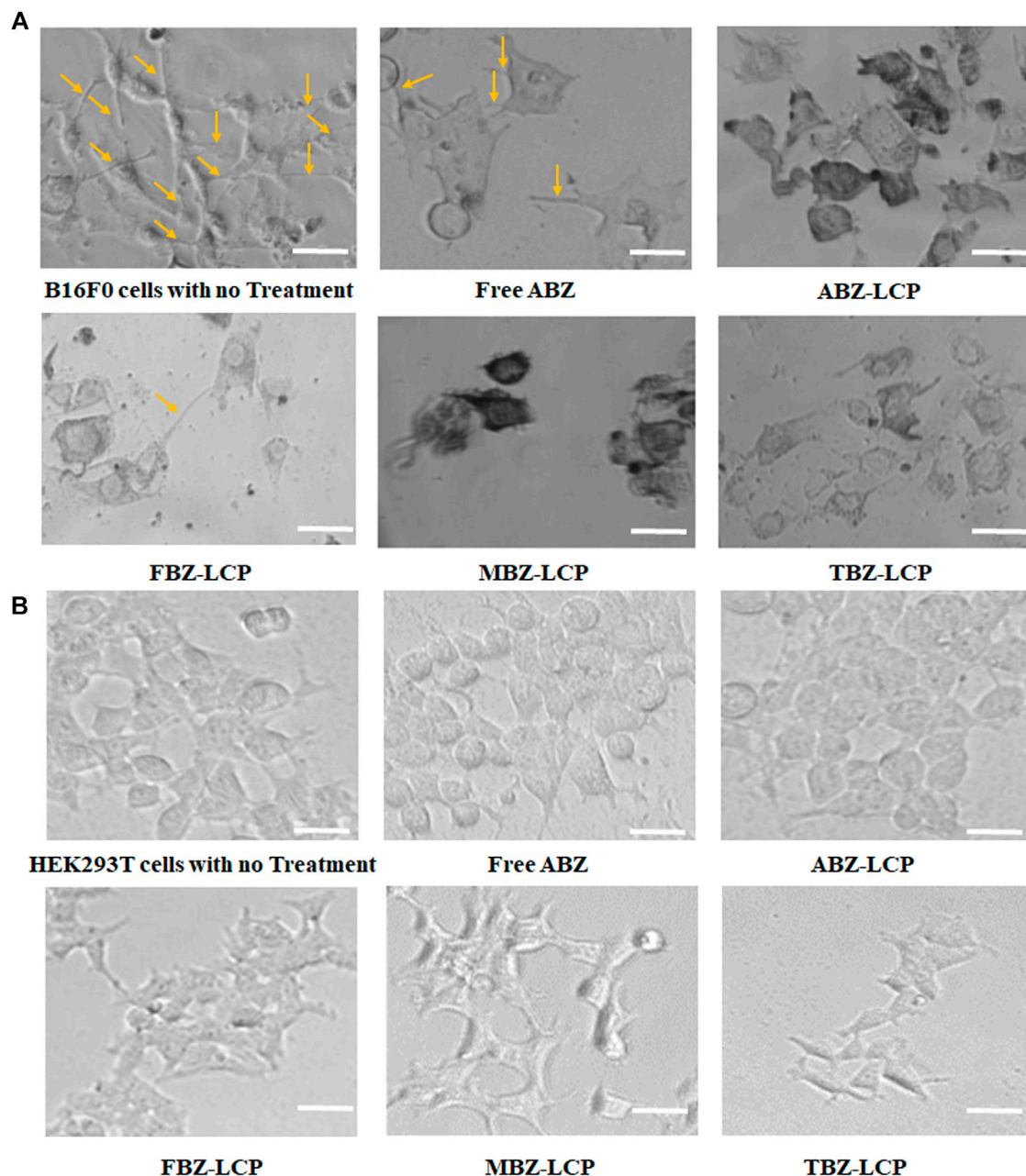


FIGURE 6 | Effect on cell morphology. **(A)** Morphology changes of B16F0 cells treated with BMZ drug-loaded LCP NPs (2.5 µg/ml); and **(B)** The similar morphology of HEK293T cells upon ABZ treatment at 2.5 µg/ml in the free and LCP forms. Scale bar = 50 µm.

drug-loaded LCP NPs, with 28–34% reduction compared to untreated cells. Since cancer cells normally overexpress Bcl-2, the pro-survival founder member of Bcl family (Campbell and Tait, 2018), and protect themselves from ROS-mediated apoptosis induction through the activation of endogenous antioxidants (Hildeman et al., 2003), Bcl-2 inhibition by BMZ drug-loaded LCP NPs further contributes to inducing apoptotic response in addition to ROS overproduction. This observation is consistent with the report that ABZ reduced the expression of apoptosis-related

proteins such as Bcl-2, Bcl-xL, Bax and Bad (Doudican et al., 2008).

Once again, no obvious alteration in the Bcl-2 level was observed in HEK293T cells (Figure 5E) upon the same treatment with BMZ drug-loaded LCP NPs, indicating the safety of the drugs to healthy cells. This is also similar to the report that melanocytes did not alter the Bcl-2 expression even after MBZ treatment (Doudican et al., 2008). Thus the comparison between B16F0 cancer cells and HEK293T healthy cells may reveal that BMZ drug treatment in free or LCP form just

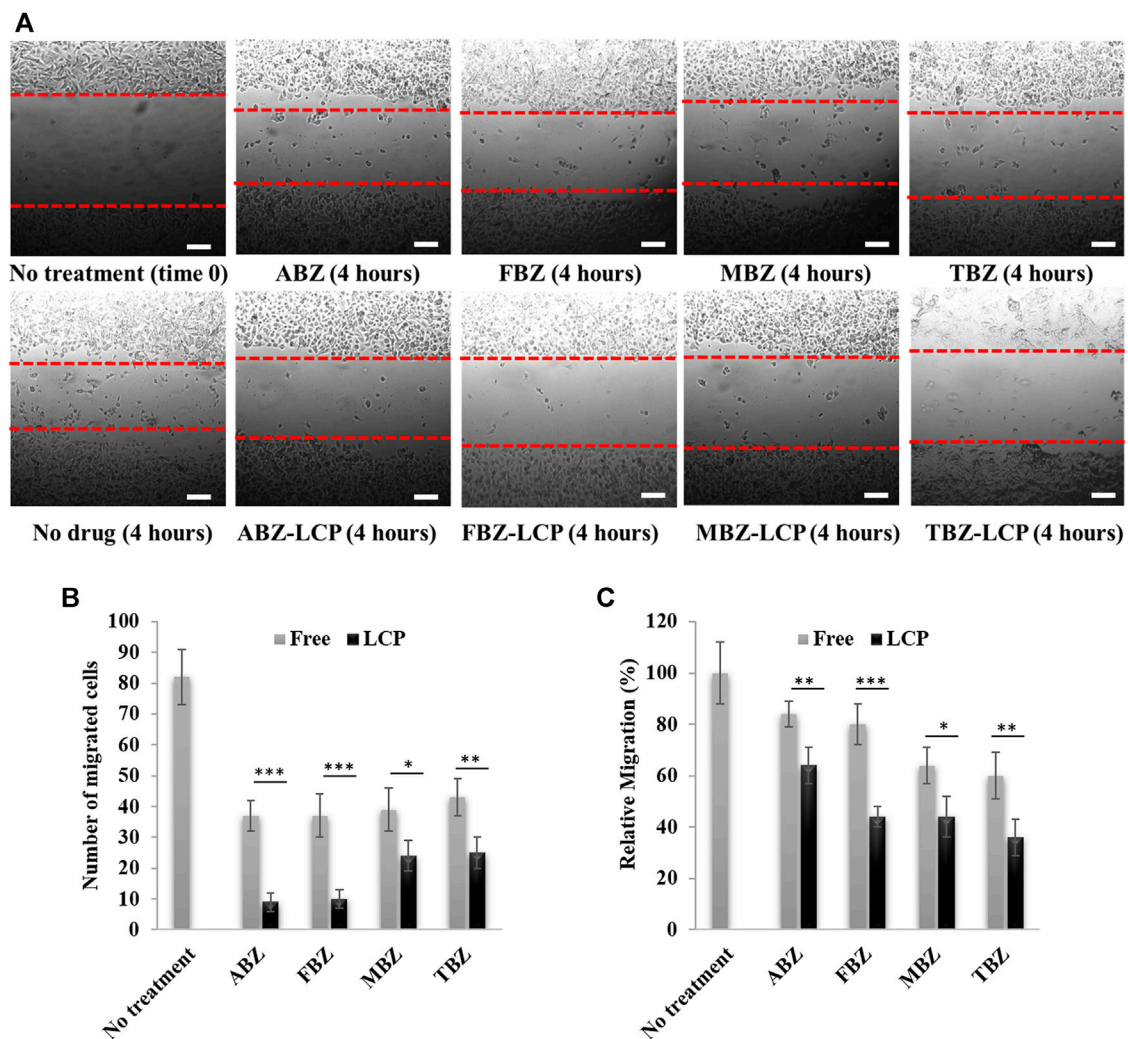


FIGURE 7 | Assessment of cell migration upon BMZ drug treatment in the free and LCP forms for 4 h. **(A)** Images of B16F0 cell migration; **(B)** number of migrated cells; and **(C)** relative migration percentage. Scale bar = 200 μ m.

inhibits the overexpressed Bcl-2 in cancer cells but does not interfere the general Bcl-2 level in the normal cell line.

Morphological Changes and Cell Migration Inhibition

Treatment of B16F0 cells with drug-loaded LCP NPs substantially changed their morphology, as illustrated in **Figure 6A**. The untreated cells showed the natural spindle-like shape, which was transformed upon the treatment of drugs either in the free form or the LCP form. Furthermore, the cellular extensions were disrupted. Disruption of tubular extensions of tumor cells limits their access to nutrients (Siemann, 2011). Thus, cellular extension disruption not only inhibits tumor growth but is also vital for tumor cell dissemination and metastasis (Ross and Hunger, 2017). Similar morphological changes in MCF7 and MDA-MB-231

cells as a result of albendazole treatment is reported (Racoviceanu et al., 2020).

Morphological transformation and extension deformation by BMZ family drugs arise from their inhibition of tubulin polymerization (Kamal et al., 2012). This inhibition has been reported to alter microtubule organization of Chinese hamster cells after exposure to TBZ (Pisano et al., 2000), inducing abnormal spindle formation and enhanced tubulin depolymerization in non-small cell lung cancer cells treated with MBZ (Sasaki et al., 2002), and disruption of mitotic bundles in 1A9 ovarian cancer cells exposed to ABZ (Chu et al., 2009). Oxidative stress caused by BMZ drug treatment can also cause morphological changes and consequently motility alteration (Alexandrova et al., 2006).

Again, no obvious morphological changes such as spindle deformation or disruption of mitotic bundle was observed in HEK293T cells upon the similar treatment with BMZ family

(Figure 6B), which may be attributed to less susceptibility to ROS (Figure 5C) (Katoh et al., 2006).

As cells with more rounded shape are supposed to have less capability of dissemination because of the microtubule destabilization (Young et al., 1985), less migratory activity of BMZ drug-treated cells was observed, as shown in Figure 7A. All free BMZ family drugs inhibited cell migration to some degree at the dose of 2.5 µg/ml. Interestingly, their LCP forms were more effective in inhibition of cell migration by 15–30% in terms of the cell number (Figure 7B) and 20–40% in terms of the relative migration (Figure 7C), due to the enhanced solubility and cellular uptake in the LCP form. This inhibition results are comparable with previous studies about cell migration inhibition by BMZ family drugs. For instance, 24 h treatment of PE/CA-PJ15 cells (human oral squamous carcinoma) with 5 µM (~1.5 µg/ml) of FBZ and MBZ reduced cell migration very significantly (Kralova et al., 2018). Treatment with 0.6 µM (0.16 µg/ml) of ABZ halved migration activity of SW1990 and PANC-1 pancreatic cancer cells after 24 h treatment (Chen et al., 2020).

Since tumor cell migration is not only a prerequisite for tumor dissemination but also a contributor to tumor metastasis (Entschladen et al., 2004), inhibition of cell migration is vital for a comprehensive cancer treatment. Thus, BMZ family drugs, especially loaded in LCP NPs, may be considered as a treatment beyond the primary tumor inhibition, without obvious side effects to healthy cells or organs. This is a sharp contrast to other conventional chemotherapeutics such as paclitaxel, doxorubicin and cisplatin that are associated with various toxicities to healthy organs such as neurotoxicity, cardiotoxicity, nephrotoxicity, hepatotoxicity and hematotoxicity (Cersosimo, 1993; Juaristi et al., 2001; Guastalla and Diéras, 2003).

CONCLUSION

LCP nanoparticles provide a versatile nanoplatform to load anti-worm BMZ family drugs and repurpose their applications for cancer treatment. Having superior cytotoxic effect on cancer cells but almost no toxicity to healthy cells, BMZ drugs were successfully loaded by LCPs and showed even more efficient

inhibition of cancer cell proliferation via enhanced solubility and cellular uptake. We further found that BMZ drug-loaded LCPs induced more later apoptosis through Bcl-2 reduction and ROS augmentation. Moreover, BMZ-LCP NPs were able to control tumor cell dissemination through induction of morphological changes and inhibition of cell migration. Overall, LCP-based nanoformulated BMZ drugs would be considered as promising nanomedicines not only to treat primary tumors but also to potentially control tumor dissemination and metastasis with minimum side effects.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FM: Experimental WG: Co-supervision Associate CS: Co-supervision ZX: Supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnano.2021.693837/full#supplementary-material>

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Recapitulating Tumorigenesis *in vitro*: Opportunities and Challenges of 3D Bioprinting

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Cancer is considered one of the most predominant diseases in the world and one of the principal causes of mortality per year. The cellular and molecular mechanisms involved in the development and establishment of solid tumors can be defined as tumorigenesis. Recent technological advances in the 3D cell culture field have enabled the recapitulation of tumorigenesis *in vitro*, including the complexity of stromal microenvironment. The establishment of these 3D solid tumor models has a crucial role in personalized medicine and drug discovery. Recently, spheroids and organoids are being largely explored as 3D solid tumor models for recreating tumorigenesis *in vitro*. In spheroids, the solid tumor can be recreated from cancer cells, cancer stem cells, stromal and immune cell lineages. Organoids must be derived from tumor biopsies, including cancer and cancer stem cells. Both models are considered as a suitable model for drug assessment and high-throughput screening. The main advantages of 3D bioprinting are its ability to engineer complex and controllable 3D tissue models in a higher resolution. Although 3D bioprinting represents a promising technology, main challenges need to be addressed to improve the results in cancer research. The aim of this review is to explore (1) the principal cell components and extracellular matrix composition of solid tumor microenvironment; (2) the recapitulation of tumorigenesis *in vitro* using spheroids and organoids as 3D culture models; and (3) the opportunities, challenges, and applications of 3D bioprinting in this area.

Keywords: tumor microenvironment, tumorigenesis, 3D cell culture, spheroids, organoids, drug assessment, high-throughput screening, 3D bioprinting

INTRODUCTION

Cancer remains one of the most predominant diseases in the world in the 21st century, affecting millions of patients per year (Roy and Saikia, 2016). Rather than responding appropriately to signals that maintain cell behavior, cancer cells grow and proliferate without control, invading normal tissues and organs, and eventually spreading throughout the organism (Chambers et al., 2002).

The cellular and molecular mechanisms involved in the development and establishment of solid tumors is known as tumorigenesis. It is widely accepted that tumorigenesis is a multistep process, depending on a sequential accumulation of mutations of tissue cells (Ashkenazi et al., 2008). The tumor microenvironment is composed of non-cancerous cells with functions in all stages of tumorigenesis by both stimulating and/or facilitating abnormal cell proliferation (Arneth, 2019).

In recent years, literature has advanced in the better understanding of tumor microenvironment (DeBerardinis, 2020). The non-cancerous cell types include fibroblasts, endothelial cells, and immune cells (Casey et al., 2015; Jarosz-Biej et al., 2019). In addition, depending on the type of tumor, organ-specific interstitial cells are also present. According to previous descriptions, these cells are denominated as “tumor stroma” and, together with the extracellular matrix (ECM), oxygen levels and pH, constitute the tumor microenvironment (Briest et al., 2012; Hirata and Sahai, 2017). This complex interaction between tumor and non-tumor cells leads to an altered metabolism and ECM production. The better understanding of tumor microenvironment is a key challenge to address, contributing to the development of new drugs and treatments (Valkenburg et al., 2018).

In this context, 3D cell culture has gained space in literature due to its advantages compared with “classical” 2D cell culture. 3D cell culture can recreate a sort of tissue microenvironment, providing more accurate data about cell-to-cell interactions, cell-to-extracellular matrix interactions, tumorigenesis, drug discovery, gene expression, metabolic profiling, and protein profiling of the cells. 3D cell culture, such as spheroids and organoids, has the potential to provide alternative models to study tumor microenvironments (Nath and Devi, 2016; Jensen and Teng, 2020). In tumor biology, spheroids are represented by cancer cell lineages self-assembled in rounded shape and organoids by cells derived from tumor biopsies, including cancer stem cells, self-assembled in amorphous shape. Furthermore, cell culture platforms of tumor spheroids and organoids start to be adapted as a model for drug assessment and high-throughput screening (HTS) (Kondo et al., 2019; Heredia-Soto et al., 2020; Renner et al., 2020).

3D bioprinting is a promising emergent bottom-up technology to develop complex tissue models *in vitro*. 3D bioprinting is a form of additive manufacturing, where cells, biomaterials, and soluble factors can be assembled layer by layer (Mandrycky et al., 2016). From 3D bioprinting, it is possible to hierarchically organize tissues, as they are found *in vivo*, and faithfully recapitulate their morphology as well as functional aspects (Datta et al., 2018). Although 3D bioprinting represents a promising technology, main challenges still remain such as the speed of bioprinters and better bioinks for improving cell survival and function in cancer research. The main objective of this review is to explore the cellular and molecular composition of solid tumor microenvironment, the recapitulation of tumorigenesis and drug assessment using spheroids and organoids, and the opportunities and challenges of 3D bioprinting in this field.

THE TUMOR MICROENVIRONMENT

Background

The tumor microenvironment is heterogeneous, composed mainly of tumor cells and endogenous stromal cells (non-cancerous) that are later recruited by the tumor itself. This microenvironment also contains extracellular components: ECM proteins, extracellular vesicles, cytokines, growth factors, and hormones nourished by a vascular network. The stromal cells are represented by endothelial cells, mesenchymal stem/stromal cells (MSCs), fibroblasts, and macrophages (Wu and Dai, 2017; O’Loughlen, 2018). During tumorigenesis, tumor cells interact greatly and evolve with this surrounding microenvironment, having profound effects on therapeutic efficacy (Bussard et al., 2016).

All tumor microenvironment components communicate continuously with each other mainly by (1) cell-to-cell interactions, (2) cell-to-extracellular matrix interactions, and (3) the network of cytokines, proteins, and chemokines that can favor the immune system or the tumor growth. Thus, any disruption in tumor microenvironment signaling will reflect changes of the balance between immune system and tumor (Hui and Chen, 2015; Merlano et al., 2019).

One of the most crucial factors for tumor microenvironment maintenance and progression to metastasis is the vascular network (Naumov et al., 2008; Quail and Joyce, 2013). Tumor vasculature is characterized as being disorganized and leaky, which is associated with altered endothelial cell adherents junction and tight junction formations, both critical to maintain vascular barrier functions. In addition, tumor cells induce programmed necrosis of endothelial cells, thus, increasing vascular leakiness and tumor cell extravasation and metastasis (Yang and Lin, 2017).

Cell Components of Tumor Microenvironment

In solid tumors, mesenchymal stem cells and fibroblasts, also named as cancer-associated fibroblasts (CAFs), are the main cellular components of the microenvironment. It is well known that in healthy tissues, fibroblasts support tissue repair and homeostasis; however, CAFs is a heterogeneous population that serves a different function compared with resident fibroblasts (Petrova et al., 2018; Ayan et al., 2020), as suggested by Sugimoto et al. (2006) and Kobayashi et al. (2019). The principal functions of CAFs in the tumor microenvironment are: (1) stimulate tumor cell proliferation by growth factor secretion, (2) modify cancer ECM, which will induce tumor progression and metastasis, and (3) modulate the inflammatory components that facilitate tumor initiation, progression, and metastasis (Servais and Erez, 2013; Raffaghello and Dazzi, 2015). Furthermore, CAFs support endothelial cells to start tumor angiogenesis. Endothelial cells offer nutritional support for tumor growth and development, showing a key role in tumor cell protection from the immune system (Arneth, 2019). Tumor endothelial cells are considered one of the main targets of anti-angiogenic therapy (Hida et al., 2013). A study published by Maishi et al. (2016) showed with

two different tumor models that endothelial cells in the tumor microenvironment are able to promote tumor metastasis by direct interaction with tumor cells.

Myo-fibroblasts are specialized fibroblasts, a subpopulation of CAFs, which express the alpha-smooth muscle actin protein and are considered major players in the development of different fibrotic diseases, mainly due to their capacity to remodel the ECM (Yazdani et al., 2017; Ribatti and Tamma, 2019). In tumors, these activated fibroblasts can enhance tumorigenesis, angiogenesis, and metastasis by secreting growth factors and cytokines. Besides fibroblasts and endothelial cells, MSCs are present in the tumor microenvironment as well, interacting with tumor cells via the secretion of growth factors or cytokines, and by transferring mitochondria or microRNAs. Residing in tumors, MSCs form a fibrovascular network by differentiating into smooth muscle cells and vascular pericytes, contributing to vascular network extension (Guo and Deng, 2018). At the beginning of tumorigenesis, MSCs have been shown to drive tumor cells toward an invasive, premetastatic state. However, some studies showed that MSCs can also have an inhibitory effect on tumor growth by reducing cytotoxicity effects, pluripotency, and even by influencing macrophage polarization (Ridge et al., 2017).

Pericytes are multipotent perivascular cells with an established role in vasculature development. Studies have already shown that these cells present immune properties and might serve as a reservoir of MSCs to influence in the *in vivo* regeneration of diverse tissues. Pericytes located in the vessels play a significant role in the homeostasis of these vessels, and when recruited, they change their activation stage to MSCs in order to participate in injury events of the tissue (Meirelles et al., 2013). In addition, pericytes are capable of realizing tumor homing and are considered an important cell component of the tumor microenvironment (Ribeiro and Okamoto, 2015). In cancer, pericytes have been explored because of their capacity to stabilize blood vessel structure and permeability. Due to this, it was discovered that pericytes can affect tumor growth and metastasis positively or negatively. The effects of tumor growth are related to establishing a stable vascular network, which will ensure a proper delivery of nutrients to allow tumor cells maintenance and proliferation. However, these cells can prevent tumor cell dissemination by maintaining the permeability of blood vessels (Barrow and Colonna, 2019). Furthermore, many studies have shown that cancer vessels are characterized by abnormal pericyte population of cells and altered pericytes/endothelial cell interactions, which can effectively contribute to metastasis process and progression of cancers, especially perivascular ones such as glomus tumor, myopericytoma, and solitary fibrous tumor/hemangiopericytoma (Mravic et al., 2014; Chen et al., 2016).

Another cell type whose role is largely explored in tumor microenvironment is the adipocyte. Adipose tissue is composed of adipocytes and non-adipocyte cells, including MSCs from adipose tissue and macrophages. These cells release a variety of molecules that enable them to play a paracrine effect in pathological processes such as breast and ovarian cancer (Robado de Lope et al., 2018).

The macrophage is the most prominent immune cell type in the tumor microenvironment (Arneth, 2019). Macrophages have an active role from early carcinogenesis to tumor progression and metastasis, constituting up to 50% of a tumor mass depending on the type of tumor. Previous studies suggest that after infiltrating tumors, macrophages polarize to a M2 phenotype, take on the functions of tumor growth and angiogenesis, tissue remodeling, and suppression of antitumor immunity (Kim and Bae, 2016). Zhang A. et al. (2017) reported that CAFs promoted M2 polarization of macrophages in pancreatic ductal adenocarcinoma, which enhanced tumor cell growth, migration, and invasion.

Another immune population of cells present in the tumor microenvironment is the natural killer cells (NK). NK cells are large granular lymphocytes that control tumor growth by interaction with tumor cells or because they can affect the function of other innate and adaptive cell populations (Melaiu et al., 2020). Interestingly, NK cells show antitumor activity as they have the efficient and fast capacity to recognize and kill tumor cells. This function is mediated through cell-surface receptors, which examine tissue microenvironments for changes in surface and secretory phenotypes, and then alerts the immune system for the presence of infection or of a malignancy agent. Therefore, this function is largely explored for cancer immunotherapy treatments (Bi and Tian, 2017; Barrow and Colonna, 2019; Zhang et al., 2020). According to Fang et al. (2017), the main approaches used for cancer immunotherapy with NK cells are based on the use of cytokines, as IL-2 and isoforms, antibodies, and the adoptive transfer of *ex vivo* NK cells.

T cells also play important functions in the tumor microenvironment, where it is common to find inhibitory receptors. These can inhibit T cell metabolism and influence T cell signaling, both directly and through release of extracellular vesicles. When isolated from tumors, T cells generally show signs of exhaustion and present distinct metabolic features (Lim et al., 2020). Other immune cells that are present and modulate the tumor microenvironment are granulocytes, such as the mastocytes. Early mastocyte cell infiltration has been reported in human and animal tumors, especially in malignant melanoma, breast, and colorectal cancer (Liu et al., 2011; Komi and Redegeld, 2020). Mastocytes have different functions in the tumor microenvironment such as: (1) modulating tumor biology, by influencing in cell proliferation, survival, angiogenesis, and metastasis; and (2) establishing crosstalk with other tumor-infiltrating cells in the microenvironment (Aponte-López and Muñoz-Cruz, 2020).

Currently, different studies discuss the concept and functions of cancer stem cells (CSC) in tumor microenvironments. These cells, also called stem-like cells or tumor-initiating cells (TICs), were first described in 1994 and are a distinct subpopulation of tumor cells. Recently, this subpopulation of cells has been described as having a unique ability to initiate tumor growth and maintenance. In this context, CSC is considered an important target for cancer immunotherapies (Nassar and Blanpain, 2016; Codd et al., 2018). The quantity of CSC in the tumor microenvironment varies according to the tumor type. These cells can be responsible for preserving tumor heterogeneity by

retaining self-renewal and differentiation properties. In addition, CSC also plays a role in innate resistance to cancer therapies, which in turn links to their persistence of the tumor in a specific tissue, which can lead to disease recurrence and metastatic spread (Albini et al., 2015). A study performed by Chen et al. (2014) demonstrated that CAFs enrich CSCs through de-differentiation process and reacquisition of stem cell-like properties in lung cancer. Briefly, the main results showed that CAFs develop a paracrine signaling that induce Nanog expression and promote stemness in cancer niche. What is interesting is that it is possible to discover new therapeutic targets to act in this paracrine signaling of CAFs to CSCs.

The Extracellular Matrix in Tumor Microenvironment

The ECM contains a diversity of proteins, which influence the cell phenotype of specific tissues due to their biochemical and biophysical properties. The principal ECM proteins secreted by cells in the tumor microenvironment are collagen, fibronectin, laminin, vitronectin, and tenascin (Cheng et al., 2020). It is well known that the ECM is highly dynamic because it is constantly being remodeled and degraded from embryogenesis until maturity. This remodeling is crucial for tissues homeostasis; however, dysregulation of ECM dynamics is common in the development of diseases as cancer (Bonnans et al., 2014; Walker et al., 2018).

In the tumor microenvironment, two main modifications are commonly observed in the ECM: stiffness (rigidity) and degradation. The increase in cross-linking between ECM proteins can cause stiffness (Najafi et al., 2019). The enhancement of tumor ECM stiffness is mainly induced by ECM deposition, remodeling by resident fibroblasts and by the transformed epithelium. In addition, the presence of chemokines and growth factors lead to an inflammation state. The inflammation state induces CAFs activation and their transdifferentiation into myofibroblasts, causing tissue desmoplasia. Then, myofibroblasts deposit ECM proteins, secrete growth factors, and apply contraction forces on the tumor ECM. In the end, newly deposited ECM proteins will generate larger and rigid fibers that turn the ECM rigid (Frantz et al., 2010). However, the disruption in the signaling between these ECM proteins will result in degradation, mainly caused by the activation of metalloproteinases (MMPs) (Najafi et al., 2019). The MMPs cleave collagen fibers of tumor ECM and reorganize them into tube-like structures to facilitate cell migration in the microenvironment (Malik et al., 2015).

The MMP genes were previously associated with increased risk and evolution of breast cancer. In the study developed by Slattery et al. (2013), the genetic variation of MMP1 (nine SNPs), MMP2 (eight SNPs), MMP3 (four SNPs), and MMP9 (three SNPs) together with breast cancer risk was evaluated in Hispanic and Non-Hispanic women. The results showed that MMPs have associations with breast cancer progression and prognosis. Overall, MMP-2 showed the strongest gene association with breast cancer development.

Regarding ECM modifications in breast cancer, another study, published by Boghaert et al. (2012) showed, with a 3D cell culture

model, that the regions where the tumor cells invaded the breast tissue more was directly correlated with a higher mechanical stress of the host epithelial tissue. The use of a 3D cell culture model to recapitulate the breast tumor microenvironment can then aid in the better understanding of *in vivo* mechanisms.

One of the first studies published correlating abnormal ECM and the progression of cancer was performed by Neglia et al. (1991), which investigated the risk of cancer in patients with cystic fibrosis. The study was developed with North American and European patients with cystic fibrosis, and the results showed that, in fact, these patients had an increased risk to develop digestive tract cancers. In cancer, the abnormal ECM affects the progression of the disease by promoting changes in host cells normal functions. In addition, ECM anomalies are also capable of (1) deregulating the behavior of stromal cells, (2) promoting angiogenesis and inflammation associated with the tumor, (3) leading to the generation and maintenance of an established tumorigenic microenvironment, and (4) can also induce metastatic dissemination (Lu et al., 2012; Seager et al., 2017).

Not only cancer cells but also CAFs lead the modification and remodeling of the ECM during cancer progression. The biochemical cross talk between the cancer cells and CAFs, and the biomechanical changes of the ECM are major contributors to tumor cell migration and invasion, which will influence tumor progression to metastatic state. Additionally, growth factors, chemokines, and metabolic changes released from the ECM contribute to the maintenance and progression of the tumor microenvironment (Erdogan and Webb, 2017; Eble and Niland, 2019).

Due to the importance of ECM modification in the tumor microenvironment, studies are being conducted in order to develop therapeutic treatments to target the cancer ECM. Van der Steen et al. (2017) explored the functionalization of drug-loaded lyophilisomes (albumin-based biocapsules) loaded with doxorubicin and functionalized with antibodies, to act in the ECM, or stroma, of ovarian carcinomas, in order to evaluate its potential to eliminate cancer cells. The principal results showed that drug-loaded lyophilisomes were effective to induce cancer cell death and can be considered as a therapeutic agent to specifically target ECM components of the tumors. In addition, Zhang et al. (2018) explored the use of cyclopamine, a special inhibitor of the hedgehog-signaling pathway, which contributes to ECM formation of pancreatic ductal adenocarcinoma, to ameliorate solid stress and improve nanomedicine delivery to tumor site. The principal results showed that the drug was able to disrupt ECM in pancreatic ductal adenocarcinoma, reduced solid stress of the tumor together with an improvement of function of tumor vessels, which allowed a better perfusion in the tumor area.

Although the drugs discovered recently to target tumor ECM might effectively reduce the number of cancer cells and reduce solid stress, there are still many challenges that ECM components in tumor microenvironment can set that could interfere with therapeutic treatments. Briefly: (1) ECM proteins act as a physical barrier, which makes drug delivery more difficult, (2) ECM proteins can de-differentiate non-CSCs into CSCs, and this can make it harder for the elimination process

of CSCs in the microenvironment, (3) the ability of ECM to modulate immune responses, and (4) complex nature of ECM, with its different molecules and isoforms (Nallanthighal et al., 2019). Therefore, the ECM in the tumor microenvironment has a considerable impact in cancer progression and further metastasis. Due to this, a better understanding of the interactions between cancer cells and ECM is needed and might only be addressed by 3D cell culture models, especially in order to have more faith in the results of drug screening to target cancer (Drost and Clevers, 2018).

3D MODELS RECAPITULATING THE TUMORIGENESIS *IN VITRO*

Background

The tumorigenesis of cancer disease is heterogeneous in growth rate, invasiveness, drug sensibility, and individual patient derived characteristics (McGrath and Swanton, 2017; Fan et al., 2019). Therefore, the *in vitro* and *in vivo* preclinical studies fail in emulating the microenvironment of the tumor to predict its sensibility or its resistance to drugs, or the metabolic and molecular pathways. This explains the low success rate of drug acceptance for oncologic drugs at 3.4% (Wong et al., 2019).

Immortalized cell lines are a valuable resource to investigate the physiological mechanisms and body-environmental interactions between healthy cells and cancerous cells due to their ease of growing and manipulating *in vitro*. Monolayer assays employing immortalized cancer cells are characterized by low cost, less complexity, and are readily employed in the HTS of drug trials and molecular biomarkers (Fan et al., 2019). However, because of the fast proliferation of the monolayers, it is likely that the culture might be affected by problems such as de-differentiation or abnormal gene expression profiles, which may influence the result of experiments as well as be contrasting to *in vivo* tests (Shah et al., 2018). Furthermore, monolayer assays glean so little about the gene expression, reorganization, and responses involved in the tumorigenesis, mainly due the absence of a tumor microenvironment (Gao and Chen, 2015).

To fill the gap between these insufficient or inappropriate models, 3D cultures arise as an urgent tool to improve the prediction system and mechanism of understanding tumorigenesis in humans. 3D cultures allow for systematic investigation into the several unidentified metabolic pathways and cascades (Sawant et al., 2016).

The classical scaffold-based approach in tissue engineering has focused on devising cells, bioactive factors, and scaffolds with biocompatible biomaterials to produce models able to maintain the tumor phenotype (Molina et al., 2020). In these models, it is possible to co-cultivate epithelial and stromal cells and observe the crosstalk of multiple cell types interacting, which regulate normal and neoplastic development (Sawant et al., 2016).

In contrast to scaffold-based methods, scaffold-free approaches emerge as 3D tumor models. The scaffold-free approaches are aggregates of cells, producing several common features that are similar to the solid tumor *in vivo* such as cellular

heterogeneity, cell-cell signaling, hypoxia, membrane protein distribution, and gene expression patterns (Zhao et al., 2019).

Tumor Spheroids

The development of 3D models such as spheroids made it possible to engineer several cancer-like microenvironments *in vitro*. Many papers claim to have developed their protocols to build tumors such as glioblastomas, colorectal, breast, liver, lungs, among others (Kelm et al., 2003; Hirschhaeuser et al., 2010; Chimenti et al., 2017; Eilenberger et al., 2018; Froehlich et al., 2018; Oraiopoulou et al., 2019; Foglietta et al., 2020; Lee et al., 2020).

The breast cell line MCF-7 is an adenocarcinoma-luminal subtype one. The cell morphology is epithelium-like resulting in their ability to self-aggregate into a steady shape, which makes it easier to maintain their viability and to use it for implantation in mice for *in vivo* studies (Do Amaral et al., 2011; Comşa et al., 2015; Froehlich et al., 2018).

HEPG-2 is an epithelial-like hepatocellular carcinoma that, due to the liver cells' role of the metabolism, is considered a valuable option to study cell genotoxicity (Luckert et al., 2017; Shah et al., 2018). 3D models using HEPG-2 can be used alone in drug screening or as a co-culture with other tumor cell lines (Lan et al., 2010; Jung et al., 2017).

Some aspects must be considered when working with spheroids. One of them is the quality of the 3D protocol, which is related to some variables such as the kind of support for the culture, the non-adherent medium used, the number of cells that are seeded, the spheroid formation technique, the temperature, and the amount of CO₂ and O₂ available (Mironov et al., 2009; Mehta et al., 2012; Däster et al., 2017). All these factors are highly changeable according to the tumor line chosen.

The role of hypoxia and the capacity of a tumor to induce neovascularization in its microenvironment using spheroid models have been debated since the early 1990s. It has been established that genetic changes can cause an “angiogenic switch” as the newly mutated cells acquire the ability to upregulate the production of angiogenic factors in comparison to healthy cells, especially in hypoxic niches (Shweiki et al., 1995; Catalano et al., 2013).

Studies using colorectal spheroids and the 5-Fluorouracil drug have indicated that hypoxia and necrosis induction is associated with tumor progression and cell resistance to chemotherapy treatments. The difference in the spheroid size is a variation that also shows its importance in determining whether the mentioned effects moderately or intensely impact the aggressiveness of the tumor (Karlsson et al., 2012; Däster et al., 2017). On the contrary, other studies developed with multicellular spheroids also have demonstrated that when hypoxia-reoxygenation is induced, the levels of vascular endothelial growth factor (VEGF) are downregulated by the tumor cells, as well as it activated DNA damage repair markers (Kondoh et al., 2013; Riffle et al., 2017).

Nevertheless, managing these elements and controlling the long-term viability of the spheroids is an arduous task due to their natural propensity of apoptosis, as a result of poor gaseous and nutrients diffusion (Zhang W. et al., 2016). One possible solution is to use microfluidic systems to allow continued flow

of the molecules needed for the spheroids to keep metabolizing and proliferating (Moshksayan et al., 2018). Human lung adenocarcinoma A549 cells, for instance, can be seeded with human endothelial cells in a collagen-I–Matrigel microfluidic device containing a micro-pump to supply the system with oxygen and nutrients. It is a useful protocol for further respiratory system cancer studies (Lee et al., 2019).

Colorectal tumors are likely to be formed at elderly ages, especially over 50 years. It is also the third cause of death among men and women in the United States (Siegel et al., 2020). The communication promoted by cells in the spheroid allows studies to explore the interactions between drugs and the 3D model (Elliott and Yuan, 2011). Concerning this approach, it was shown through spheroid models that the anticancer drug KP1339 triggers an immune cell death *in vitro*, which matches arrays that showed preclinical activity *in vivo* (Wernitznig et al., 2019).

Coming up with a model that mimics the microenvironment of mammary tissue requires a complex mixture of several cell types and tissues, as well as functional ECM and long-term sustainable cell–cell and cell–ECM interactions. In this regard, adipose tissue might work well when co-cultured with mammary cell lines (Kim et al., 2004; Picollet-D'hahan et al., 2016). As a complex tissue, adipose is constituted of several populations of cells such as adipocytes, MSCs, endothelial progenitor cells, pre-adipocytes, lymphocytes, pericytes, and macrophages (Schäffler and Büchler, 2007; Hu and Polyak, 2008).

Studies with co-culture between MCF7 line and MSCs have shown that this mesenchymal population can improve tumor aggressiveness *in vivo* in comparison with MCF7 culture alone. Similar to immune cells, MSCs demonstrate tropism for spots consisting of damaged tissue including tumor microenvironmental sites, cooperating with migration and metastasis (Koellensperger et al., 2017; Chen et al., 2019).

Tumor Organoids

Different from spheroids, tumor organoids must be derived from human tumor biopsies (Drost and Clevers, 2018; Wang et al., 2020). The advantages and applications of tumor organoids are related to the tissue-specific mutagenic processes accumulating specific types of somatic mutations during malignant transformation in patients. Single stem cell-derived and long-term-cultured organoids were used to determine the genome-wide mutation patterns in distinct healthy stem cells (Wang et al., 2020).

The ability to grow organoids with high efficiency from healthy human adult stem cells has paved the way to grow tumor tissue patient-derived organoids (PDO). So far, long-term organoid cultures have been established from primary colon, esophagus, pancreas, stomach, liver, endometrium, and breast cancer tissues, as well as from metastatic colon, prostate, and breast cancer biopsy samples (Drost and Clevers, 2018).

Another 3D model is the cultivation and testing of the patient-derived tumor xenografts (PDX) generated in animal models. PDX is about the implantation of small pieces of tumors from human biopsies into highly immunodeficient mice. After tumor growth, the tumor is transferred into secondary recipient mice. PDXs often maintain the structures of the original tumors at

molecular, cellular, and tissue levels (Drost and Clevers, 2018). Thus, it is able to recapitulate the heterogeneity of the tumor and its native microenvironment; however, it is more incompatible to HTS due to its expensive, time consuming and complex procedure (Hidalgo et al., 2014). Besides PDX, it is also possible to induce the tumor directly into animal models. However, animals present great phylogenetic distance to humans, have different metabolism, size, and lifespan, which all misdirect the drug development during human clinical trials (Wang, 2019).

The generation of cancer spheroids and organoids, like PDO are low cost, fast compared with PDX, can be adapted to HTS and allow investigation of the alterations occurring during the initiation and progression of tumorigenesis (Fatehullah et al., 2016; Fan et al., 2019). This is one of the reasons why tumor organoids have been increasingly used as a faithful *in vitro* model system to study cancer metastasis (Fan et al., 2019).

Tumor organoids keep the main pathophysiological features required to identify the critical factors in the acquisition of cancer metastatic potential, which may elucidate mechanisms involved in the metastasis cascade (Fan et al., 2019). On the other hand, one of the intrinsic limitations is the lack of stroma, blood vessels, and immune cells in cultured organoids, especially the immune cells (Wang et al., 2020) due to their regulatory roles in epithelial cell growth and differentiation, invasion, and metastasis (Mueller and Fusenig, 2002; Sawant et al., 2016).

One very interesting strategy when studying tumor organoids is to associate healthy organoids with tumor ones in a fluidic platform called organ-on-a-chip aiming to study metastasis via the circulatory system. These devices mentioned before are microfabricated to emulate a precise microenvironment, controlled, with continuous flow perfusion culture, and high-throughput format (Fan et al., 2019). Jeon et al. (2015) studied 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation, while Xu et al. (2016) projected a four-organ chip to assess lung cancer metastasis. Huang et al. (2009) found out that the laminar flow properties of microfluidic devices have been leveraged to compartmentalize human mammary fibroblasts in an ECM gel side-by-side with another ECM gel containing breast ductal carcinoma *in situ* cells; this setup revealed that the fibroblasts had to be in contact with the tumor cells to induce the transition to the invasive phenotype (Huang et al., 2009; Benam et al., 2015).

High-Throughput Screening and 3D Models

Pre-clinical studies fail around 85% in the oncological drug trials, not demonstrating sufficient safety or efficacy (Gao et al., 2015). To overcome this issue, the approaches that enable high-throughput (thousands of cells per experiment) are best suited to efficiently sample the complex cellular diversity in organoids and to understand organoid-to-organoid variability (Brazovskaja et al., 2019).

High-throughput screening provides a practical method to investigate large numbers of pharmaceutical compounds in *in vitro* monolayers assays, being a universal assay in pharmaceutical and Biotech industries (Pereira and Williams,

2007). It has also spawned a billion-dollar industry that supports the increasing demands for speed, capacity, and cost-effective screening of vast libraries of compounds (Pereira and Williams, 2007). The accessibility of HTS data merged with the ToxCastTM/Tox21 databases allows for elucidative toxicological considerations seen below (Suh et al., 2018).

Based on the advantages of tissue engineering scaffold-free approaches in recapitulating the tumor microenvironment, mainly represented by spheroids and organoids, a paradigm shift in HTS placing them at the forefront of drug discovery (Li et al., 2016) together with the need to adapt the protocols for the HTS. Spheroids have been adapted for use with several HTS technologies. On the other hand, organoids represent a challenge, mainly due to the presence of hydrogels and their heterogeneity of shape (Figure 1). Furthermore, the most common read-out of HTS technologies is still based on imaging systems making spheroids and organoid depths and their associated light scattering a technical challenge (Li et al., 2016).

So far, tumor spheroids, tumor organoids, and PDTXs are allowed for testing of multiple individual drugs prior to *in vivo* analysis (Beshiri et al., 2018). Gao et al. (2015) established ~1,000 PDXs with a diverse set of driver mutations against 62 treatments across six indications. Mateo et al. (2015) showed the presence of a homologous recombination deficiency genotype in Metastatic castrate-resistant prostate cancer and predicted responsiveness to Olaparib, which is the first genomic biomarker-driven therapy on track for FDA approval. Another example is for human kidney organoids, where Czerniecki et al. (2018) produced automated organoids and assessed drug effects by HTS.

Liu et al. (2020) review that Kita et al. (2019) screened 2,098 compounds in bladder cancer organoid cell lines. They also discovered that Disulfiram, an anti-alcoholism drug, and cisplatin had a cooperative effect. Lampis et al. (2018), after screening 484 compounds in six cholangiocarcinoma's organoid cell lines, presented that the sensitivity of HSP90 inhibitors was related to the mutation of MIR21 gene. Kondo and Inoue (2019) reported an advanced system for the HTS of 2,427 drugs using the cancer tissue-originated spheroid; those lines exhibited diverse sensitivities to the hit compounds, demonstrating the usefulness of this system for investigating highly heterogeneous disease.

There is now increasing evidence that the tumor microenvironment affects the efficacy of drugs on the cancer cells (Lal-Nag et al., 2017). Several complex ovarian cancer models have already been published, such as the 3D omental mesothelium model and models that include microfluidics, which demonstrates this (Watters et al., 2018). Currently, the mesothelium model is the only 3D organotypic microenvironment model of ovarian cancer that is used by multiple research groups (Kenny et al., 2007). The mesothelium model recapitulates the main physiological aspects of ovarian cancer cells in the mesothelium lining (Watters et al., 2018). Lal-Nag et al. (2017) proved that several classes of targets were more efficacious in cancer cells growing in the absence of the metastatic microenvironment, and other target classes were less efficacious in cancer cells in pre-formed spheres compared with forming spheroids cultures. These methods were adapted to HTS and to more than 100,000 small-molecule compounds that

can potentially identify novel treatments (Watters et al., 2018). Hasan and group reported the use of bioprinting for *in vitro* ovarian cancer tissue modeling for research applicable to HTS. Human ovarian cancer was printed on MatrigelTM to form multicellular acini (Hasan et al., 2011). This approach allows for physiologically relevant cell fabrications and can also provide an alternative to animal testing (Matai et al., 2020).

Tumor Organoids and Personalized Medicine

As explained in the sections before, tumor organoids must be derived from human biopsies. This outstanding characteristic from tumor organoids has given rise to the creation of tumor biobanks highlighting the concept of personalized medicine to predict effective drugs before the start of the treatment. One crucial challenge to be addressed related to drug testing for cancer models is that the majority of drugs show intratumor heterogeneity, while others are uniformly toxic in all cases. Furthermore, as organoids can be produced from a patient's own cells, the genetic analyses and drug screening results will be specific to the patient's tumor (Tellez-Gabriel et al., 2018; Kondo et al., 2019). Some examples are described below.

An organoid biobank of breast cancer tissues from >100 patients was established (Sachs et al., 2018). These organoids represented genetic and histopathological features of breast cancer and maintained the expression of breast cancer biomarkers. This means organoid biobanks have predictive value for drug efficacy in the treatment of individual patients (Wang, 2019), allowing personalized cancer treatment.

Van de Wetering et al. (2015) established tumor organoid cultures from 20 consecutive colorectal carcinoma patients. The results showed that organoids were able to resemble the original tumor characteristics, and gene expression analysis indicated that the majority of consecutive colorectal carcinoma molecular subtypes were properly represented.

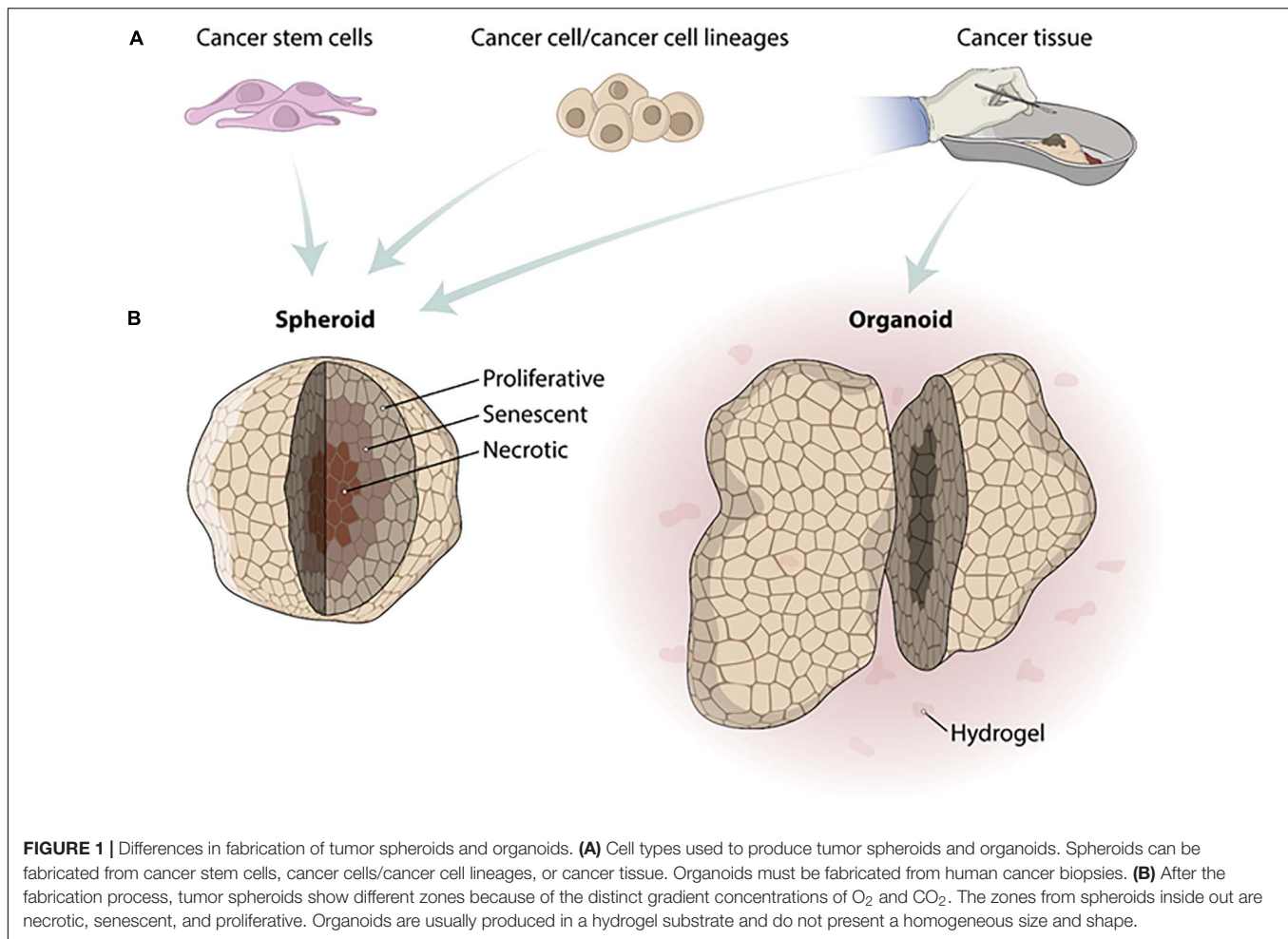
Sachs et al. (2018) described a protocol to produce a biobank of human mammary epithelial organoids. The organoids were able to recapitulate the diversity of the disease. Additionally, histological, hormonal, and gene expression analysis resembled the status of the original tumor. Furthermore, the organoids allowed proper drug screening tests when compared with *in vivo* xeno-transplantations.

Yan et al. (2017) developed a primary gastric cancer organoid biobank that comprises normal, dysplastic, cancer, and lymph node metastases from 34 patients. The results showed that organoids were able to closely mimic the morphology, transcriptome, and gene expression profiles when compared with *in vivo* original tumors. It was also seen that organoids were sensitive to unexpected drugs (recently approved or in clinical trials) after drug screening tests.

3D BIOPRINTING

Background

As discussed previously, 3D cell culture models as spheroids and organoids are capable of better mimicking the tumor



microenvironment that is found *in vivo* by recapitulating cellular and molecular events. However, spheroids and organoids follow a non-guided spontaneous formation of tissues and organs by self-assembly mechanism. In this context, because of the ability to precisely guide and organize the position of different cell types and growth factors and also perfusable networks, 3D bioprinting has a potential to improve current models and guide recapitulation of the tumor microenvironment (Datta et al., 2020). The ability to engineer controllable cancer tissue models in high resolution can considerably accelerate cancer research and improve personalized medicine, improving the treatment and life expectancy of cancer patients in the future (Knowlton et al., 2015; Belgodere et al., 2018; Langer et al., 2019).

3D bioprinting is one of the most widely used technologies in tissue engineering and regenerative medicine to develop complex tissues and organs that mimic their native microenvironment (Murphy and Atala, 2014; Moroni et al., 2018). As 3D bioprinting is a process where bioinks, usually composed of hydrogels, and cells are turned into functional tissue-engineered constructs from digital models, it is constantly showing more advantages compared with classical scaffold-based tissue engineering. One of the principal aims of using

3D bioprinting techniques so far is to biofabricate vascular structures (Vijayavenkataraman et al., 2018). This technique integrates biomaterials, living cells, and automated controlled systems to create complex microstructures and precise control over the structures developed compared with other currently available methods (Mandrycky et al., 2016).

Usually, 3D bioprinting begins with a computer-assisted process in order to deposit biologically relevant biomaterials, growth factors, and living cells to generate a desired tissue or organ model. Basically, it is possible to divide the 3D bioprinting process in three: (1) pre-processing for acquiring the 3D computer-aided design (CAD) model of the tissue to be bioprinted, (2) automated deposition of cells, spheroids, biomaterials, or other biological component of interest, and (3) maturation of the tissue constructs (Zhang Y. S. et al., 2017; Datta et al., 2018).

The principal 3D bioprinting techniques are (1) inkjet, (2) extrusion-based, and (3) laser-assisted bioprinting (Huang et al., 2017). In inkjet bioprinting, it is possible to precisely control both the size of the desired tissue pattern, as well as the generated droplets. In this way, it is possible to determine the volume, size, and quantity of a sample to be bioprinted. In terms of precision, it is possible to control the number of the cells per

droplet, which is an advantage when scaffolds are being used (Zhang and Zhang, 2015).

Extrusion-based bioprinting is the most used technique that uses the principles of a fluid-dispensing system with a robotic one for extruding materials, which can then be applied to different 3D bioprinting approaches. The fluid-dispensing system can be directed by pneumatic, mechanical, or solenoid forces. Through extrusion-based bioprinting, it is possible to precisely deposit cells, which can be encapsulated in a pre-established design of geometrical filaments and then bioprinted (Ozbolat and Hospodiuk, 2016). However, one of the biggest challenges of this technique is the resolution level that can be reached (Ning and Chen, 2017).

Laser-assisted bioprinting is based on the laser-induced forward transfer (LIFT) principle and is considered a “direct-write” method, which can precisely control the virtual deposition of cells, growth factors, and biomaterial containing droplets at a MHz range speed. Therefore, with this technique, it is possible to achieve high resolution (Devillard et al., 2014). However, the principal disadvantage is the use of the laser directly on the cells, which can damage cell viability (Derakhshanfar et al., 2018).

In order to authentically develop the desired tissue construct, the hydrogel choice is crucial, mainly because the hydrogel will provide the physical and biochemical properties to guide cell proliferation, differentiation, and the final maturation of the engineered construct. In this way, the hydrogel must contain similar properties of the desired tissue when *in vivo* (Gopinathan and Noh, 2018). Several hydrogel formulations have been developed, such as decellularized ECM, alginate, gelatin, hyaluronic acid, and polymers (such as methacrylated gelatin, polyethylene glycol and poly lactic acid) to serve as functional bioinks (Parak et al., 2019).

Scaffold-Free 3D Bioprinting

The use of bioinks is the foundation of bioprinting. This approach is based on cells and/or biomaterials with specific formulations for each type of cell (Hospodiuk et al., 2017). The ideal formulation of bioinks should meet each cell type's biological requirements without toxicity to the cells (Gungor-Ozkerim et al., 2018). Their desired properties include printing, mechanical properties, biodegradation, and post-bioprinter maturation (Hong et al., 2018). These properties depend on different parameters such as solution viscosity, surface tension of the bioink, the ability to interconnect on its own, and the properties of the printer nozzle surface. The living cells encapsulated in the bioink grow and occupy the space to form predefined tissue structures (Huang et al., 2017; Gungor-Ozkerim et al., 2018).

However, an important limitation of this approach is that, although cells can be manipulated individually, they do not form mechanically stable assemblies in many cases unless intercellular adhesions are made very strong, possibly by chemical means, which is not ideal for mimicking the tissue microenvironment (Goulart et al., 2019). Additional structural cohesion needs to be produced by the cells, like their own secreted ECM. However, this is a long-term process and depends on the cell type and the ECM deposition quality (Ong et al., 2018; Heo et al., 2020).

The alternative approach of using cells with a pre-assembly of spheroids has been widely studied, as it improves the production capacity of its ECM, in addition to providing greater biomechanical cohesion in larger-scale constructs for bioprinting (Swaminathan et al., 2019). Also, spheroid-based methods are generally milder and, therefore, induce much less or no cell damage during bioprinting (De Moor et al., 2018). Another attractive feature of spheroid bioprinting is its efficiency, as the speed of bioprinting can be increased using large building blocks such as spheroids (Gutzweiler et al., 2017).

An alternative method, still considered to be scaffold-free, can provide temporary support to the spheroids and, thus, facilitate their fusion and maturation in tissue models using a set of microneedles (“Kenzan”). The Kenzan bioprinting method provides a high-resolution biofabrication process, facilitating the fusion of spheroids into larger tissue constructions in a needle matrix removed after spheroid fusion. This method is used in the Bio-3D Regenova Printer marketed by Cyfuse Biomedical (Moldovan, 2018; Murata et al., 2020).

Recently, bioinks were developed using formulations composed only of spheroids with several thousand cells. Studies have shown that spheroids can form tissue threads up to 8 cm in length with rapid spheroid fusion without using aggressive chemicals as crosslinkers or as support materials (Bakirci et al., 2017; Ji and Guvendiren, 2017; Osidak et al., 2019). The spheroid bioinks showed better results than the individual cells because they preserved the integrity of the ECM. The use of bioinks without structure, composed only of cells, has been attracting more and more attention as a bioprinting method for the 3D construction of complex tissues, through which the application of ball-beading constructions is widely addressed (Skardal et al., 2016).

Several spheroid bioprinting techniques have been reported in the literature. One of the first techniques widely explored was extrusion-based bioprinting, in which the spheroids were loaded into a syringe cylinder and extruded into a controlled distribution gel medium. However, the spheroid tips easily deform in the syringe and are subject to breakage during the extrusion process. Simultaneously, the support structures need to be printed in 3D to facilitate the aggregation of extruded ball tips (Mandrycky et al., 2016). A significant advance was made using the Kenzan method. However, the method has limitations inherent to the accuracy of the 3D bioprinting process (Moldovan et al., 2017). To overcome some of the greatest challenges of the current techniques, recent studies have shown that aspiration-assisted bioprinting allows accurate bioprinting of spheroids over sacrificial or functional gel substrates (Chimene et al., 2016). In a mold of sacrificial material, such as alginate or agarose, the material is discarded as the bioprinted tissue matures and subsequently deposits its components in the ECM (Ayan et al., 2020).

Although methods using sacrificial gel substrates do not present the common problems of inkjet and microextrusion (such as nozzle clogging), they still have their technical limitations (Vijayavenkataraman et al., 2018; Adhikari et al., 2021). One of these limitations is the time necessary to form and mature spheroids prior to bioprinting. In addition,

the development of specific bioinks compatible with the characteristics of most spheroid types is essential for the viability and correct maturation of each tissue (Li et al., 2016).

Large-scale production is also still a great challenge (Datta et al., 2020). Studies on standardization and automation of spheroid production are essential for building more complex and genuine-sized tissues in the future. Moreover, an important implication in biofabrication is training for the unique skills and techniques required of this technology's users and operators. Finally, the bioprinting of 3D cell constructs originating from spheroids composed of various types of cells has been studied to increase the functionality of these 3D constructs (Sasmal et al., 2018; Swaminathan et al., 2019).

3D Bioprinting of Tumor Models

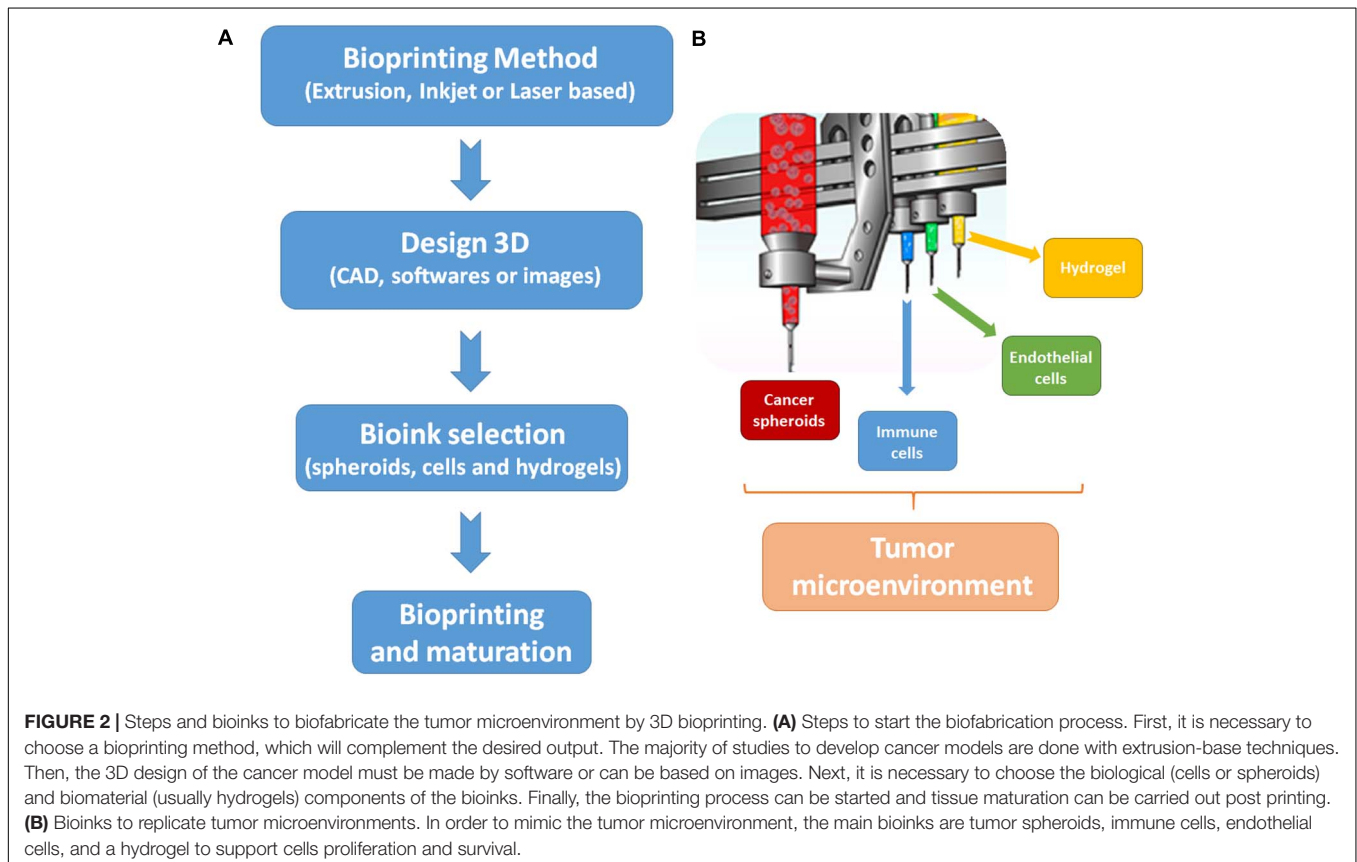
For recreating the tumor microenvironment, there is a need of tumor ECM reconstitution and the recreation of tumor vasculature (Liu et al., 2020). In **Figure 2**, the essential steps and bioinks to recreate the tumor microenvironment by 3D bioprinting are proposed. The ECM of the tumor microenvironment is composed of different proteins and stromal cells, but it is known that the composition of tumor ECM is tumor and patient specific. In addition, the biomechanical properties of tumor ECM can regulate tumor behavior and progression (Zhang Y. S. et al., 2016).

Recently, a considerable number of studies were performed to develop tumor models by 3D bioprinting. **Table 1** reviews

some of these studies. Dai et al. (2017) focused on replicating tumor microenvironments by improving tumor and stromal cell interactions in 3D bioprinted constructs. Their strategy relied on the self-assembly of multicellular heterogeneous brain tumor cell fibers by extrusion-based bioprinting. These fibers were part of the tumor ECM of the brain tumor. The morphological results showed that the construct was viable, proliferative, and presented tumor-stromal cell interactions. Hermida et al. (2020) used extrusion-based bioprinting to engineer glioblastoma models made of cancer, microglia, and stromal cells bioprinted within alginate modified with RGDS cell adhesion peptides, hyaluronic acid, and type I collagen. The glioblastoma cells presented more resistance to chemotherapeutic drugs in 3D engineered bioprinted constructs compared with monolayer cultures.

Despite the development described above for spheroid bioprinting strategies, several studies have shown the spontaneous formation of spheroids after 3D bioprinting, reaching the mimicry of specific cancer types. Jiang et al. (2017) developed a proof of concept study by bioprinting a cross-linked alginate/gelatin hydrogel composed of breast cancer lineage cells and fibroblasts. After 1 week in culture, breast cancer cells formed viable spheroids that increased in size over time and attracted migrating fibroblasts through a matrix region of the hydrogel, which infiltrated the breast cancer spheroids.

Using the technique of 3D bioprinting named "laser direct write," Kingsley et al. (2019) used microbeads to allow the formation and growth of multicellular tumor spheroids with



homogeneous size and shape. The decellularized rat and human breast tissue ECM was used as a bioink for organoid formation by 3D bioprinting (Mollica et al., 2019). The principal advantage in this strategy is that these ECM hydrogels keep the structural and signaling cues of the breast cancer environment, which can determine a cell's fate. The results showed that the hydrogel supported the production of breast cancer cell organoids allowing their use to engineer more complex organoids models to pre-clinical assays.

Schmidt et al. (2019) compared the interaction of different bioprinted hydrogels with melanoma cells. In total, five hydrogels were tested: matrigel and two different types of commercially available bioinks, with or without RGD sequence/laminin mixture. In Matrigel, melanoma cells were able to spread,

proliferate, and produce networks in the construct, while in gelatin methacrylate melanoma cells grow in clusters. As expected, the choice of the bioink is crucial for the behavior of cancer cells in engineered constructs.

Human breast epithelial cell lines can be bioprinted as a cell suspension or as formed spheroids in alginate-based bioinks. These cells only formed spheroids in Matrigel-based bioinks and pre-formed spheroids kept their morphology and viability after bioprinting. When spheroids were formed, breast cancer cells were more resistant to drug assessment, replicating the tumor microenvironment (Swaminathan et al., 2019).

Maloney et al. (2020) used an immersion printing technique approach to perform the 3D bioprinting of tissue organoids in 96-well plates. The results showed that the bioink allowed the

TABLE 1 | Biofabrication of cancer models by 3D bioprinting.

Authors and year	Aim	Bioprinting technique	Bioink	Main result	Article title and journal
Dai et al., 2017	Improve tumor and stromal cell interactions by the development of 3D bioprinted constructs	Extrusion	Cancer cells within alginate, gelatin and fibrin	The construct was viable and resembled properly tumor and stromal cell interactions found in the <i>in vivo</i> tumor	Coaxial 3D bioprinting of self-assembled multicellular heterogeneous tumor fibers. <i>Scientific reports</i> .
Hermida et al., 2020	Engineer a 3D construct of a glioblastoma model by bioprinting	Extrusion	Cancer, microglia and stromal cells within alginate/RGDS, hyaluronic acid and collagen I hydrogels	The biofabricated glioblastoma model was functional and showed resistance to drugs	Three dimensional <i>in vitro</i> models of cancer: Bioprinting multilineage glioblastoma models. <i>Advances in biological regulation</i> .
Jiang et al., 2017	3D bioprinting of cells to produce complex spheroids models	Extrusion	Cancer and fibroblasts cells within alginate/gelatin hydrogel	The biofabricated spheroids were viable and increased in size over time	Directing the Self-assembly of Tumor Spheroids by Bioprinting Cellular Heterogeneous Models within Alginate/Gelatin Hydrogels. <i>Scientific reports</i> .
Kingsley et al., 2019	Biofabrication of tumor organoids by 3D bioprinting	Laser-assisted	Decellularized rat and human breast tissue extracellular matrix	The hydrogel supported the biofabrication of breast cancer cell organoids	Laser-based 3D bioprinting for spatial and size control of tumor spheroids and embryoid bodies. <i>Acta Biomaterialia</i> .
Schmidt et al., 2019	Biofabrication of melanoma constructs	Extrusion	Melanoma cells within matrigel, two different types of commercially available bioinks, with or without RGD sequence/laminin-mixture	The melanoma cells were able to spread, proliferate and create networks in the hydrogels	Tumor Cells Develop Defined Cellular Phenotypes After 3D-Bioprinting in Different Bioinks. <i>Cells</i> .
Swaminathan et al., 2019	Biofabrication of breast cancer spheroids by 3D bioprinting	Extrusion	Breast epithelial cells and alginate	Spheroids were formed and resistant to drugs, replicating better the tumor microenvironment	Bioprinting of 3D breast epithelial spheroids for human cancer models. <i>Biofabrication</i> .
Maloney et al., 2020	Biofabrication of glioblastoma and sarcoma organoids by 3D bioprinting	FRESH	Cancer cells within hyaluronic acid and collagen	The organoids were biofabricated, presented a spherical shape and can be used for drug screening tests	Immersion Bioprinting of Tumor Organoids in Multi-Well Plates for Increasing Chemotherapy Screening Throughput. <i>Micromachines</i> .
Han et al., 2020	Recapitulate tumor microenvironment with spheroids by 3D bioprinting	Extrusion	Fibroblasts and endothelial cells in gelatine, alginate and fibrinogen	Microvessel sprouting in the construct, increase of spheroids size and efficacy in drug screening tests	3D Bioprinted Vascularized Tumor for Drug Testing. <i>International journal of molecular sciences</i> .

TABLE 2 | Current 3D models for recapitulating tumor microenvironment.

3D models	Materials for 3D models preparation	Cell types	Mimicry level
Spheroids	- Plates and rotors for cultivation such as spinner flasks, rotary cell culture systems, liquid overlay, micropatterned plates, low binding plates, microfluidics device - Culture in the presence or absence of fetal bovine serum (FBS)	- Can be obtained from cancer stem cell (CSC) population, tissue-derived tumor spheres such as lung, bladder, prostate, or breast cancer tissue and uveal melanoma, including cell lineages - Some tumor cells form spheres spontaneously, while others require additional manipulations	+
Organoids	Cultured on diverse matrices such as Matrigel, collagen type I, HA (hyaluronic acid) hydrogel, PEG hydrogel, fibrin/laminin hydrogel	- Can be obtained from tumor cells isolated from tumor tissue such as metastatic colorectal carcinoma tissue, cervical carcinoma biopsy tissue, tumors of the gastrointestinal tract, prostate tumor cell lines - Can also be obtained from non-tumor organoids using gene-editing techniques	++
Bioprinting	- The 3D computer models containing information such as complex 3D geometries surface information can be created using MRI or CT scans - Bioreactors for tissue maturation in post-processing	Multiple types of cancer cells including primary cancer cells, circulating tumor cells, and stromal cells including fibroblasts, endothelial cells and stem cells can be used for printing personalized tumor construct	+++
3D models	Advantages	Disadvantages	
Spheroids	- Presence of gas, nutrient and pH gradients - Co-culture - Cultures without expensive cultivation methods - Reproduction of cell-cell and cell-ECM interactions - The screening of personalized drug can be performed with very small quantities of chemotherapeutic candidates	- Gradient structure complicates drug testing - Fragile structure - Difficulty of forming homogeneous spheroids - Cannot completely recapitulate the cellular and microenvironmental heterogeneity of physiological tumor tissue	
Organoids	- Reproduction of cell-cell and cell-ECM interactions - Co-culture Primary tumor cells - Long-term cultivation - Stable at passaging	- Gradient of gases, nutrients and pH is not always reproducible - Therapeutic responses may depend on the matrix - High cost method	
Bioprinting	- Enables the generation of cell laden cancer tissue constructs that can recapitulate the features of various types of cancers - Expressed characteristics of <i>in vivo</i> tumor tissues, such as high growth rates of cancer cells, aggressive invasiveness, angiogenesis, metastasis, high resistance to anticancer drugs. - Can supplement animal xenograft models because they maintain cancer-stromal cell interactions. - Can integrate perfusable vascular networks, automation and high-throughput testing - The inkjet bioprinting have low cost, fast printing and widely accessible - Non-contact and high cell viability in the Laser-assisted bioprinting (LAB) - Deposition of high-density cells in the Extrusion bioprinting	- A single bioprinting method cannot yet produce synthetic tissues and organs at all scales and complexities. - The inkjet bioprinting has drawbacks in terms of material viscosity. - The microextrusion bioprinting may need materials having crosslinking mechanisms or shear reduction properties not to affect cell viability - The difficulty in developing well-established vascular network within tumors - Require a labor-intensive and high cost - The inkjet bioprinting may have Nozzle clogging - Complex operation and time consuming preparation in laser-assisted bioprinting (LAB) - Low cell viability in extrusion bioprinting	
3D models	Adaptable to the HTS system	References	
Spheroids	+++	Sutherland, 1988; Wartenberg et al., 2001; Del Duca et al., 2004; Mazzoleni et al., 2009; Hardelauf et al., 2011; Li et al., 2011; Vinci et al., 2012; Froehlich et al., 2018; Ruiz et al., 2019	
Organoids	+	Fujii et al., 2016; Nanki et al., 2018; Xu et al., 2018; Fan et al., 2019; Lin et al., 2019; Nunes et al., 2019; Fiorini et al., 2020	
Bioprinting	N/A	Hopp et al., 2005; Koch et al., 2010; Gruene et al., 2011; Murphy and Atala, 2014; Orloff et al., 2014; Mandrycky et al., 2016; Zhu et al., 2016; Peng et al., 2017; Mohammadi and Rabbani, 2018; Datta et al., 2020; Emmermacher et al., 2020	

+, means less mimicry; ++, means intermediary mimicry; +++, higher mimicry.

maintenance of the organoid structure. In the study, the bioink was composed of hyaluronic acid and collagen and was printed in a support bath made of gelatin. This innovative strategy, named as “Freeform Reversible Embedding of Suspended Hydrogels” (FRESH) is being largely explored to bioprint soft tissues without

a scaffold, because it allows the maintenance of the biological structure after the removal of the support bath. To the best of our knowledge, this is the only technique at the moment that can be used to bioprint organoids. More importantly, the authors proved with patient-derived glioblastoma and sarcoma organoids

that it is possible to use the method for drug screening tests *in vitro*.

Han et al. (2020) used 3D bioprinting to recapitulate the tumor microenvironment using spheroids. The method consisted of the biofabrication of a blood vessel layer engineered by fibroblasts and endothelial cells in gelatin, alginate, and fibrinogen, followed by the seeding of multicellular tumor spheroids of glioblastoma cells onto this blood vessel layer. The main results showed the sprouting of blood vessels with an increase in spheroid size. In addition, drug testing was performed and the biofabricated construct was sensitive to the treatment, showing that it can be used for drug efficacy tests *in vitro*.

However, there is an important limitation of these models that use hydrogels for drug testing. HTS analysis based on luminescence/fluorescence cannot be applied to these models due to the presence of hydrogels which are high viscous biomaterials. Another issue related to hydrogels is the small volume used in some applications because it can impair HTS tests (Yu et al., 2018). **Table 2** summarizes the main characteristics, advantages, and disadvantages of 3D models described in this review.

PERSPECTIVES

Some studies already used 3D bioprinting to develop successful tumor models; however, to the best of our knowledge, the use of spheroids as a printable bioink to biofabricate tumor models has not been largely explored yet. As spheroids are a 3D model with complex cell-to-cell and cell-to-extracellular matrix interactions, it would be advantageous to use them as the main component of the bioink associated with the stromal components and immune cells. Tumor organoids show the main advantage of being derived from human cancer biopsies; however, their 3D bioprinting is still in its infancy due to their shape heterogeneity, lack of reproducibility, and complexity.

Furthermore, patient-derived 3D bioprinted tumor models could be successfully used for *in vitro* drug screening of anticancer drugs in large scale. However, some challenges need to be addressed before this step, especially related to the hydrogel composition. Some studies are already exploring how to optimize the hydrogel to not impair HTS tests and analysis (Barata et al., 2016; Sarkar and Kumar, 2016; Lee et al., 2018). In addition, ongoing studies are focusing on the development of combined microfluidic/bioprinted constructs to minimize the cost and facilitate HTS of a large number of cancer drugs for a particular patient in order to improve personalized medicine approaches (Augustine et al., 2021).

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CONCLUSION

3D bioprinting is a recent and innovative approach that offers the ability to create highly complex hierarchical 3D constructs with cells, biomaterials, and growth factors. Bioprinting methods have been developed and optimized in recent years in order to accurately replicate the morphology, functions, and physiology of a specific tissue and their *in vivo* microenvironment. As tumor microenvironments are complex in cell and extracellular matrix composition, 3D bioprinting holds great potential for applications in cancer research, in order to mimic more reliable tumor models and their vasculature (Knowlton et al., 2015; Albritton and Miller, 2017).

The use of 3D bioprinting can allow the positioning of tumor spheroids or organoids and the surrounding stromal and immune cells, commonly associated with this complex tumor microenvironment. The recapitulation of tumorigenesis will provide more reliable results to drug screening tests (Satpathy et al., 2018; Meng et al., 2019) and personalized medicine (Ma et al., 2018).

AUTHOR CONTRIBUTIONS

GK and LB contributed to conception and design of the manuscript. GK, GM, RT, and LB wrote the first draft of the manuscript. GK, GM, RT, and UK wrote sections of the manuscript. BM designed the table and performed the formatting of the manuscript. LB performed the main corrections and revised the manuscript. All the authors contributed to manuscript revision, read, and approved the submitted version.

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Cancer Nanomedicine and Immune System—Interactions and Challenges

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Nanoparticles have tremendous therapeutic potential in the treatment of cancer as they increase drug delivery, attenuate drug toxicity, and protect drugs from rapid clearance. Since Doxil®, the first FDA-approved nanomedicine, several other cancer nanomedicines have been approved and have successfully increased the efficacy over their free drug counterparts. Although their mechanisms of action are well established, their effects towards our immune system, particularly in the tumor microenvironment (TME), still warrant further investigation. Herein, we review the interactions between an approved cancer nanomedicine with TME immunology. We also discuss the challenges that need to be addressed for the full clinical potential of ongoing cancer nanomedicines despite the encouraging preclinical data.

Keywords: tumor microenvironment, immunogenicity, hypersensitivity, cytotoxicity, drug development

TUMOR IMMUNOLOGY

Tumor immunology is the interaction between cells of the immune system with tumor cells which lead to our understanding in the mechanisms of both tumor rejection and tumor progression (Copier and Dalglish, 2013). In cancer, tumors may undergo “spontaneous regression” in which a tumor disappears on its own. This phenomenon can be attributed to the active immune system that is triggered by a secondary immune stimulation such as an active infection, which can then initiate an antitumor cell immune response (Tadmor, 2019).

In principle, our immune system protects us against cancer through three primary roles which are 1) elimination of the potentially virus-induced tumor infection, 2) prompt resolution of inflammation that is conducive for tumorigenesis, and 3) identification and elimination of tumor cells based on their expression of tumor-specific antigens (Swann and Smyth, 2007). The third process is called immune surveillance that ideally eliminates all tumors promptly upon identification of their antigen. However, some malignancies appear to escape immune surveillance by either inducing tolerance rather than an active immune response or the immune system eventually is too overwhelmed and hence the tumor progresses (Ostrand-Rosenberg, 2008; Mak et al., 2014).

In immune surveillance, tumor antigens (TAs) play important parts in the development of the tumor microenvironment (TME). They generally fall into two classes, tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs), TAs are presented by major histocompatibility complex (MHC) I and II on the surface of tumor cells and trigger immune response in the host (Mak et al.,

2014). TAAs are normal proteins or carbohydrates expressed in a way that is abnormal relative to its status in the healthy, fully differentiated cells in the surrounding tissue of origin. For example, they may be expressed in abnormal concentrations and at wrong locations and times. Meanwhile, TSAs are new macromolecules that are unique to the tumor and are not produced by any type of normal cells. Due to their non-self nature, TSAs constitute true immunogens capable of eliciting an immune response. Overall, TAs can be categorized into several types including oncofetal, oncoviral, overexpressed or accumulated, cancer-testis, lineage-restricted, mutated, post-translationally altered, and idiotypic (Zarour et al., 2003). Hence, identification of TAAs and TSAs serve as a reliable biomarker for tumor diagnosis as well as a target for the development of cancer vaccines (Aly, 2012).

In the TME, there are two possible interactions that might happen. First is the antitumor immunity that works to prevent tumorigenesis in the first place (Munhoz and Postow, 2016). In antitumor immunity, both innate and adaptive immune responses are activated by TAs leading to tumor control. In this immunity, leukocytes such as tumor-infiltrating lymphocytes (TILs) which are mature CD4⁺ or CD8⁺ or B cells directly respond to the presence of a tumor cell (Mak et al., 2014). The second interaction is the evasion of antitumor immunity or immune escape as the immune system does not always succeed in controlling tumorigenesis. It is widely accepted that tumor immunoediting is a dynamic process that not only involves antitumor immunity, but shapes the immunogenicity of developing tumors as well. There are three distinct phases of tumor immunoediting which are elimination, equilibrium, and escape (Muenst et al., 2016). All three phases of tumor immunoediting are manifested through metabolic and cellular changes, in which the differences influence different types of cancer (Teng et al., 2008; Wenbo and Wang, 2017).

Elimination is a phase where evolving tumors are successfully rejected by the innate and adaptive immune response through various mechanisms (IFN γ , Perforin, TRAIL, IFN α/β , NKG2D) (Swann and Smyth, 2007). Then, some of the tumor cells that are not completely eliminated may enter the equilibrium phase when the immune system controls tumor outgrowth and tumor cells enter a dormant state or continue to evolve over a period of time (Dunn et al., 2004). In this phase, the constant interaction of tumor cells with the immune system over a period of time may edit the phenotype of the developing tumor into a less immunogenic state (Teng et al., 2008). Being in this state, the tumor cells are no longer susceptible to immune attack and this is where the tumor cells may escape from immune control and proliferate in an unrestricted manner, leading to clinically apparent tumors (Muenst et al., 2016). According to Mak et al., 2014, there are two forms of escape from immune control that are thought to be associated with all TMEs, regardless of which leukocytes respond to the malignancy. First is the abnormal property of the tumor vasculature comprised of capillaries that wind in and out of a tumor mass that hinder leukocyte extravasation into the tumor site. The second form of escape is from the elevated levels of plasma TGF β that is established to promote malignant transformation of

fibroblasts and stimulate angiogenesis within the tumor, termed as immunosuppression.

DEVELOPMENT OF APPROVED NANOMEDICINE

Over the years, the Food and Drug Administration (FDA) in the US and its equivalent in the EU, the European Medicines Agency (EMA), have certified a number of nanomedicine-based drugs for cancer diagnostic and therapeutic purposes, and many other formulations are currently being evaluated (Martinelli et al., 2019). Worldwide, nearly 250 formulations based on the nanotechnology platform have been approved for the market or are in various clinical stages for evaluation (Bremer-Hoffmann et al., 2018) (Table 1). The approval process for nanomedicine in humans regulated by the FDA is essentially the same as for any other regulated drug, device, or biologic (Eifler and Thaxton, 2011). According to the FDA, development of a drug and its approval is categorized into three major phases as outlined in Figure 1. Following discovery of the material, the pre-clinical phase of testing usually involves animal studies to demonstrate the efficacy, safety, and toxicity profile and to identify appropriate dose ranges (Tinkle et al., 2014). The FDA approval process is time consuming, labor intensive, and rigorous, hence it is estimated that it takes approximately 10–15 years to develop a new medicine (DiMasi et al., 2003). For nanomedicine, the important aspect regarding its R&D, highlighted by the FDA, is the comprehensive characterization of the nanomaterial considering its efficacy, toxicity, and physiochemical properties (Bobo et al., 2016). These findings are compiled into an Investigational New Drug (IND) application for FDA consideration. Upon approval of an IND, clinical trials, which are divided into three phases, are conducted to determine the safety and efficacy of the new nanomedicine. Since 2005, more than 30 new and abbreviated drug applications involving nanomaterials have been approved by the FDA (D'Mello et al., 2017). This is remarkable for a newly developing field. By comparison, for recombinant proteins and for antibody-based therapeutics, it took almost 2 decades of developments before the first drugs started to make it to the market (Reichert, 2003). More than 50 drug products containing nanomaterials are FDA approved for clinical use and more than a dozen of them have been approved in the last decade (Bobo et al., 2016; D'Mello et al., 2017).

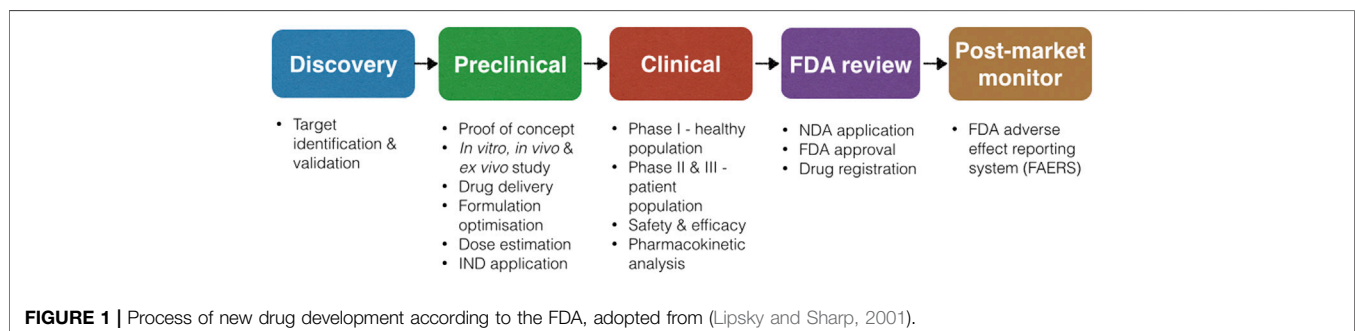
EFFECT OF NANOMEDICINE TOWARDS IMMUNE RESPONSES

Previously, most cancer therapies were designed to directly killed/removed tumor cells either by pharmacological agents, surgery, or radiotherapy. Then it moved to targeted therapy when specific drugs with some molecular targets such as selective kinase inhibitors and monoclonal antibodies were developed (Falzone et al., 2018). While these therapies significantly improved quality of life as well as survival of cancer patients, variable efficacy and

TABLE 1 | Approved nanomedicine in cancer.

Type	Nano medicine	Nanomaterial	Active substance	Indication	Approval year	Advantage	Reference
Liposome	Doxil/Caelyx	PEGylated liposome	Doxorubicin	Ovarian cancer	2005	Improved delivery	Barenholz (2012), Tejada-Berges et al. (2014)
				Multiple myeloma	2008	Decrease systemic toxicity	
	DaunoXome	Liposome	Daunorubicin	HIV-related kaposi sarcoma	1995	Less cardiotoxic	Pillai (2014)
				HIV-related kaposi sarcoma	1996	Improved delivery	
	Myocet	Non-PEGylated liposome	Doxorubicin	Metastatic breast cancer	2002	Decrease systemic toxicity	Batist et al. (2005)
	Marqibo	Liposome	Vincristine sulfate	ALL	2012	Less cardiotoxic	Pillai and Ceballos-Coronel (2013)
	Mepact	Liposome	Mifamurtide	Bone sarcoma	2009	Improved delivery	Hartmann et al. (2013)
	Onivyde	Liposome	Irinotecan	Pancreatic cancer	2015	Reduced AE	Havel et al. (2016)
Inorganic and metallic	Vyxos	Liposome	Cytarabine	AML	2017	Improved OS	Krauss et al. (2019)
				AML	2017	Improved OS	
	CPX-351	Liposome	Daunorubicin	Lymphomatous malignant meningitis	1999	Improved delivery	Patra et al. (2018)
	Depocyt	Liposome	Cytarabine	Lymphomatous malignant meningitis	1999	Decrease systemic toxicity	
	NanoTherm	SPION	Aminosilane	Glioblastoma	2010	Less invasive ablation therapy	Massadeh and Al Aamery (2016)
						Reduce risk of overtreatment	
	Protein	Abraxane	Albumin	Paclitaxel	Breast cancer	Increased solubility	Gradishar et al. (2005), Fu et al. (2009)
						Reduced IR	
Protein	Ontak	Recombinant DNA-derived cytotoxic protein	IL-2 and diphtheria toxin	Cutaneous T cell lymphoma	1999	Targeted delivery	Ventola (2017)
	Oncaspar	PEGylated protein conjugate	L-asparaginase	Acute lymphoblastic leukemia	2006	Improved stability of drug load	Brandenburg et al. (2020)
Polymer	SMANCS	Polymeric conjugate	Neocarzinostatin	Hepatocellular carcinoma	1994	Decrease toxicity	Maeda (2001)
	Genexol-PM	Polymeric micelle	Paclitaxel	NSCLC	2006	Controlled drug release	Guo et al. (2016)
	Eligard	Polymeric NPs	Leuprolide acetate	Breast cancer Ovarian cancer Advanced prostate cancer	2002	Targeted delivery — Controlled drug release Longer circulation time	Sartor (2003)

HIV—human immunodeficiency virus, SPION—superparamagnetic iron oxide nanoparticle, NSCLC—non-small cell lung cancer, AML—acute myeloid leukemia, ALL—acute lymphoid leukemia, PEG—polyethylene glycol, AE—adverse event, OS—overall survival, IR—immune response.



safety issues persistently limited the full capacity of cancer therapies. Nanomedicine offers these therapies a better targeting approach that would increase drug accumulation into a tumor without affecting other healthy cells, thus reducing systemic toxicities (Gao et al., 2019). Furthermore, nanomedicine is established to address several issues with current cancer therapies including the low response rate of free drugs as well as the emergence of drug resistance. Like the drug itself, introduction of NPs would induce a different interaction in the body, particularly with the immune system, either targeted or spontaneous.

Immunogenicity

Cancer chemotherapy is often immunosuppressive and drug resistance usually occurs after a short period of tumor shrinkage. Certain chemotherapeutic drugs such as doxorubicin have the potential to increase tumor immunogenicity through activation of immunogenic cell death (ICD). ICD is defined as the chronic exposure of damage-associated molecular patterns (DAMPs) in the TME, which provide long-lasting antitumor immunity (Zhou et al., 2019). Doxil is shown to increase the expression of CD80 on mature dendritic cells which activate an anti-tumor T cell response (Rios-Doria et al., 2015), improve macrophage immunostimulatory (M1) content in tumor tissue and efficacy of immune checkpoint blocking antibodies anti-CTLA-4/anti-PD-1 (Panagi et al., 2020), upregulate MHC-1 and Fas, and sensitize CTL killing and Fas-mediated death *in vitro* (Alagkiozidis et al., 2009). Meanwhile, Abraxane is taken up by macrophages *via* macropinocytosis which induces M1 cytokine expression and promotes nitric oxide synthase expression, thus increasing cytotoxicity towards tumor cells (Cullis et al., 2017). Furthermore, Abraxane is shown to enhance drug uptake and penetration into tumors *in vitro*, hence the superior efficacy in numerous cancer types compared to Taxol alone (Yuan et al., 2020).

Mepact is a liposome conjugated to a synthetic analog of a bacterial cell wall component and is used as an adjuvant in standard chemotherapy. This potent, non-specific immunomodulator mediates the activation of monocytes and macrophages, thus modulating the balance of immune responses such as increased circulating TNF and IL-6 (Punzo et al., 2020). Not only that, Mepact is demonstrated to be a possible anti-resorption agent by reducing pro-osteoporotic markers, thus explaining the improved overall survival from osteosarcoma (Ando et al., 2011; Bellini et al., 2017). Oncaspar, a PEGylated form of native *Escheria coli*-asparaginase is indicated for treatment of acute lymphoblastic leukemia. The PEGylation showed diminished asparaginase immunogenicity without affecting its enzymatic properties (Heo et al., 2019). Reduction of Oncaspar's immunogenicity is portrayed by the decrease of neutralizing antibodies that may induce hypersensitivity and/or loss of enzyme activity. Ontak is an engineered fusion protein of IL-2 and diphtheria toxin that targets the IL-2 receptor, such as CD25 on tumor-infiltrating cells regulatory T cells (Tregs), the internalization releases diphtheria toxin, causing apoptosis (Foss, 2006). The effect of Ontak on immunosuppressive Tregs further enhances anticancer immune responses. Furthermore, CD25 that can be targeted by the IL-2 fusion protein on Ontak is also present

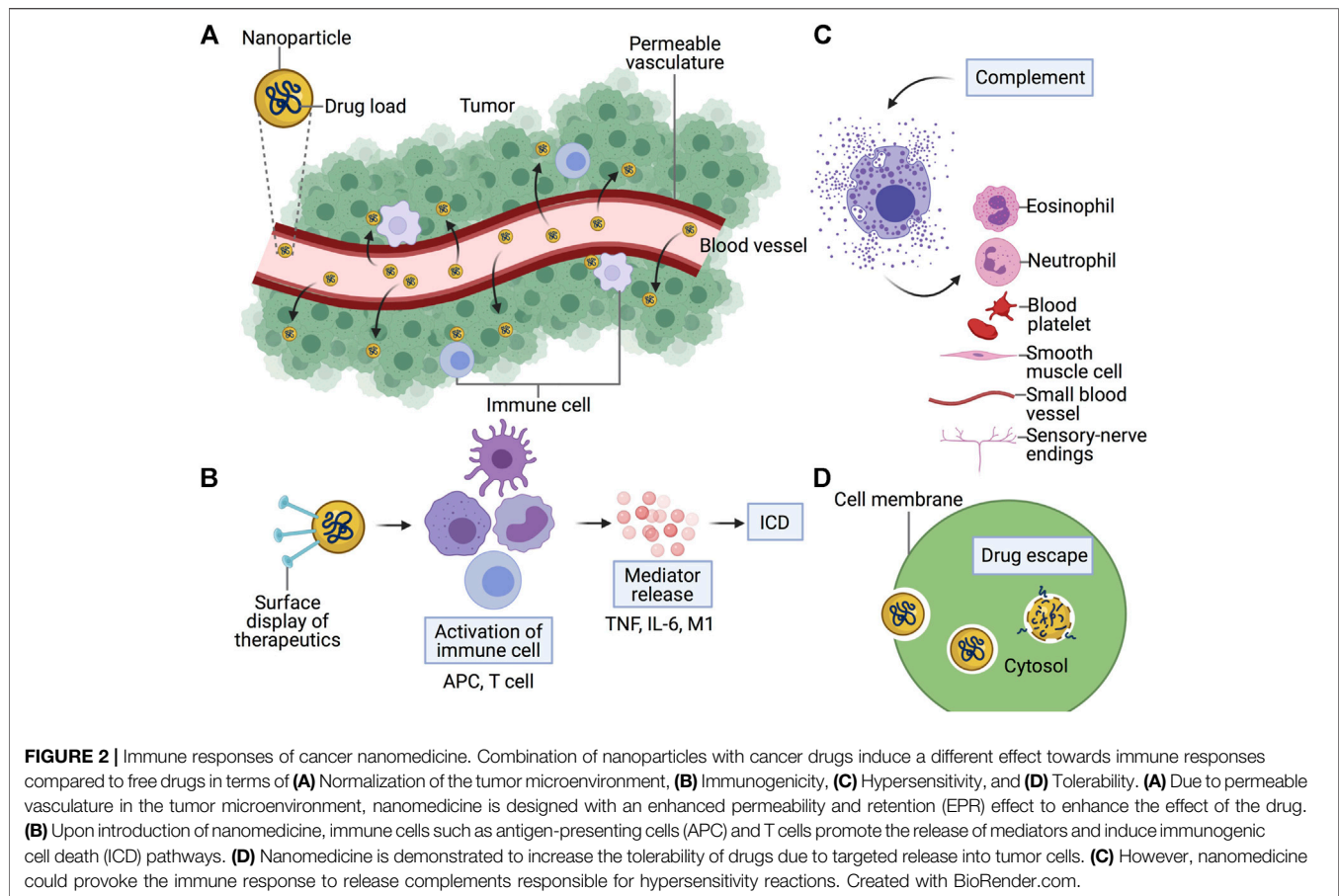
on lymphoid tumor cells and dendritic cells effector T cells, making this recombinant protein a great pharmacological intervention strategy (Lutz et al., 2014). However, due to production issues related to bacterial immunotoxin, Ontak was discontinued in 2014 although currently there are several Ontak-like formulations under development that use other bacterial expression systems (Shafiee et al., 2019).

TME Normalization

The tumor microenvironment (TME) consists of a complex ecosystem with blood vessels, immune cells, fibroblast, extracellular matrix, cytokines, and hormones that promote the growth of cancer. So, the normalization of the TME to a normal tissue environment may inhibit the growth of cancer and improve cancer therapeutics including checkpoint blockers and TNFR agonists. In *in vitro* studies, nanoparticles such as gold have been demonstrated to facilitate TME normalization, increase blood perfusion, and reduce hypoxia (Li et al., 2016; Li et al., 2017; Xiao et al., 2017). Instead of playing a role in TME normalization directly, the efficacy of nanomedicine is enhanced when adjuvanted with several approaches of TME normalization including anti-inflammatory agents, immune checkpoint blockade, and stromal and tumor vessel normalization (Zheng and Gao, 2019). Furthermore, studies showed that TME normalization improves the delivery of nanomedicine in a size-dependent manner (Chauhan et al., 2012). Delivery of Doxil, with a diameter of ~100 nm is hindered upon normalization of blood vessels by the VEGFR-2 blocker while enhanced delivery of the smaller diameter Abraxane was demonstrated, hence greater accumulation within the TME. Meanwhile, Onivyde, liposomal irinotecan, is shown to enhance accumulation of active metabolites within the TME, thus improving its antitumor activity with minimal systemic toxicity (Zhang, 2016). Another issue in pharmacological intervention in cancer that needs to be addressed is their defective vasculature. Due to this, macromolecules such as drugs could not be retained in tumor cells and leak out into interstitial space, limiting its efficacy. Nanoparticles, due to their physiochemical properties, could be utilized to address this issue using the principle of enhanced permeability and retention (EPR) (Maeda, 2017). SMANCS, a conjugate of a hydrophobic polymer with antitumor Neocarzinostin is the first nanomedicine using this EPR principle, was developed to selectively deliver drugs to solid tumors and prolong intratumoral concentration of the drug (Maeda, 2012).

Tolerability

Chemotherapy is known to induce several side effects such as myelosuppression, cardiotoxicity, and even skin toxicity which is a dose-limiting factor that often limits drug efficacy. Since chemotherapy suppresses the hematopoietic system and impairs its protective mechanism, neutropenia is one of the serious adverse events associated with the risk of life-threatening infections. Doxil is reported to be much less toxic to the immune system than free doxorubicin with comparable efficacy (O'Brien et al., 2004). In a systematic review, Abraxane is demonstrated to induce a higher number of hematological toxic effects (neutropenia, leucopenia, increased alanine aminotransferase and aspartate aminotransferase) and frequent



non-hematological toxic effects (peripheral sensory neuropathy) compared to the free drug-paclitaxel group (Zong et al., 2017). Abraxane is also reported to cause drug-induced immune hemolytic anemia, a rare but fatal adverse event that affects only one patient in a one million population (Thomas and Shillingburg, 2015). Marqibo is designed to overcome the dosing and pharmacokinetic limitation of Vincristine. Marqibo is demonstrated to increase the circulation time with targeted and intense delivery of Vincristine without augmented toxicities including hematologic toxicity (Deitcher et al., 2014). Most common adverse events for Onivyde and Vyxeos are neutropenia, abdominal pain, and diarrhea that are considered manageable, except for prolonged severe neutropenia in patients receiving Vyxeos (Zhang, 2016; Tzogani et al., 2020). Cardiotoxicity is another toxicity induced by chemotherapeutic drugs including nanomedicine. Doxil and Daunoxome are both demonstrated to reduce the rate of cardiotoxicity compared to their free drugs, Doxorubicin and Daunorubicin, which are significantly limited by dose-dependent cardiotoxicity (O'Brien et al., 2004; Fassas and Anagnostopoulos, 2009). Palmar-plantar erythrodysesthesia or hand-foot syndrome is a type of skin toxicity that could develop from some cancer treatments. This type of skin toxicity is demonstrated to often occur in patients receiving PEGylated liposomal doxorubicin such as Doxil (Huang et al., 2018; Ni et al., 2020). Polymer-based nanomedicine

including Genexol and Eligard are demonstrated to show good safety profiles in terms of the absence of increased toxicities and occurrence of adverse events (Sartor, 2003; Kim et al., 2004).

Infusion-Related Reaction

Hypersensitivity upon administration of a variety of drugs is common, including nanomedicine formulation. Doxil is reported to cause hypersensitivity, which is a non IgE-mediated allergy caused by activation of a complement referred to as complement activation-related pseudo allergy (CARPA) (Chanan-Khan et al., 2003). The mechanism of CARPA upon administration of Doxil is partly associated with some pre-existing anti-PEG antibodies (Neun et al., 2018). Since solvent-based taxane administration such as paclitaxel induces a high rate of hypersensitivity, albumin-bound paclitaxel, Abraxane, represents a valid treatment option as fewer hypersensitivity reactions towards Abraxane compared to free paclitaxel have been reported (Zong et al., 2017; Parisi et al., 2019). Unlike PEG, the absence of cross-reactivity between a previous history of hypersensitivity towards taxanes and Abraxane indicate the advantageous safety profile of this nanomedicine (Pellegrino et al., 2017). However, another approach of PEGylation of *E. coli* asparaginase in Oncaspar successfully reduced immunogenicity of the enzyme, which subsequently reduced the occurrence of hypersensitivity (Heo et al., 2019). As an immunomodulator, Mepact could activate immune responses

TABLE 2 | Advantages and challenges of nanoparticles to be translated into nanomedicine.

Advantages	Challenges
- As a non-invasive therapeutic vehicle or agent or device for theranostic application on human diseases Poirot-Mazeres (2011) - A smaller size of NPs helps in boosting the theranostic purpose in terms of increasing the drug dissolution rate, saturation solubility, and intracellular uptake of drugs in the human body Bawa (2011), Galvin (2012) - Enhancing bioavailability of drugs at specific sites in the right proportion for a prolonged period of time Galvin (2012) - Targeting only the diseased cells without affecting normal healthy cells Sajja et al. (2009)	- Less value was given to toxicity and safety of the patients Seigneuric et al. (2010) - Theranostic NPs can present unexpected toxic effects compared to usefulness Seigneuric et al. (2010) - Induction of oxidative stress and formation of free radicals lead to further damage of lipids, proteins, DNA, and other biological components through oxidation Bhaskar et al. (2010) - Accumulation, storage, and slow clearance of NPs from the body will lead to toxicity of the organs such as liver and spleen Seigneuric et al. (2010), Galvin (2012)

with a standby effect, thus causing a hypersensitivity reaction such as pericardial effusion (Şimşek et al., 2020). The mechanism of this reaction might be due to both the active and inactive ingredients of Mepact that target immune cells in the lungs (Anderson et al., 2010). The most common related effects for Mepact are chills, fever, and headache in the initial dose and delayed fatigue in the subsequent doses (Jimmy et al., 2017).

In conclusion, incorporation of NPs with cancer drugs induce a different effect towards host immune responses compared to free drugs, either intended or spontaneous. Its immunogenicity, normalization of the TME, tolerability, and other infusion-related reactions could be due to NPs' own physiochemical characteristics or interaction between the drugs (Figure 2).

CHALLENGES FOR CLINICAL USE

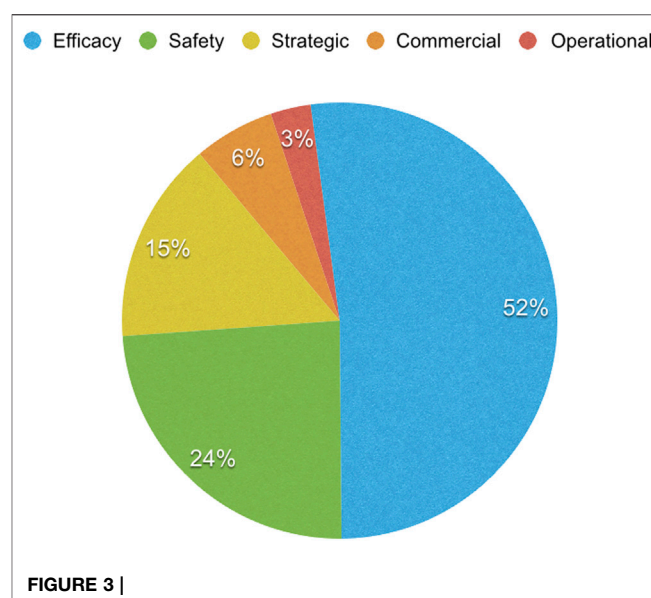
Although involvement of NPs in human clinical settings increased a decade ago, extensive research to improve biocompatibility and efficacy of NPs is still needed. Despite several challenges that need to be addressed in the application of NPs as a nanomedicine, its advantages outweigh those challenges, making NPs a highly potential tool (Table 2).

Despite the abundance of encouraging experimental data on NPs for medical purposes, only a few reach clinical use. This statement is supported by Greish *et al.*, who explored more than 20,000 scientific papers published on nanomedicine, and found of these, only 15 nanoparticle-based anti-cancer drugs had reached the market as of 2017. It is clear that the number of publications claiming to have found new, effective, and safe anticancer formulations, compared to the number of compounds that actually reached the clinic, is remarkably small (Greish et al., 2018). In their review, Greish *et al.* discuss different biological aspects that hinder the clinical progression of cancer nanomedicine, which include misconception of EPR phenomenon, overlooking the acquired pharmacokinetics and clearance of nanomedicine through reticuloendothelial system, and accelerated blood clearance. The limitations of animal models and heterogeneity of human tumors further restricted the clinical application of formulated nanomedicines.

Safety is the most important aspect in the development of new drugs. Although the size of nanoparticles represents their strength,

for some nanomedicines it has also brought some shortcomings. The small size of NPs cause some of these particles to accumulate in the spleen and liver, which is a major safety concern in patients (Resnik and Tinkle, 2007). In some cases, the injected doses of nanosized molecules are cleared by reticuloendothelial system cells with a minimal percentage of the drug dose reaching the tumors which lowers the efficacy of the treatment.

Even when some studies reach clinical validation, logistics issues including mass production, consistency, and reproducibility of complex nanomedicine systems are the main hurdles. Furthermore, the controlled and scale-up manufacture of each component, batch-to-batch reproducibility, and stability of designed nanomedicines are essential for approval by the regulatory authorities (Greish et al., 2018). Not only that, regulation and standards for nanomedicine by regulatory bodies are severely lacking and could be geographically differed as one nanomedicine is approved in one country but not in others (Zhang et al., 2020). Due to these challenges (Figure 3), hundreds of nanomedicine formulations have failed in different phases of clinical trials, or even worse, some are withdrawn from the market even after its approval.

**FIGURE 3 |**

To overcome these issues, several solutions can be proposed. In order to address the biological challenges of nanomedicine in cancer that is a heterogeneous disease, thorough designation of nanomedicine and identification of the right animal models and patients in preclinical investigations should be in mind when designing a new drug entity. Good laboratory practice (GLP) is a standard to ensure the safety and quality of new therapeutics during clinical transition by many countries. However, GLP for nanomedicine has not been made available yet, hence it is imperative to formulate GLP for nanomedicine to enhance its success rate in the market (Zhang et al., 2020). In addressing logistic issues, careful examination of the cost-benefit analysis should be done during the early stage of nanomedicine development.

CONCLUSION

Despite challenges, the latest technologies and advantages of nanoparticles continue to encourage research communities to develop new, better nanomedicines. It is recognized as a proven strategy to alleviate the side effects of cancer therapies and enhance their efficacies. Nevertheless, development of nanomedicine should always accentuate their interactions with

host immune responses, as in cancer, it's tangibly interlinked between one another. Although there are several aversions to nanomedicine due to the induction of unwanted hypersensitivity, available findings suggested that targeted approach of nanomedicine provides a favorable effect in the immune system, from its immunogenicity and interaction in the TME to its tolerability. With this understanding of the interaction of nanomedicine with the immune system, the future of nanomedicine is promising as long as the shift to improve the clinical impact of nanomedicine moves alongside it.

AUTHOR CONTRIBUTIONS

SA, RI, WW, and KP wrote this paper. RM, JB, MP, JJ, and JL supervised this work and revised the manuscript. RM acquired funding for this work. All authors contributed to the paper and approved the submitted version.

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Nanoparticles and Gut Microbiota in Colorectal Cancer

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Recent years have witnessed an unprecedented growth in the research area of nanomedicine. There is an increasing optimism that nanotechnology applied to medicine will bring significant advances in the diagnosis and treatment of various diseases, including colorectal cancer (CRC), a type of neoplasm affecting cells in the colon or the rectum. Recent findings suggest that the role of microbiota is crucial in the development of CRC and its progression. Dysbiosis is a condition that disturbs the normal microbial environment in the gut and is often observed in CRC patients. In order to detect and treat precancerous lesions, new tools such as nanotechnology-based theranostics, provide a promising option for targeted marker detection or therapy for CRC. Because the presence of gut microbiota influences the route of biomarker detection and the route of the interaction of nanoparticle/drug complexes with target cells, the development of nanoparticles with appropriate sizes, morphologies, chemical compositions and concentrations might overcome this fundamental barrier. Metallic particles are good candidates for nanoparticle-induced intestinal dysbiosis, but this aspect has been poorly explored to date. Herein, we focus on reviewing and discussing nanotechnologies with potential applications in CRC through the involvement of gut microbiota and highlight the clinical areas that would benefit from these new medical technologies.

Keywords: dysbiosis, microbiota, nanoparticle, immunomodulation, colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in females and third most common cancer in males worldwide. Over time, it has become a leading cause of morbidity and mortality. There is a broad geographical variation in the incidence of CRC globally, and there has been a rapid rise in its incidence in Asian countries for the past few years (Siegel et al., 2020). In Malaysia, for example, the National Cancer Patient Registry has reported that CRC is the second most common cancer in both males and females, with a total number of 4,501 cases diagnosed from 2008 to 2013 (Abu Hassan et al., 2016). In general, CRC incidence is higher in the developed countries as compared to the developing ones; however, the burden of this disease is rising globally, including that in the low-to-middle-income nations (Siegel et al., 2020).

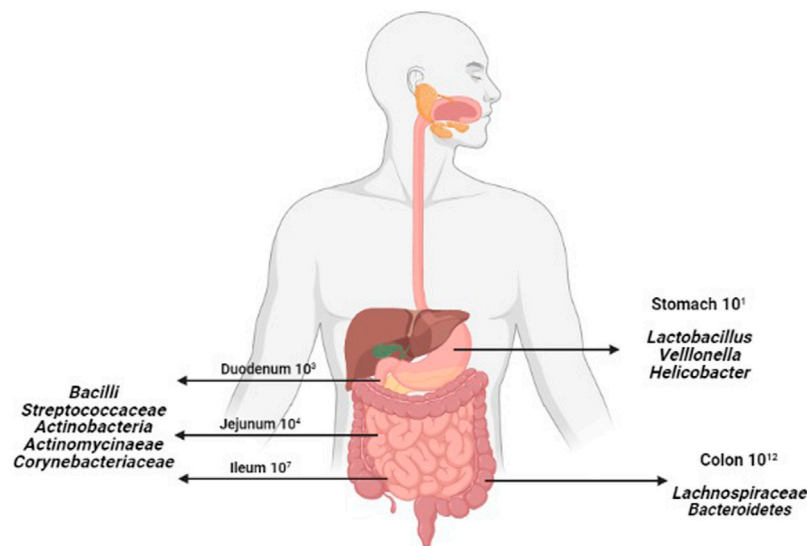


FIGURE 1 | Variations in the number and composition of microbiota across the GIT.

Currently, ample research is being conducted in order to find the definite cause of CRC. Based on the actual findings, it is theorized that the bacteria present in the human colon are linked to the development of carcinogenesis (Saus et al., 2019). Large numbers of bacterial cells live in commensal relationships with the host. However, once the gastric ecosystem is altered, various bacterial species become prone to develop pathogenic phenotypes (Sekirov et al., 2010). In recent years, there has been a surge of interest in assessing the relationship between the gut microbiota and the gut modifications that eventually lead to CRC. From here on, modification in the composition of the gut microbe is suggested as the cause underlying the development of colorectal malignancies (Saus et al., 2019).

Nanomedicine can be broadly defined as comprehensive monitoring, control, construction, repair, defense and/or improvement of all human biological systems, working from the molecular level to more complex wholes, with the use of nanomaterials. Nanoparticles (NPs) have been increasingly applied in the disease diagnosis and treatment during the last few decades. This use of NPs for medical purposes has led to encouraging prospects of their use for the betterment of human health (Riaz Rajoka et al., 2021). Diagnostic and therapeutic research on nanomedicine formulations has brought about a number of effective platforms, including those for combined diagnosis, targeted drug delivery and therapy (Saini et al., 2010). Still, despite the immense prospect of nanomedicine for improving human health, the prognosis for advanced stages of CRC is still relatively poor. The current treatment method for CRC includes surgery and/or chemotherapy. Although CRC is curable, the survival rates are still low. With the current advancements in the world of nanomedicine, it is expected that utilization of NPs will be a vital future approach in CRC theragnostics (Werner and Heinemann, 2016). Correspondingly, this review primarily elaborates on the dysbiosis condition that leads to the development of CRC, and the clinical aspect of NPs and their limitations based on published research.

MICROBIOTA

Human body is inhabited by a vast number of bacteria, archaea, viruses and unicellular eukaryotes. The collection of microorganisms that live in peaceful coexistence with their hosts has been referred to as the microbiota, microflora or normal flora (Dieterich et al., 2018). By far the most heavily colonized organ is the gastrointestinal tract (GIT); the colon alone is estimated to host over 70% of all the microbes in the human body (Jandhyala et al., 2015). The concentration of microbiota increases steadily along the GIT. Hence, lesser concentrations of microbiota are found in our stomach and significantly higher in the colon (Anselmo and Mitragotri, 2019), as shown in **Figure 1**.

The GIT shelters trillions of microbes which mostly live in a harmonized relationship with the host. In spite of the fact that most of the microbiome shows a favorable symbiotic relationship with the host, when the microbiome composition and function get disturbed, preconditions for various diseases arise, including cancers, obesity, metabolic diseases, diabetes, allergies, depression and disorders of the immune system (Quigley, 2013). Here, a selective group of bacteria such as *Enterococcus faecalis*, *Bacteroides fragilis*, *Escherichia coli* and *Fusobacterium nucleatum* are seen as fundamental causes of the pathogenesis of CRC (Kong and Cai, 2019). These bacteria are listed in **Table 1**.

Immunomodulation Through Host-Microbiota Interactions

Host-microbiota interactions are fundamental for the development of immune system. The early life colonization of the human gut mucosal surfaces plays a pivotal role in the maturation of the human immune system (Zheng et al., 2020). Gut microbial community composition is modified by many environmental factors, such as geographical location, host diet, and administration of antibiotics and other medicines. Local

TABLE 1 | Comparison of the gut microbiota.

Bacteria	Characteristic	Shape	Probiotics	Symbiotic relationship	Medicinal value	Other pathological condition	Molecular condition leads to CRC	References
<i>Enterococcus faecalis</i>	Gram-positive, facultative anaerobic	Cocci	Yes	Commensal in GIT	Treats chronic sinusitis and bronchitis	Leads to urinary tract infection, endocarditis, persistent endodontic disease bacteraemia, chronic periodontitis	<i>E. faecalis</i> shows harmful activities due to its ability to damage colonic epithelial cell of DNA which sooner or later leads to development of CRC	de Almeida et al. (2018), Fiore et al. (2019), Kong and Cai (2019)
<i>Bacteroides fragilis</i>	Gram-negative, obligately anaerobic	Rod	Yes	Commensal in GIT and opportunistic pathogen	Contribute to the host's nutritional status as well as mucosal and systemic immunity	Abscess formation in multiple body site abdomen, brain, liver, pelvis and lungs and able to act as opportunistic pathogens	<i>B. fragilis</i> able to induce alterations in mucosal permeability, which favors the translocation of bacteria and bacterial toxins, causing gut inflammatory response that contributes to the development and progression of CRC	Wexler (2007), Wick and Sears (2010), Sears and Garrett (2014), Sears et al. (2014), Purcell et al. (2017)
<i>Escherichia coli</i>	Gram-negative, facultative anaerobic	Rod	Yes	Commensal in GIT	Promoting normal intestinal homeostasis and preventing colonization by pathogens	Rarely causes disease except in immunocompromised hosts	<i>E. coli</i> polyketide synthetase, genomic permit the synthesis of colibactin, a genotoxic protein which has been linked to DNA damage and mutation, cell cycle arrest and chromosomal instability in human cells	Kaper et al. (2004), Delmas and Bonnet (2015), Wassenaar (2018), Iyadorai et al. (2020)
<i>Fusobacterium nucleatum</i>	Gram-negative, obligately anaerobic	Rod	No	Commensal in oral and opportunistic pathogen	None	Leads to sinusitis, endocarditis, septic arthritis, tonsillitis and abscesses of the brain, skin and liver	<i>F. nucleatum</i> stimulates CRC cancer growth by modulating the E-cadherin/b catenin signaling via its unique FadA adhesin	Allen-Vercoe et al. (2011), Han (2015), Brennan and Garrett (2019)

immune responses are triggered by microbes via interactions with immune cells that express pattern recognition receptors (PRRs). Local dendritic cells (DCs) get activated by microbes or microbe-derived elements such as constituents, products, metabolites via interactions with PRRs. Activated DCs travel from the gastrointestinal tract to mesenteric lymph nodes (mLNs), where they present microbe-derived antigens and subsequently induce the differentiation of immature T cells into effector T cells, particularly regulatory T cells (Tregs) and T helper 17 (Th17) cells (Inamura, 2021). A subset of these effector T cells migrates back to the gastrointestinal tract and influences local immune

responses. The remaining population enters the systemic circulation and influences systemic immunity. The conversion of the immune system from a pro-inflammatory to an anti-inflammatory state occurs through the release of anti-inflammatory cytokines such as IL-10, TGF- β or the engagement of DCs mediated by Tregs. Conversely, Th17 cells mediate the conversion of the immune system to a pro-inflammatory state by secreting immunostimulatory cytokines (e.g., IL-17) or by activating and recruiting neutrophils. This intriguing relationship strongly suggests the vital role of microbes in Th17 cell activation (Inamura 2021).

Impact of Nanoparticles and Gut Microbiota

NPs could be present in food in the form as additives, supplements and packaging and directly impact the composition and/or metabolic activities of the gut microbiota (Lamas et al., 2020). Furthermore, inorganic NPs, particularly, such as silver, titanium dioxide, silicon dioxide and zinc oxide have been shown to affect gut microbiota through their interaction with immune system. This alteration in gut microbiota-immune axis is associated with many chronic diseases such as inflammatory bowel disease (IBD), diabetes and even colorectal cancer (Ni et al., 2017; Meijnikman et al., 2018; Richard et al., 2018). Silver NPs are used in hundreds of commercial products due to their anti-microbial properties and intentional and accidental uptake of silver NPs, which might affect the gut microbiome, are underestimated. Dahiya et al. (2018) have reviewed evidence from both animal and human studies and concluded that silver NPs altered the gut microbiota, thus making the host susceptible to certain diseases. They also illustrated that the mucus present in the gut lining prevents absorption of silver NPs into intestinal cells, hence the interaction with gut microbiota. Meanwhile, titanium dioxide NPs, which are commonly available in daily products, also cause changes in both gut microbiota morphology and metabolism (Juan et al., 2018; Mao et al., 2019; Zhangjian et al., 2019). Specifically, a mix of commensal and pathogenic bacteria was modulated upon administration of titanium dioxide NPs and manifested as oxidative stress and inflammatory responses in the intestine (Zhangjian et al., 2019). However, another study indicates that titanium dioxide NPs have limited effect on gut microbiota compared to silver NPs that drastically changes the commensal density. In this study, titanium dioxide NPs formed large agglomerates that appeared to loosely interact with microbial cells, whereas Silver NPs were found both within and outside of microbial cells. This explains the significantly lower effect of titanium dioxide NPs on community growth (Agans et al., 2019). There is also optimistic opportunity of NPs in their interaction with gut microbiota. Selenium NPs are shown to be a promising tool in livestock industry due to their efficacy in intestinal pathogen control (Gangadoo et al., 2019). These NPs are observed to reduce emerging poultry pathogen, *Enterococcus cecorum*, without any significant disturbance to the gut total commensal community. Another type of NPs, fullereneol NPs with good biocompatibility properties, markedly increased the production of short-chain fatty acids (SCFAs)-producing bacteria *in vivo* (Li et al., 2018). Fullereneol NPs contain furan- and pyran-like structure which is similar to those of polysaccharides in dietary fiber such as inulin, thus the capacity to promote this kind of gut microbes. The increase of this gut main metabolites-producing bacteria is accompanied with anti-hyperlipidemic effect of fullereneol NPs when both triglycerides and total cholesterol levels in liver and blood were decreased. Interestingly, fabrication of fullereneol NPs to form carbonyls in low pH would induce a peroxidase-like activity and eradicate *Helicobacter pylori* both *in vivo* and *in vitro* (Zhang C. et al., 2020). In addition, approach to design NPs with natural sources and line up into a systematic drug delivery tool is demonstrated to be beneficial to gut microbiota. Curcumin- and

ginger-derived NPs are shown to improve absorption by gut microbiota, so that they can produce their respective effects (Ohno et al., 2017; Teng et al., 2018). Curcumin NPs are shown to suppress development of mouse colitis through the increase of butyrate-producing bacteria and regulatory T cells (Tregs) while ginger NPs is design to contain microRNA that could ameliorate mouse colitis. Another natural sources such as milk is developed as extracellular vesicles and these NPs modulated intestinal SCFAs metabolites and increased intestinal immunity (Tong et al., 2020).

NANOMEDICINES AND COLORECTAL CANCER

The unique optical, magnetic, and electrical properties of metallic NPs such as gold and silver have rendered these systems a great tool for the detection of several markers of cancer. For example, fluorescent gold NPs have been used to detect mechano-growth factor (MGF), a unique marker of colon cancer (Kasprzak and Szaflarski, 2020). Another study demonstrated that the incorporation of theragnostic gold nanorods as cores inside tumour-specific antibody shells as allowed to make the distinction between the different types of tumors and destroy the surrounding tumor cells (Lee et al., 2015). A non-invasive NP-based breath sensor was also shown to distinguish between the healthy and cancerous patients, including those with CRC (Peng et al., 2010). This nanomaterial-based sensor array would analyze volatile organic compounds associated with and differently regulated in different diseases using cross-reactive absorption sites on nanomaterials such as gold NPs, thus producing a unique breath fingerprint (Xu et al., 2013). Local staging of colon cancer is routinely done with computerized tomography (CT) scans and magnetic resonance imaging (MRI), which both benefit from the use of NPs, which can improve the sensitivity for the detection of tumor invasion (Nerad et al., 2017). In MRI, superparamagnetic iron oxide (γ -Fe₂O₃ or Fe₃O₄) NPs coated with a polymer are demonstrated as promising contrast agent for MRI, whereas conjugation with doxorubicin further displayed the multimodality of these NPs in both diagnostic and therapeutic assays.

Conjugation of these NPs to chemotherapeutic drugs did not only effectively kill tumor cells via active targeting, but also inhibited tumor growth using near-infrared (NIR) irradiation due to the photothermal activity of the NPs (Fan et al., 2019). In another study, Fe₂O₃-gold NPs were observed to accumulate within the tumor mass instead of other organs in a colon cancer model, while also enhancing the tumor detection selectivity as MRI contrast agents in a pancreatic cancer model (Kumagai et al., 2010). Conventional chemotherapy has been reported to be associated with severe side effects, mainly due to the nonspecific biodistribution, whereby both the cancer cells and the healthy cells are affected by the therapy. In CRC, common side effects over the course of chemotherapy include fatigue, diarrhea, constipation and dyspnoea, as reported in routine healthcare settings (Pearce et al., 2017). However, over the past three decades, the concept of enhanced permeability

retention (EPR) effect conditioned by the hypervascularity in solid tumors, as proposed by Matsumura and Maeda (1986), greatly improved the logistics of cancer treatments using NPs (Matsumura and Maeda, 1986). Nowadays, the application of NPs in cancer therapeutics enhances the efficacy of the conventional cancer management, particularly relative to that of chemotherapy. This enhanced efficacy is largely owing to the passive targeting of tumors by the NPs, which are able to pass through the tumor vascular pores and accumulate the nanomedicine in the tumor zone. Furthermore, the poor response to anticancer drugs due to the development of multidrug resistance (MDR) phenotypes has shown improvement following the incorporation of NPs in various chemo-photothermal therapies (Wang et al., 2017; Jiang et al., 2020).

Various preclinical and clinical studies have explored the potential of nanomedicines, including metallic NPs, in the CRC treatment. Drugs such as 5-fluorouracil (5-FU) and doxorubicin (DOX) have been incorporated into NP delivery systems to enhance the efficacy of the treatment of CRC. One example of a recent *in vitro* study using NPs was that of enhancing the antitumor efficacy in a CRC model, as reported by Jiang et al. (2020). In this study, DOX-resistant SW620/Ad300 cells treated with a nanocomposite based on mesoporous silica-coated gold nanorods (GNRs/mSiO₂) loaded with DOX showed a higher toxicity compared to the free DOX treatment, the reason being the increased intracellular DOX accumulation achieved by the NP drug carrier. Apart from biocompatibility, the GNRs exhibit a highly efficient photothermal conversion, which enabled the chemo-photothermal therapy on CRC cells through functionalization with anticancer drugs (Zhou et al., 2017). In another study, manganese-based (Mn) NPs stabilized by L-arginine were developed as a nanocarrier system to deliver 5-FU to colon cancer cell lines (Jain et al., 2020). In that case, cell viability was significantly reduced after the treatment with 5-FU-loaded Mn-based NPs as compared to the treatment with free 5-FU, being twice lower in the former specimen group than in the latter.

Previously, CRLX101, a 30–40 nm NP consisting of camptothecin (CPT), a topoisomerase 1 (topo-1) and hypoxia-inducible factor 1- α (HIF-1 α) inhibitor, conjugated to cyclodextrin and polyethylene glycol (PEG) was designed to enhance the CPT efficacy against CRC (Weiss et al., 2013). Promising findings of CRLX101 in a human-phase trial with advanced solid tumors has led to further studies in other *in vitro* and *in vivo* systems to support or complement the ongoing clinical trials. Nonetheless, the local recurrence of tumors has been a major concern in these tumor treatments. Thus, CRLX101 with 5-FU incorporation was investigated in a subcutaneous mouse xenograft model of CRC following radiotherapy (Tian et al., 2017). It was reported that the nano-formulation had the highest therapeutic effect by eradicating tumor repopulation after chemoradiotherapy. Meanwhile, a comparative study involving the use of gold-based NPs in a radio-iodide cancer treatment reported that iodine-131 alone reduced the tumor growth by 15%, while the incorporation of iodine-131 inside the NPs reduced the tumors by 50%, suggesting that functionalized NPs may open up

a new venue for radiopharmaceutical therapies in cancer management (Le Goas et al., 2019).

The branch of nanomedicine is emerging into the biomimetic NPs, particularly cell membrane-based NPs to avoid the immune system clearance and, thereby, increase their therapeutic effects. This biomimetic approach mimics the native cells to mediate the interactions at the nano-bio interface as the fate of NPs *in vivo* is governed by their physicochemical surface (Sushnitha et al., 2020). The presence of natural cell membrane coatings to camouflage NPs offers the ability to remain the NPs in circulation and eventually to achieve targeted delivery. Red blood cell (RBC) membrane-coated NPs have been one of the most studied cell membrane-based nanocarriers in drug targeting delivery to enhance tumor cell death. Fabricating NPs with RBC membrane suited *in vivo* circulation due to the expression of CD47 protein on their surface to ensure self-recognition by the RES to allow longer circulation (Liu et al., 2019). In addition, RBCs have limited nuclei and are easily isolated from the blood donor (Zhang et al., 2017). Poly(lactic-co-glycolic acid) (PLGA) NPs loaded with gambogic acid (GA) possessed antitumor potential when covered with RBC membrane obtained by BALB/c mice. They found that RBC membrane-GA/PLGA NPs showed significant inhibition of tumor growth in CRC *in vivo* model via necrosis. Comparing GA toxicity with RBC membrane-coated NPs to the free GA without NPs, they observed the higher median survival rate recorded at 30 days compared to the free-GA group that lasted for 4 days only. It should be noted that, RBC membrane-coated NPs also improved the poor aqueous solubility of GA, just as with the poor water solubility of the available chemotherapy drug in the market such as paclitaxel.

Still, to date, limited numbers of clinical trials have been conducted on CRC treatments using nanomedicine. The most prominent NPs used in these studies have been polymer-based. For instance, a phase I clinical study using PEGylated liposomal mitomycin C (NCT01705002) and PEG conjugate of SN38 (NCT00931840) showed that both nano-formulations were well-tolerated at higher doses compared to the free antitumor drugs (Norris et al., 2014; Golan et al., 2015). Nevertheless, further studies are warranted to evaluate therapeutic efficacies of nanomedicine formulations in CRC management. However, to our knowledge, no clinical trials using metallic NPs in CRC treatments have been reported yet. Still, considering the number of promising preclinical findings, the metallic NPs are expected to be utilized in human-phase trials with CRC patients in due time.

ADVANTAGES AND CHALLENGES IN NANOMEDICINE

Nanomedicine aims to provide more efficient tools for prevention and treatment of various diseases as it possess potential advantages (Desai, 2012), and might someday provide answers to long lasting problems in medical research, such as poor drug solubility and lack of target specificity for therapeutic compounds (Bawa, 2011). This field give new perspectives for the biomedical

area as well as clinical for prevention, diagnosis, and treatment of severe diseases such as cancer (Chow and Ho, 2013). Making remarkable enhancements in drug delivery systems (Butcher, Mortimer et al., 2016), medical imaging and diagnosis platforms have been accounted as nanotechnology effects in health and medicine (Bayford et al., 2017).

Biological Barrier

NPs impart a range of properties to drugs that they carry into a living organism. Due to specific characteristics such as efficient transport through capillary blood vessels, longer circulation duration, higher binding capacity to biomolecules, higher accumulation in target tissues, and reduced inflammatory and oxidative stress in tissue, various nanomedicines have been developed and commercially applied in clinical and non-clinical areas as their characteristics are differ from those of conventional medicine (Liu et al., 2011). This range of improved properties often translates to a greater therapeutic efficacy of nanomedicines than that of conventional medicines. These features also endow nanomedicines with lower toxicity, improved bioavailability, and enhanced pharmacokinetics compared to the conventional drug therapies. Thus, nanomedicines overcome obstacles that limit conventional drugs, and have the potential to meet future market demands for disease treatments (Wang et al., 2018).

Specific Surface Area

Nanomedicines often exhibit greater therapeutic efficacy than conventional small molecular drugs due to their size, large specific surface area, and flexibility of surface functionalization. These features endow nanomedicines with low toxicity, improved bioavailability, and enhanced pharmacokinetics and therapeutics effect. Thus, nanomedicines overcome obstacles that limit conventional drugs, and have the potential to meet future market demands for disease treatments (Wang et al., 2018). Nanomedicine is used in versatile aspects of the disease management process, including diagnosis, treatment and monitoring (Sajja et al., 2009). In view of the ability of NP carriers to slow down the release of drugs and prolong the pharmacokinetic half-life for many of them, nanomedicine has a key role in the controlled delivery of drugs or other biological products to improve the human health.

Solubility and Permeability

NPs are also capable of ensuring the solubility and permeability of insoluble drugs across biological barriers. Drugs with low bioavailability can thus be delivered directly to the target site (Galvin et al., 2012). Moreover, the large surface area and a higher reactivity of nanomedicines may allow for the dose reduction of a drug, which can improve toxicity profiles and patient compliance (Bawa, 2011; Galvin et al., 2012). The large surface area of nanomedicines can also increase the dissolution rate, the saturation solubility, and the intracellular uptake of the drugs, thereby improving *in vivo* performance (Bawa, 2011). In addition, combining encapsulation, release modalities, and surface modifications to improve targeting or bioavailability could improve the therapeutic efficacy (Bharali and Mousa, 2010).

Currently, 65% of nanomedicines undergoing clinical trials focus on cancer applications, as nanomedicine can offer great contributions for a better treatment and early diagnosis of this disease (Germain et al., 2020). In addition to the cancer treatments, nanomedicine has also been used to combat infectious, ophthalmic, neurological and other disease, as well as to facilitate gene editing, immunotherapies, and so on.

Challenges

The development of nanomedicines is growing rapidly; however, each of the nanomedicines has its own challenges such as safety, biological challenges, scale-up, cost, and regulation (Grenha, 2011). In addition, the development of nanomedicines requires massive preclinical research, carefully conducted clinical trials, and appropriate clinical indications (Etheridge et al., 2013). The drug development process takes years, and any mistakes or negative results may delay these process (Etheridge et al., 2013). Thus, to understand the current situation of nanomedicine, including common challenges and future demands for nanomedicines development (Moghimi et al., 2005) is important.

Specific and Precise Targeting

One of the highest priorities for the next generation of nanomedicines is to create precise and highly efficient drugs that accumulate in targeted pathological tissues and not effect the healthy tissue. There are many nanomedicines that cannot avoid damage to healthy tissues because they do not specifically target the pathological tissues. Recently, smart drug delivery systems that possess active targeting and stimuli responsiveness are actively being developed. These smart drug delivery systems represent a promising strategy to eliminate pathological tissues while protecting normal tissues (Zhang J. et al., 2020).

Safety and Quality

It is imperative to understand the toxicology of the drugs, the physicochemical properties of NPs, and the properties of nanocarriers since safety is the crucial issues for nanomedicines, even after a nanomedicine is approved for clinical use, it can be withdrawn from the market due to safety issues. In human body, nanomedicines get exposed to biological environments such as the blood, extracellular matrix, cytoplasm, and cellular organelles, which may alter their biological performance, but also elicit undesired toxicities. Currently, there is a lack of standardized methods for evaluation of the safety of nanomedicines. It should be emphasized that the methods used for traditional drugs cannot accurately evaluate the safety of nanomedicines (Wolfram et al., 2015). For example, drugs such as paclitaxel or doxorubicin, which have been extensively studied, still facing the complex toxicity issues when used with novel nanomedicines and nanocarriers (Hua et al., 2018). To increase the safety and quality, many countries and organizations have enacted and implemented good laboratory and manufacturing practices. It is important to formulate good laboratory practices for nanomedicines to improve the success rate and to promote the development of safe and effective nanomedicines (Zhang J. et al., 2020).

Biodistribution of Drugs

Other challenges involve the drug biodistribution and biological barrier penetration which aim to enhance the accumulation of the drug at the target site and reduced the accumulation in healthy tissues (Lu et al., 2021). Biodistribution is affected by interactions with external and internal biological barriers, for which reason the interaction between nanomedicines and biological barriers must be well understood (Elsababy and Wooley, 2012). Furthermore, nanomedicines often do not directly interact with living cells, but instead become coated with a protein corona, which alters the biological effects of the NPs and influences the cell uptake, biodistribution, clearance, toxicity, and immune response. Therefore, it is important to also focus on the protein coronae formed around NPs and the resulting biological responses to nanomedicines (Mahmoudi et al., 2016).

Scale up, Cost and Regulations

Additionally, nanomedicines may fail to enter the market because unmeet the requirements of scale-up synthesis and reproducibility. In both laboratory or preclinical investigations and in clinical studies, nanomedicines have been mainly synthesized in small batches (Anselmo and Mitragotri, 2019). Not only the manufacturing cost of nanomedicines scale-up high, but the cost of preclinical and clinical development is also increasing. Acquiring regulatory approvals is difficult for new nanomedicines, especially when existing products on the market have the same target indication (Ventola, 2017). The lack of regulation and standards for nanomedicines in manufacturing practices, quality control, safety, and efficacy evaluation are all barriers for the development of nanomedicines. There are currently no global regulatory standards specific to clinical translation of nanotherapeutics. Only initial guidance documents for nanotechnology products have been issued by regulatory authorities such as the FDA to provide guidance. There are also significant differences between different geographical regions in addressing the application of

nanomedicines, so that nanomedicines approved in one country may not be approved in other countries. Strengthening of regulatory standards is a major challenge but must be overcome to achieve efficient nanomedicine development (Mühlebach, 2018).

CONCLUSION

This review has highlighted the existing use of diverse nanomedicines for the diagnosis and treatment of CRC. Despite the fact that the field is young and growing, a number of studies have reported on how combining metallic NPs and multiple targeting strategies establishes conditions for the improved treatment and diagnosis of CRC. Although considerable challenges and issues remain to be addressed, the potential impact of nanomedicine on the diagnosis and treatment of CRC is extensively recognized. As discussed through several examples in this review, careful modification and characterization of metallic NPs is needed in order to exploit the potential impact of nanomedicine on the clinical management of CRC and advance its use in the CRC treatment and early diagnosis.

AUTHOR CONTRIBUTIONS

KP, SA, MM-Z, and WNWH wrote this paper. ZI, J-LS, KF, VU, JJ, and RM supervised this work and revised the manuscript. All authors contributed to the paper and approved the submitted version.

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Nanotechnology in Colorectal Cancer for Precision Diagnosis and Therapy

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Colorectal cancer (CRC) is the third most frequently occurring tumor in the human population. CRCs are usually adenocarcinomatous and originate as a polyp on the inner wall of the colon or rectum which may become malignant in the due course of time. Although the therapeutic options of CRC are limited, the early diagnosis of CRC may play an important role in preventive and therapeutic interventions to decrease the mortality rate. The CRC-affected tissues exhibit several molecular markers that may be exploited as the novel strategy to develop newer approaches for the treatment of the disease. Nanotechnology consists of a wide array of innovative and astonishing nanomaterials with both diagnostics and therapeutic potential. Several nanomaterials and nano formulations such as Carbon nanotubes, Dendrimer, Liposomes, Silica Nanoparticles, Gold nanoparticles, Metal-organic frameworks, Core-shell polymeric nano-formulations, Nano-emulsion System, etc can be used to targeted anticancer drug delivery and diagnostic purposes in CRC. The light-sensitive photosensitizer drugs loaded gold and silica nanoparticles can be used to diagnose as well as the killing of CRC cells by the targeted delivery of anticancer drugs to cancer cells. This review is focused on the recent advancement of nanotechnology in the diagnosis and treatment of CRC.

Keywords: colorectal cancer, nanotechnology, detection, treatment, targeted therapy

INTRODUCTION

Colorectal cancer (CRC) is a severe health problem and has the third-highest occurrence within the cancer-causing conditions that influence the populations of developing and developed countries (da Paz et al., 2012). Among populations of western countries, a major cause of death and morbidity is CRC (Arvelo et al., 2015). Various routine factors and the growing age are the common reason of CRC with only a few cases being the outcome of genetic disorders (Bours et al., 2015; Gulbake et al., 2016). It generally starts in the bowel lining and can extend into the wall of the bowel and beneath muscle layers if not treated properly at the earliest (Datta et al., 2016). In addition to these, there are genetic and environmental factors that can interact in various ways to enhance carcinogenesis (Bours et al., 2015). Regarding the CRC pathogenesis four central theories have been established. In the first theory, epigenetic and genetic variation generate the formation of colon cancer promotes CRC. Second, cancer emerges due to a multistep process at both molecular and morphological levels. The third and crucial molecular step is the loss of genetic stability in cancer formation. Fourth, hereditary

cancer diseases usually belong to germline forms of an important genetic disorder, for which somatic occurrences force the manifestation of sporadic colon cancers (Kheirleiseid et al., 2013). However, various factors that contribute to and affect the start and establishment of disease, continue to increase the number of potential targets (Hutchinson et al., 2015). Ultimately, this targeting potential will show the way for the success of an effective technique for disease management and will provide better treatments to the patients. Several decades of research have been concentrating on the establishment of novel methods and techniques in cancer research, especially in the areas of detection, giving rise to earlier cancer therapy to decrease mortality.

CRC is the most devastating disease identified among the most common malignancies and can be recorded as the second leading reason for cancer-associated deaths in women and the third-highest common disease in men worldwide (Huyghe et al., 2020). Current situations have been shown that up to 2030, the chances and mortality rate of CRC will enhance by 60% worldwide (Brand et al., 2017; Huyghe et al., 2020). With the existing diagnostic techniques and treatments, patients suffer long invasive techniques with unwanted side effects to remove CRC. Hence, a new detection method requires further research to improve their specificity and affectivity (Bray et al., 2019).

Nanotechnology has opened up a new way to the development of novel and efficiently organized nanomaterials with the capability of enhanced performance in screening, detection, and treatment of CRC cancer along with other types of tumors (Gulbake et al., 2016; Bray et al., 2019). The role of nanotechnology interventions to CRC includes the nanomaterial-based screening of tumors, customization of targeted drug delivery systems, and advanced treatment modalities (Upendra et al., 2016; Minakshi et al., 2017; Minakshi et al., 2018; Minakshi et al., 2020). Currently, nanotechnologies have found worldwide consideration due to their capability to improve existing standards and methods for the screening, diagnosis, and treatment of CRC. The present review has the main emphasis on the discussion of nanotechnology-based precision methods for screening, detection, and treatment of colorectal cancer.

During the preparation of this review, recent and appropriate information was collected using several scientific search engines including PubMed, Medline, Google Scholar. Several research and review articles were collected, thoroughly studied and a comprehensive manuscript is prepared. Under this review article several aspects of colorectal cancer (CRC) including their different clinical stages, recent diagnostic and therapeutic approaches using several nano-formulations such as Quantum dots, Iron oxide, Carbon nanotubes, Liposomes, Silica Nanoparticles, Nanoemulsion, Gold nanoparticles, etc. have been discussed. Apart from the use of individual medicines, the efficacy of combinatorial nanomedicine against CRC has also been explored.

Colorectal Cancer Stages

The survival rate in CRC mainly depends upon the stages of CRC disease and it usually ranges from 90% in the case of localized stage to 10% in the case of patients diagnosed with metastatic

cancer. If the stage of diagnosis is earlier, the higher the chance of survival rate (Hagggar and Boushey, 2009). The exact stage of the tumor, which explains the level of cancer in a patient's body, is one of the very significant factors in deciding which therapy is useful and how successful therapy might be (Schroy et al., 2016). **Figure 1** describes the various stages of colorectal cancer. Abnormal cells arise from colonic wall mucosa, may become tumors, and divides in stage 0. During stage I, cancer has formed in the colon wall mucosa and emerges into submucosa as well as muscularis propria. Stage II cancer further progresses from muscularis propria into pericorectal tissues (IIA), and then emerges to the visceral peritoneum (IIB), then directly penetrates into the attached organs (IIC). Stage III cancer emerges into muscularis propria metastases in nearby tissues or 1–3 regional lymph nodes and spreads in submucosa with metastases in 4–6 lymph nodes in IIIB and 7 or more regional lymph nodes (IIIC). Metastasis confines to one organ such as liver ovary, lung, regional node, etc. in stage IV, and again stage IV cancer is further divided into IV A and IV B stages (Xynos et al., 2013; Lai et al., 2016). Five years survival rate percent researches showed 90% survival in the case of stage I CRC and 10% survival in the case of stage IV CRC (Pesta et al., 2016). Physical inactivity, eating more processed meat, long-term smoking, obesity, alcohol addiction, and a diet with fewer vegetables and fruits are the most common lifestyle-related threats and major causes of CRC. Family history is also connected with CRC, a person with chronic inflammatory bowel disease, type 2 diabetes, or genetic disease likelynch syndrome has been linked with augmented risk (Young et al., 2014). Stages 0, I, II, and III may be curable but stage IV CRC is not often curable but it may be managed based on growth and spreading disease (Young et al., 2014; Sun et al., 2016).

Existing Screening Methods for Colorectal Cancer

Various screening methods have been established to screen CRC before symptoms start, when it may be easily treatable. Few of the screening methods that identify polyps and adenomas at an early stage may assist in the detection and early removal of tumor growth which might otherwise lead to further progression of cancers. Hence early detection of colorectal cancer may be another method of cancer prevention. Different tests are prevalent for suitable screening of colorectal cancer (**Table 1**). Stool tests detect minute quantities of blood that cannot be possibly seen visually from both adenomas and polyp cancers (Imperiale et al., 2004; Burch et al., 2007; Ansa et al., 2018; Qaseem et al., 2019).

In the colonoscopy entire colon and rectum are screened for cancer using a colonoscope, having a flexible tube light with a lens for seeing and a tool for excising the abnormal tissue (Kahi et al., 2016). A complex analysis of six studies showed that screening using colonoscopy reduces the chances of establishment and dying of people from CRC (Brenner et al., 2014). Conversely, the test is costly and unpleasant. Virtual colonoscopy or computed tomographic (CT) colonography and double-contrast barium enema are the optional choices for patients, who cannot go through a routine colonography due to the

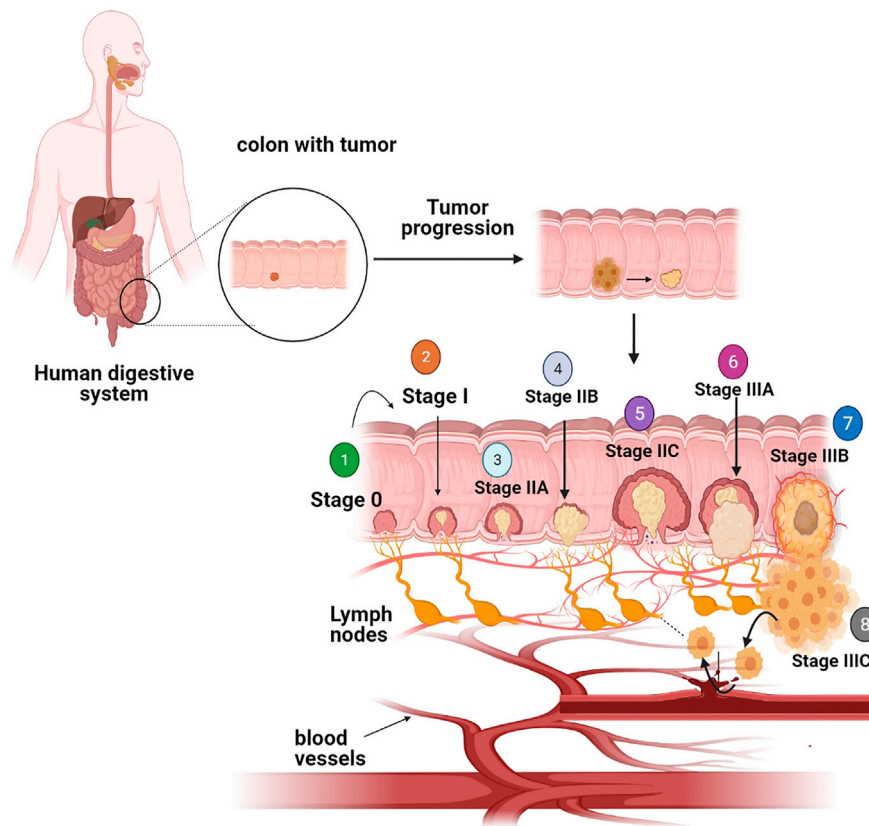


FIGURE 1 | Schematic representation of different stages of CRC.

anesthesia risk, which prevents a complete examination. In both the methods, x-rays are used to observe the colon from outside the body (Pickhardt, 2013; Kuipers and Spaander, 2015). The occurrence of colorectal neoplasm was detected using double-contrast barium enema (DBEM) screening in people of Thailand and reported a diagnostic outcome of 0.4% for CRC and 0.7% for advanced adenoma (Lohsiriwat et al., 2013). In sigmoidoscopy test for checking cancer, polyps, and other abnormalities using a flexible tube having light at the end is inserted into the lower colon and rectum (Guo et al., 2015). Many of the clinical trials reported that sigmoidoscopy reduces the chances of establishment and prevents persons from dying from CRC (Atkin et al., 2010; Segnan et al., 2011; Elmunzer et al., 2012; Schoen et al., 2012; Holme Kalager, 2014). All these screening tests for CRC have differed in respect to their preparation requirements, patient expediency, amount of colon and rectum evaluated along with their limitations.

Along with these general tests, the CellSerch[®] assay (Janssen Diagnostics, LLC, South Raritan, NJ, United States) which is based on circulating tumor cell (CTC) diagnostic technology is used for the diagnosis of meta-static colorectal cancer along with other cancers such as breast and prostate cancers. US FDA cleared circulating tumor cell (CTC) diagnostic technology for metastatic CRC along with other cancers such as prostate and breast cancers (Allen et al., 2014). It gives prediction-related information in metastatic CRC, regardless of the metastatic site. Currently, various new systems such

as MagSweeper, Cynvenio, IsoFlux, VerIFAS^t, AdnaGen, and magnetic sifters have been established to further enhance the identification speed and efficiency (Mostert et al., 2015). Most of the current screening methods are very expensive and not easy at the point of care. Hence, the development of more sensitive, fast, low cost, and specific screening of CRC is very essential.

Recent Advancements of Nanotechnology in Cancer Theranostics

Recent development in nano-sciences has allowed fabrication of several nanoparticles (NPs)-based systems for therapeutics and diagnostics. Although the clinical applications of nano-based theranostics are still limited probably due to their complex pharmacokinetics, NPs can improve the knowledge of biochemical and physiological principles of several diseases and their treatments (Siddique and Chow, 2020). NPs have been used in the enhancement of capability of several imaging techniques such as positron emission tomography (PET) by use of radioisotope chelator-free NPs, and iron oxide-based NPs in magnetic resonance imaging (MRI) (Rosado-de-Castro et al., 2018). Similarly, in the optical imaging system, persistent luminescence nanoparticles (PLNPs) have been used as a novel optical nanoprobe to utilize the characteristic long-lasting near-infrared (NIR) luminescence (Lecuyer et al., 2016) which allows the functioning of optical imaging without constant

TABLE 1 | Different methods for CRC screening.

Method	Explanation	Advantages	Disadvantages	References
Stool tests	Stool tests are kits that can detect abnormal blood or DNA markers. In gFOBT stool tests a chemical is used to detect heme from blood protein. The FIT test method uses antibodies for the detection of specifically hemoglobin protein. The FIT-DNA test identifies hemoglobin with certain DNA biomarkers for the detection of CRC.	Colon cleansing is not essential before the sample is taken. No dietary requirements are needed in FIT. Samples can be collected at home. The Stool test is a low-cost method as compared with other bowel tumor-screening methods	Stool test cannot detect nonbleeding cancers	Ansa et al. (2018), Bibbins-Domingo and Grossman (2016), Shapiro et al. (2017), Collins et al. (2005)
Colonoscopy	It allows visualization of the entire inner lining of the colon and rectum of a person for the detection of the tumor	This method detects all minute polyps along with large polyps and cancers, subsequently reducing the risk of development and death due to CRC.	It requires sedation It may lead to serious bleeding or a tear of the intestinal wall	Brenner et al. (2014), Kahi et al. (2016)
CT colonography	CT scanner is used in this technique for capturing two- and three-dimensional images of the entire colon. These images allow a radiologist to screen if cancers or polyps are present	Sedation is not required in this method. It does not require sedation. CT colonography is noninvasive and the entire colon can be examined for CRC.	Colon cleansing is not essential before the test. It may screen other abnormalities along with colon cancer and polyp	Ouyang et al. (2005), Kuipers and Spaander (2015), Pickhardt (2013)
Sigmoidoscopy	It allows the physician to directly examine the lining of the rectum and the lower section of the colon	It detects the cancers and polyps in the descending rectum and colon with a high degree of accuracy	It cannot screen the cancers or polyps present on the right side	Holme and Kalager (2014), Elmunzer et al. (2012)
Double-contrast barium enema	In this method, barium sulphate is introduced into the rectum with air via a flexible tube and x-ray images are then captured	This test mainly allows the examination of the whole colon and the rectum. Sedation is not required and complications are rare	Colon cleansing is very necessary for this method; otherwise, it will give false-positive results	Lohsiriwat et al. (2013)

excitation and autofluorescence (Liu et al., 2019). The NPs act as excellent contrast agents because of their small size, high sensitivity, and specific chemical composition. Similarly, NPs are also widely used for the therapeutic purpose of cancer. In therapeutic use, it improves the accumulation and release of pharmacologically active compounds at the pathological site leading to enhanced therapeutic efficacy and reduced toxic side effects. Moreover, recently developed NPs have the capability for integrating therapeutic and diagnostic agents into a single NP that can be easily used for theranostic purposes (Siddique and Chow, 2020). Studies have also revealed that theranostic NPs may have the capability for future use as personalized nanomedicine-based therapies (Baetke et al., 2015). In the coming future, multifunctional NPs can be designed by using various functional materials leading to the development of simultaneous diagnosis and therapeutics known as theranostics (Kim et al., 2017).

Existing Treatment Methods for Colorectal Cancer

At present, various treatment methods with their merits and demerits are available (Table 2). The most general method for the treatment of CRC is surgery and most commonly known as surgical resection. Surgery is the most common treatment for CRC and is often called surgical resection. It is a significant first line of the

protective system against any particularly well-defined tumor or any cancer (Lee, 2009; Chu, 2012). During surgical treatment in CRC, a portion of the healthy rectum or colon will also be excised. In addition to the surgical resection, other surgical options for CRC comprise colostomy for rectal cancer, radiofrequency ablation, laparoscopic surgery, etc. Radiation therapy is most widely used for the treatment of rectal cancer for the reason that this cancer tends to continue to the site where it grows initially. Radiation therapy is given to the patient in a scheduled manner comprising a defined number of treatments at defined time intervals (Joye and Haustermans, 2014). Stomach upset, mild skin infections, fatigue, etc. are the main side effects of radiation therapy. In the chemotherapy treatment method, drugs are used to deactivate cancer cells, generally by stopping the activity of tumor cells to grow and multiply. At present various approved drugs are used for the treatment of CRC (Jayakumar et al., 2015). Vomiting, nausea, neuropathy, mouth sores, and diarrhea are the main causes of chemotherapy. Treatment that targets the defined genes of tumor cells or defined tissue environment is known as targeted therapy. Such type of treatment inhibits the growth and division of the cancerous cells although limiting the harm to normal cells. To find out an efficient and reliable treatment of CRC, new methods should be established for effective detection of proteins, genes, and additional factors in a patient's cancer. For CRC treatment epidermal growth factor receptor (EGFR) inhibitor therapy, anti-angiogenesis therapy is an option. The most

TABLE 2 | Various existing methods with merits and demerits for the treatment of CRC.

Method	Description	Advantages	Disadvantages	References
Surgery	It is a very common method for the treatment of CRC and generally known surgical resection. It includes colostomy for renal cancer, laparoscopic surgery, and radiofrequency ablation	Surgical excision of the cancer is one of the important first lines of treatment against CRC, specifically when cancer is well defined	Surgery has been identified to enhance the risk of death due to metastasis in confined cancer patients by mechanical disruption of cancer integrity. Tenderness and pain in the part of operation are other disadvantages	Chu (2012), Lee (2009)
Radiation therapy	It uses high-energy X-rays to destroy tumor cells	For CRC, it is used before surgery and known as neo adjuvant therapy, to shrink cancer so that it can be easily removed. It may also be used after surgery for the death of remaining tumor cells. Both the methods have worked for the treatment of disease. It may also be used after surgery to destroy any remaining cancer cells	It may also damage the healthy cells and also cause DNA damage, which is very harmful. It may cause damage to the healthy cells	Joye and Haustermans (2014)
Chemotherapy	It uses drugs to destroy the tumor cells, which usually block the capability of CRC cells to grow and multiply	It is a well-established treatment modality	It may cause nausea, vomiting, mouth sores, or neuropathy	Jayakumar et al. (2015)
Targeted therapy	It is a treatment that targets the tumor-specific gene or tissue environment that contributes cancer growth and survival	It stops the growth and dividing of cancer cells while limiting damage to uninfected cells	Targeted treatments have side effects such as rash on the upper body and face	Xie et al. (2020)
Immunotherapy	Immunotherapies used for the treatment of CRC include monoclonal antibodies, cancer vaccines, immune modulators, adjuvants, and cytokines	This system uses body's self-immune system and fewer side effects and can provide long-term survival by 30%	Some of the immunotherapy drugs have high cost, severe side effects, and possible short-term efficacy	Johdi and Sukor (2020)

common side effects of the targeted therapy include rashes on upper body parts and the face.

During the treatment of CRC, poor drug response to chemotherapy is frequently reported which might be primarily due to the appearance of the multidrug resistance (MDR) in tumor cells (Wu et al., 2018). Several studies have shown the mechanism of drug resistance in colorectal cancer cells. Some of the studies revealed that the effect of cetuximab and panitumumab can be inactivated by the mutations in PIK3CA or BRAF gene, or due to gene amplification of MET, KRAS, and ERBB2 resulting in the prevention of EGFR signaling (Dienstmann et al., 2015). The overexpression of dihydropyrimidine dehydrogenase (DPD) gene may lead to resistance against 5-FU, because DPD may convert the 5-FU into the inactive metabolite (DeNardo and DeNardo, 2012). In addition, decreased expression of RFC-1 (reduced folate transporter 1) and PCFT (proton-coupled folate transporter) may block the LV (Odin et al., 2015). Similarly, altered levels of metallothioneins and glutathione reductase (GR) may affect the response of oxaliplatin (OXA). Moreover, the increased activity of UDP-glucuronosyltransferases (UGT) or induced DNA damage repair mechanism such as TDP1 (tyrosyl-DNA phosphodiesterase 1) may limit the effect of IRI (Jensen et al., 2015). To overcome the multidrug resistance, nanomedicine appears as a recent strategy to enhance the prognosis in CRC patients.

Applications of Nanotechnology in Screening, Detection, and Treatment of Colorectal Cancer

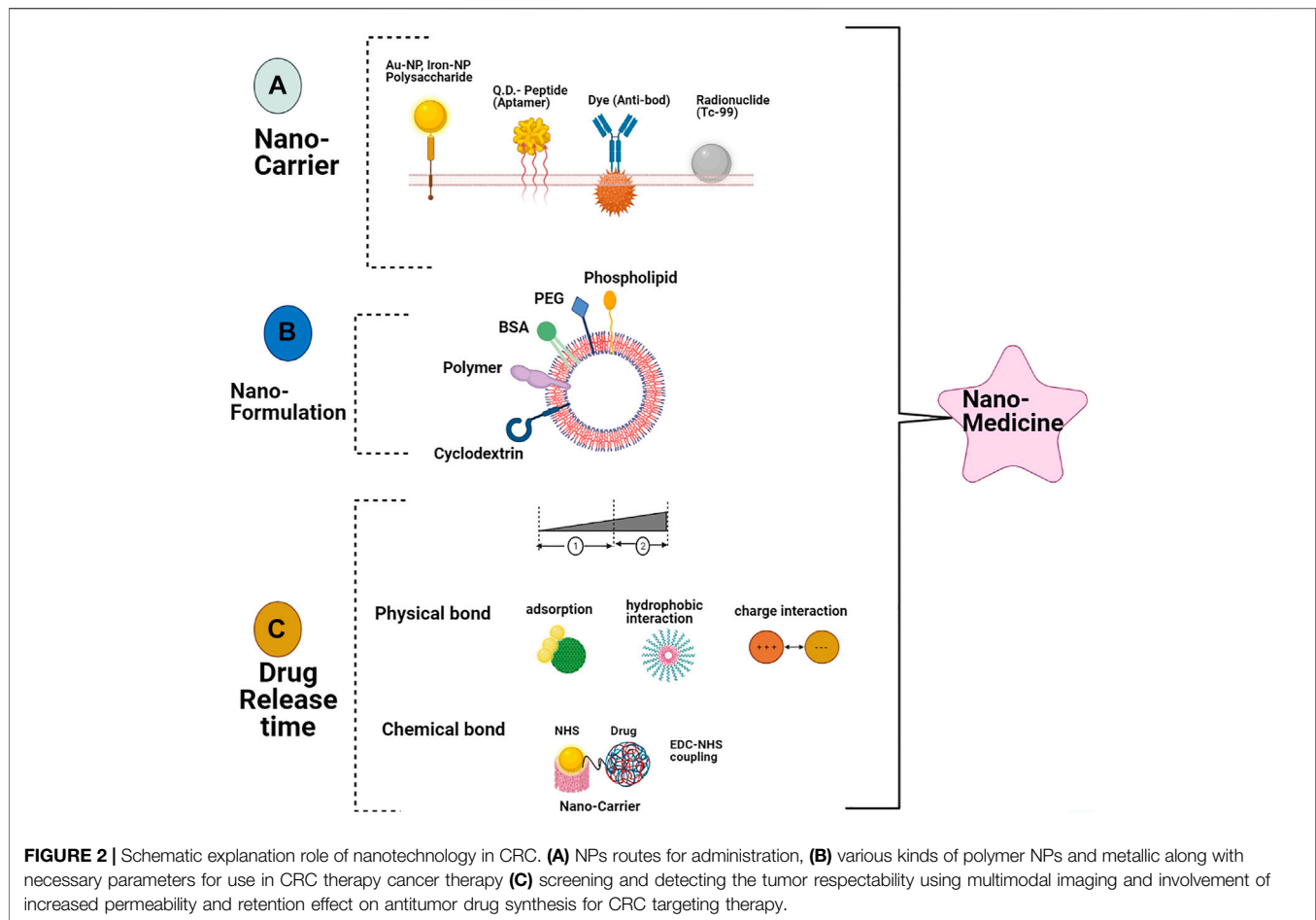
More than four decades ago application of nanotechnology foundations is laid down for their diagnostic and therapeutic

uses in a very precise manner (Fortina et al., 2007). Nanotechnology is a most important branch of research which plays a very important role in screening, detection, and treatment of day-by-day increasing diseases (Upendra et al., 2016). It plays a major role in the discovery and delivery of drugs with specific action at a specific target site with an enhanced success rate. The development of these nano-formulations includes liposomes, quantum dots, silica NPs, gold NPs, liposomes, dendrimers, nano-emulsions as a coating material in imaging at pathological sites, and nano-drug delivery (Bose et al., 2015; Yallapu et al., 2015).

Various important nanotechnological applications have been verified in cancer biology, comprising early screening and detection of cancers and establishment of new treatment approaches that cannot be gained using the existing conventional methods (Laroui et al., 2013). In *de facto*, in certain tumors, nano-sized particles of different shapes and compositions have emerged out as important and promising novel tools for colorectal cancer staging, diagnosis, and therapeutics (Figure 2) (Dong et al., 2016). Early screening and detection of CRC is the main solution for impediments and it can impact the long-lasting survival of CRC patients. Early detection of CRC is the key, and it can impact the long-term survival of patients with CRC. In this review, we discuss the current achievements of NPs that provide a new way for early screening and flourishing treatment of CRC.

Quantum Dots

Quantum dots (QDs) are semiconductor nanocrystals which fluoresce with light excitation and have special optical features, comprising high brightness, the ability to emit fluorescence at



varying wavelengths, and resistance to photo-bleaching. Owing to QDs chemical and optical advantages, QD-based nanotechnology is acting as a promising platform for studying various tumors including the CRC (Fang et al., 2012; Pericleou et al., 2012; Zeng et al., 2015). QD-based immune histochemistry (QD-IHC) has been used for qualitatively analyzing the expression of large external antigens from CRC tissue samples and compared with conventional-IHC (Wang et al., 2016), QD-IHC offered various detectable advantages for quantification of protein marker. These showed simpler operation, higher sensitivity, less human interference, and better capability for concurrent multifactor analysis; would produce more accurate clinical detections. Therefore, QD-IHC is a better option for conventional IHC in therapeutic applications. QD-based immune cytochemistry using QD-probes studies mainly focuses on marker identification in tissue sections, IHC combined with imaging quantitative analysis for screening of large external antigen in living cells showed results that were the same as obtained using flow cytometry and expanding the applications of QD-probes for clinical applications (Wang et al., 2016). In another study, a sensitive method was developed to identify Aldo-keto reductase family 1 member B10 (AKR1B10) in the serum that acts as a therapeutic target and prognostic predictor for CRC using QDs. The QDs possess stability against photo bleaching along with size-controlled

luminescence activity that makes QDs a suitable agent for photoelectrochemical-based tumor marker detection from biological samples. However, QDs are still not used for the detection of AKR1B10 from serum samples (Wang et al., 2015; Liu et al., 2021). This technique played a great role in the early detection of CRC with high specificity and sensitivity.

A highly sensitive and specific technology was developed, which enables concurrent detection of biochemical fluorescence and morphological changes during CRC progression and development using optical coherence tomography or laser-induced tomography that allows nondestructive internal visualization of CRC (Carbary-Ganz et al., 2015). A probe QDot655 specific to vascular endothelial growth factor receptor 2 (QD655-GEGFR2) restricted to the colon used in carcinogen-treated mice and provides significantly high contrast between infected and healthy tissues with specificity and sensitivity *ex-vivo* (Carbary-Ganz et al., 2015). These probes act for *in vivo* magnetic resonance imaging (MRI) and further biopsy of CRC, an exceptional cell-specific, paramagnetic double-signal fluorescent molecular multipurpose nanoprobe (GdDTPA-BSA@QDs-PCAb) was designed using surface engineering of QDs with DTPA-BSA-Gd3+ large molecular complex by ultrasonic conditions. These nanoprobes act as promising tools for use in contrast-enhanced

MRI diagnosis of CRC and have no cytotoxic effects in MTT assay (Xing et al., 2015). QDs-bevacizumab nanoprobe have been successfully used for targeted CRC detection in both *in-vitro* and *in-vivo* systems. During detection, they enhanced the tumor-specific signal after subsequent injection of the QDs and act as a significant achievement in VEGF-directed noninvasive imaging systems during clinical practices (Gazouli et al., 2014).

Under defined conditions subtractive Cell-SELEX technology is used for the production of a panel of seven aptamers (Apts) that precisely attach metastatic CRC cells (LoVo) along with other metastatic tumor cells with great affinity, and hence provides a broad spectrum specific detection of metastatic tumor cells (Li et al., 2014b). This research showed that Apts selected from a single Cell-SELEX can alone act as functionalized for several purposes based on target biochemical properties, thereby increasing the applications of the Cell-SEARCH approach. This receptor-specific Apt W14 was used as the targeted carrier for doxorubicin-specific delivery to defined cells to reduce the toxicity of this drug (Li et al., 2014b).

QDs conjugated with non-membrane receptor binding Apt W3 were used as a molecular probe for specified imaging of metastatic tumor-bearing sections, metastatic tumor cell lines, and formalin-fixed paraffin-embedded tissues from the CRC persons (Li et al., 2014). Hence QDs are great technological modalities in progress that can reform the CRC treatment and diagnosis.

IRON OXIDENANO-FORMULATIONS

Iron oxide NPs have the two-fold capability to work as both photo-thermal and magnetic agents for human applications as MRI agents. They also have brilliant biodegradability *in vivo* and after dissolution, iron ions can be adjusted by the body by a highly regulated physiological phenomenon (Espinosa et al., 2016). A smart multi functional magnetic nano vehicles encapsulating an antibody targeting peptide AP1 (MPVA AP1), which are consistent in size and also water soluble, anti-cancerous drug has been developed (Kuo and Liu, 2016). These nano-vehicles are easily dispersed in aqueous solutions and exhibit no cytotoxic effects in L929 fibroblasts, and showed their capability for therapeutic applications (Kuo and Liu, 2016). A CRC cell (CT26-IL4R) trial discovered that the MPVA-AP1 showed outstanding selectivity and targeting. A steady storage test revealed no leakage of encapsulated drugs without the stimulus of the magnetic field. In disparity, nano-vehicles loaded with doxorubicin burst upon treatment of high frequency of magnetic field, which is fast, accurate, and controlled release. Furthermore, *in vivo* investigations recognized that magnetic nano-vehicles exhibited obvious chemotherapeutic and thermotherapeutic effects. Hence, smart magnetic nano-vehicles e.g. MPVA-AP1 have the noteworthy capability for specified doses and precise controlled release in antitumor applications (Kuo and Liu, 2016).

The usefulness of polylactide-co-glycolic acid (PLGA) nanoparticle as 5-fluorouracil (5-FU) vehicles with or without an iron oxide and hyperthermia at DNA damage point in an HT-

29 colon tumor cell line spheroid culture model by alkaline comet assay (Esmaelbeygi et al., 2015) and found that less DNA damage in case 5-Fu loaded nanoparticles as compared with hyperthermia. Hence hyperthermia is a damaging agent and NPs are an efficient drug delivery system to CRC. The iron oxide NPs enhanced the effect of hyperthermia and could be extremely beneficial in the diagnosis of CRC (Esmaelbeygi et al., 2015).

The paclitaxel (PTX), as well as super-paramagnetic iron oxide (SPIO), is encapsulated inside the core of PEAL Ca micelles and studied for significant tumor therapy (Feng et al., 2014). The drug release in this research showed that PTX in the micelles was released with a lower rate at neutral pH and a faster rate at pH 5.0. Cell culture studies also showed that PTX-SPIO-PEALCa was successfully absorbed by the CRC LoVo cells and PTX was likely internalized by lysosomal cells. Additionally, successful inhibition of CRC LoVo cell growth was verified. Hence, micelles play a great role in MRI visible drug release methods for CRC treatment (Feng et al., 2014).

The lectin-Fe₂O₃@AuNPs are synthesized by joining lectins on the Fe₂O₃@AuNPs via bifunctional polyethylene glycol (PEG) NHS ester disulfide (NHS-PEG-S-S-PEG-NHS) linkers. Both *in vitro* and *in vivo* studies are done for checking the activity of lectins- Fe₂O₃@AuNPs and found that it could be useful for dual-mode MRI and X-ray CT imaging of CRC. Hence results obtained proposed that lectins could be applicable as cancer-targeting ligands in nano-formulations based on contrast agents (He et al., 2014).

Poly Lactic-co-Glycolic Acid Nanoparticles

Eco-friendly Poly lactic-co-glycolic acid (PLGA) NPs have been used as carriers for proteins, peptides, vaccines, drugs, and nucleotides. These can help in shielding drug moieties from breakdown and subsequently ensure the effective release of drugs (Sah et al., 2013) and are extensively studied for their potential use in tumor therapy, specifically for CRC. Using the emulsion-solvent evaporation method curcumin-loaded PLGA NPs (C-PNPs) are successfully synthesized for colon delivery (Akl et al., 2016). C-PNPs exhibit significantly higher cellular uptake in HT-29 cells in comparison with pure curcumin solution due to their sustained release, greater colloidal stability in gastrointestinal fluids, and smaller size. Therefore, C-PNPs have great potential as an early platform for the further establishment of a sufficient oral targeted drug system to the colon, mainly if it is additionally functionalized with a specific targeting ligand (Akl et al., 2016).

Scientists also prepared chitosan polymeric NPs using the solvent extraction emulsification technique, with different ratios of polymer, and showed potential application in the successful delivery of active pharmaceutical components to the CRCs (Tummala et al., 2015). Total health center complex N-38 is an effective treatment against many cancers but the delivery system is not easy due to its low solubility. SN-38 encapsulated in poly(D,L-lactide-co-glycolide) NPs were prepared by the spontaneous emulsification solvent diffusion method to improve its solubility, stability, and cellular uptake (Essa et al., 2015). To study cellular uptake and cytotoxicity of

SN-38 encapsulated NPs, the colorectal adenocarcinoma cell line-205 (COLO-205) was used and found considerably reduced cell proliferation and death. Hence, SN-38 loaded NPs are potentially essential drug delivery systems for the treatment of CRC (Essa et al., 2015).

Carbon Nanotubes

Carbon nanotubes (CNTs), allotropes of carbon cylindrical in shape, have rolled graphene sheets with a diameter of fewer than 1 μm and a few nanometers in length (Rastogi et al., 2014). Owing to CNT chemical and physical properties such as high surface area, needle-like structure, heat conductivity, and chemical stability enable its potential uses in several fields such as immunotherapy, diagnosis, gene therapy, and a carrier in the drug delivery system (Hampel et al., 2008).

Various researches showed that several strategies have been developed in which CNTs have been used for carrying antitumor drugs. For the delivery of paclitaxel in Caco-2 cells single-walled carbon nanotubes conjugated with a synthetic polyampholyte were used. Paclitaxel-SWCNT-treated Caco-2 and HT-29 cells showed greater anticancer effects when compared with alone paclitaxel (Lee and Geckeler, 2012; Wu et al., 2015). The same types of results have been found in Eudragit®-irinotecan loaded CNTs (Zhou et al., 2014). Oxaliplatin and mitomycin C-coated CNTs, induced by infrared light rays in colon cell lines, found considerably higher drug delivery and localization in cancer using thermal treatment of cell membrane (Levi-Polyachenko et al., 2009). One study revealed that SWCNTs modified with TRAIL (a ligand that binds to specific receptors and causes apoptosis process in cancer) increased the cell death 10 times in comparison with alone delivery of TRAIL in carcinoma cell lines (Zakaria et al., 2015).

Dendrimer

They are three-dimensional macromolecular structures. They possess central core molecules surrounded by consecutive layers. The dendrimer exhibit a high degree of molecular consistency along with shaping characteristics, fine molecular weight distribution, and multivalency. The specific physicochemical properties along with biodegradable backbones facilitate several applications of dendrimer in nanopharmaceuticals development (Abbasi et al., 2014; Huang et al., 2015a; Wu et al., 2015). Some of the studies revealed that dual antibody conjugates can provide advantages of capturing circulating tumor cells (CTCs) in contrast to single-antibody counterparts (Xie et al., 2015). The study also revealed that the surface-active dendrimers can be successively shielded with two antibodies against the human colorectal CTCs surface biomarkers Slex and EpCAM. Dual antibody-coated dendrimers exhibit improved specificity in the detection of CTCs in both patient blood and nude mice in comparison to single antibody-coated dendrimers. Moreover, dual antibody-coated conjugates may downregulate the captured CTCs. Thus, theoretically, it can be assumed that biocompatible two antibodies conjugated to a nanomaterial may have the capability to capture and

downregulate the CTCs that can be used as a new strategy for the prevention of metastasis (Xie et al., 2015).

Similarly in another study, HT29 cells (colon cancer cells) were successfully captured by employing Sialyl Lewis X antibodies (aSlex)-conjugated Poly(amidoamine) dendrimers (Xie et al., 2015b). The colon cancer cells were characterized using aSlex-coated dendrimer conjugate and examined by flow cytometry and microscopy. The study revealed that conjugate possessed an enhanced capacity to capture HT29 cells in a concentration-dependent manner. The aSlex-coated dendrimer conjugate showed optimum potential in capturing and detaining CTCs in the blood (Xie et al., 2015b). The maximum capture competence was obtained within 1 h of exposure.

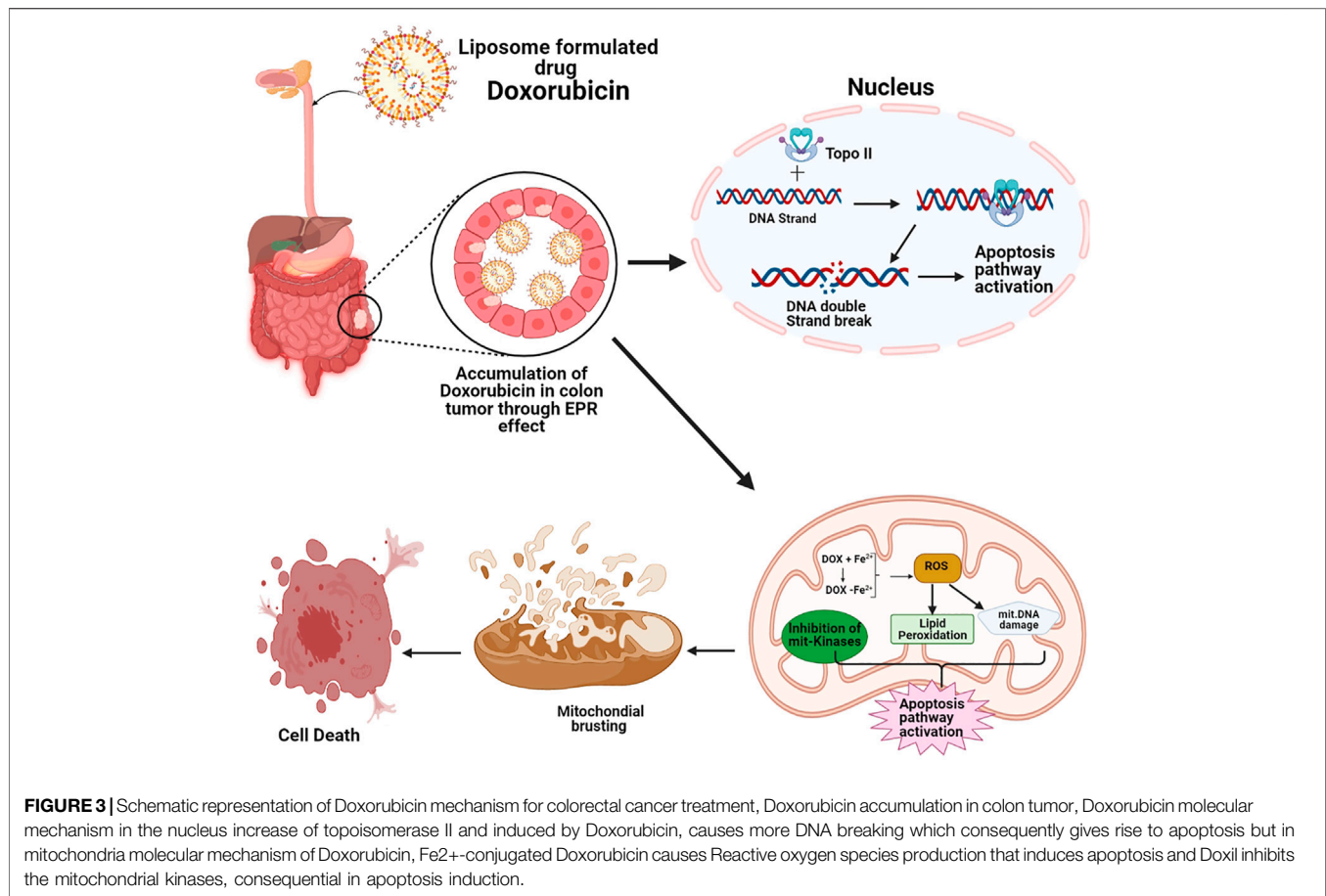
Apart from the diagnostic value, dendrimer may also be used for therapeutic purposes. In one of the studies, telodendrimers were suspended with linear PEG-blocking dendritic oligomer of vitamin E and cholic acid, designed for the delivery of gambogic acid (GA) and other natural anticancer compounds were used (Huang et al., 2015a). The study revealed that high GA-loading ability and subsequent drug release were obtained with these optimized telodendrimers. Moreover, these novel nano-formulations of GA were found to exhibit similar *in vitro* cytotoxic activity as the free drug against the colon cancer cells (Huang et al., 2015a).

Later, a new platform for drug delivery was developed with the reengineering of nano-scale dendrimers for the capture of CTCs in the blood (Xie et al., 2014). These nano-scale dendrimers were lodged with dual antibodies to aim the two surface biomarkers specific to colorectal CTCs with the capacity to particularly recognize and bind CTCs and downregulate the activity of CTCs by arresting cell division in the S phase. Moreover, dual-antibody conjugates revealed enhanced specificity and competence in controlling the CTCs *in vitro* as well as *in vivo* in comparison with their single-antibody counterparts. Thus, these studies revealed an innovative way of effective prevention of metastatic initiation by binding and restraining CTCs that were usually achieved by the traditional cytotoxic killing of cancer cells (Xie et al., 2014).

Liposomes

Chemically, liposomes are lipid-based vesicles that act as artificial carriers with a small and spherical aqueous core that are nontoxic (Silva et al., 2011). Owing to their smaller size, ability to incorporate various substances, and phospholipid bilayer in nature, they are considered the most effective drug delivery systems into cells with decreased side effects (Suntres, 2011; Patil and Jadhav, 2014). In 1961, liposome was described as the first nanoparticle platform for drug delivery in clinical medicine (Bangham et al., 1965). It is one of the most used for drug delivery systems especially for peptides, nucleic acids, and proteins as nano-liposomes (Abreu et al., 2011).

Liposomes can be divided into three categories viz., long-circulating liposomes, active targeting liposomes, and liposomes with special properties that include thermo-sensitive, pH-sensitive, magnetic, and positive (Akbarzadeh et al., 2013; Nag



and Awasthi, 2013; Noble et al., 2014). For clinical practice, liposome formulation carrying the drug such as daunorubicin (DaunoXome®) and Doxorubicin (Doxil®) has been approved by FDA in the mid-1990s (Barenholz, 2012). The study revealed that Doxil is approximately 100 nm in size and has much less gastrointestinal and cardiac toxicity (Rivera, 2003). Later, FDA has approved Marqibo® as a liposomal drug that is a cell cycle-dependent anticancer drug (Lammers et al., 2008; Lam and Ho, 2009; Allen and Cullis, 2013).

Drug resistance is a common problem in anticancer therapy. Several efforts have been made to fight drug resistance obtained when administrating liposome-based drugs such as Doxorubicin. In one of the studies during the administration of high dosages of an antitumor drug such as Doxorubicin (DOX) an aptamer-based drug delivery system assists in delivering high dosages of the active drug toward the target cancer cells (Li et al., 2014). Similarly, Thermodox® (thermo-sensitive liposome Doxorubicin) is used for colorectal liver metastases treatment in combination with radiofrequency ablation (Figure 3). In this treatment procedure, liposomal Doxorubicin formulation releases the active drug in response to the mild hyperthermic signal (Stang et al., 2012). The study revealed that Thermodox can effectively deliver twenty-five-fold more Doxorubicin to tumor cells than ordinary intravenous. Doxorubicin dose is

five fold more effective than to standard liposomal formulations in animal models.

Silica Nanoparticles

Silica materials are broadly categorized as xerogels and mesoporous silica nanoparticles (MSN). Silica nanoparticles possess numerous advantages such as a highly porous framework, biocompatibility, and easy functionalization (Amato, 2010; Wei et al., 2010). MSNs consist of a bee's-hive-like porous structure that assists in packing large amounts of bioactive molecules. Moreover, MSN also possesses important features such as adjustable size of cavities in the range of 50–300 and 2–6 nm respectively (Stang et al., 2012), very low level of toxicity, easy endocytosis, the ability of large quantity of medicine loading along with resistance to heat and pH (Bharti et al., 2015). For more precise drug delivery and action to cancer cells, amesoporous silica nanoparticle-protamine hybrid system (MSN-PRM) was also used which can selectively release the drugs in the proximity of cancer cells and get activated with specific enzymes to trigger the anticancer activity (Radhakrishnan et al., 2013). In another study conjugated the hyaluronic acid to MSNs was done as HA-MSN and it was proved that the amount of DOX loading into HA-MSNs increases significantly than bare MSNs (Yu et al., 2013). Moreover, the cellular uptake of DOX-HA-MSNs conjugate was also increased which was reflected by

the enhanced cytotoxicity to the human colon carcinoma cell line (HCT-116 cell line) (Gidding et al., 1999). Similarly, in another study, MSNs were functionalized with polyethyleneimine-polyethylene glycol (PEI-PEG) and polyethylene glycol (PEG) which was resulted in an increased amount of loading of Epirubicin hydrochloride (EPI) and exhibited improved antitumor activity (Hanafi-Bojd et al., 2015). Silica nanoparticles can also be used along with photosensitizer chemicals to kill the CRC cells. Researchers at the University of Buffalo and Roswell Park Cancer Institute have developed a silica-based nano shell that encases photosensitizer molecules. The nano shells are designed in such a way that they are easily taken up by the tumor cells and, upon exposure of cells to light the photosensitizers are activated and release the reactive oxygen molecules to kill the cancer cells (Advances in Colorectal Cancer Research, 2016). Currently, this technique is under clinical trials. Moreover, the second-generation photosensitizer-loaded nano shell is also in the developing phase where tumor-targeting and imaging agents will be incorporated to deliver at the tumor-specific sites that will assist in image-guided therapy for cancer (Advances in Colorectal Cancer Research, 2016). Thus, nanoparticles may also be allowed for molecular imaging of cancer cells followed by earlier diagnosis and targeted drug delivery.

Nano-Emulsion System

It is a transparent solution consisting of oil, water, and surfactant. It possessed thermodynamically uniform and stable physical properties. Nano-emulsions possess specific features such as facilitation of transferring drugs and drug combinations protection against external factors such as heat and pH (Bilensoy et al., 2009), lower toxicity, higher stability, and better efficiency along with the dissolution of nonpolar compounds (Patil and Jadhav, 2014). In one of the studies, the synergistic effect of gold nanoparticles (AuNPs) and lycopene (LP) on the HT-29 colon cancer cell line was studied. The experiment was designed in such a way that the first case consisted of a system of nano-emulsion having Tween 80 as emulsifiers along with LP and AuNPs and the second system consisted of a mixture of AuNPs and LP without any emulsion. The results revealed that the nano-emulsion system consists of dosages of LP and AuNPs as 250 and 125 times respectively lesser in comparison with their mixture mode, but the apoptosis induced by nano-emulsion was found three times higher than the mixture model (Chen et al., 2015; Huang et al., 2015b). Similarly, in another study, an ion-pairing complex of Oxaliplatin (OXA) with a deoxycholic acid derivative (Na-deoxycholy-1-lysyl-methylester, DCK) (OXA/DCK) was prepared for oral delivery of OXA and 5-fluorouracil (5-FU) to the colorectal cancer patient. The study revealed that in CT26 tumor-bearing mice, the tumor was inhibited by 73.9, 48.5, and 38.1% in comparison with tumor volumes in the control group and the oral OXA and 5-FU groups, respectively, which indicated the application of OXA/DCK and 5-FU as an oral combination therapy for CRC (Pangeni et al., 2016).

Core-Shell Polymeric Nano-Formulations

Nowadays there is an increasing interest in the manufacturing of core/shell nanoparticles with two or more materials (Zhou et al., 1994; Kumar et al., 2020). Such nanomaterials possess a core-shell structure where the outer surface atoms differ from those of the interior core atoms. The surface chemistry of core-shell type nanomaterials is usually characterized by multiple techniques such as secondary ion mass spectroscopy and X-ray photoelectron spectroscopy (Simonet and Valcárcel, 2009; Zielińska et al., 2020). In core/shell nanoparticles combinations of different materials such as organic/organic, organic/inorganic, inorganic/inorganic can be used (Ghosh and Paria, 2012) with specific purposes including increasing the stability, functionality, and dispersibility of the core particles. Moreover, specifically designed core-shell particles may also provide a controlled release of the core and therefore, allow the reduced consumption of precious materials (Kalele et al., 2006). The core/shell particles possess specific applications in the biomedical field such as bio-imaging for cell labeling, controlled drug delivery, and tissue engineering practices (Bai et al., 2006; Sounderya and Zhang, 2008; Stanciu et al., 2009). Some other types of nanomaterial such as *Lactobacillus reuteri* biofilm coated with zinc gallogermanate (ZGGO) mesoporous silica may also be used as a bacterial inspired bio-nanoparticle system (ZGGO@SiO₂@LRM). This nano system was found possessed with the unique property of targeted delivery of 5-FU to the tumor of colorectum area. Further study revealed that in comparison with 5-FU alone, the ZGGO@SiO₂@LRM nanosystem decreased the tumor number per mouse to one-half during *in-vivo* chemotherapy. Additionally, this system was found capable of tolerating the digestion of gastric acid and thus may support the targeted drug delivery of oral medicines into the colorectum region. Moreover, ZGGO also assists in the hassle-free photo luminescence (PL) bioimaging where LRM coating accurately targets the tumor of the colorectum region (Wang et al., 2019). Similarly, other silica-based core-shell nanoparticles such as mesoporous silica nanoparticles coated with PEG or hydrochloride dopamine along with epithelial cell adhesion molecule aptamer (MSN@PDA-PEGapt) were also used for targeted delivery of maytansine derivative (DM1) with 94% drug loading efficiency to treat colorectal cancer in mice (Li et al., 2017).

Metal-Organic Frameworks

Metal-organic frameworks (MOFs) are manufactured using metal nodes and organic linkers (Jiao et al., 2018). The MOFs-based biosensors have currently been applied for the detection of various targets, such as heavy metal ions (Zhang et al., 2017), hazard molecules (Liu et al., 2017), and living cancer cells (Gu et al., 2019). Most of the biosensors have been designed either for the detection of cancer markers (Jayanthi et al., 2017; Huang et al., 2018) or small biomolecules released from cancer cells for the early diagnosis such as the PBA(Ni-Fe):MoS₂ hollow nanocubes for hydrogen peroxide detection (Zhang et al., 2019) and 3D bimetallic Au/Pt nanoflowers for the identification of cellular ATP secretion (Zhu et al., 2020).

Later on, integrated MOFs with electrochemically active other components have also become an efficient strategy to exploit the

MOF-based cytosensors. In one such study bimetallic TbFe-MOFs have been developed using MOF on MOF strategy and was used for the anchoring of carbohydrate antigen 125 aptamers for identification of living Michigan Cancer Foundation-7 (MCF-7) cells (Li et al., 2020). Similarly, the bimetallic ZrHf-MOF doped with carbon dots was used in the identification of human epidermal growth factor receptor-2 (HER2) and HER2 expressed in MCF-7 cells (Gu et al., 2019). In another study, multicomponent Zr-MOFs were used for the detection of MCF-7 cells with an extremely low limit of detection as 31 cell ml⁻¹ (Li et al., 2020).

Later, combinations of MOFs with different types of nanomaterials such as metal oxide, metal nanoparticles, and carbon-based nanomaterials were used to create newer forms of multifunctional composites/hybrids (Liang et al., 2018; Al-Sagur et al., 2019). Several organic compounds such as metallophthalocyanines (MPs) (e.g., CuPc, FePc, CoPc, and NiPc) possess electro catalysis activity for the oxidation of biological compounds (Liu et al., 2017). The biological tissue of CRC possessed CT26 cells. Therefore, it is essential to detect the living CT26 cells for the early diagnosis of CRC (Peng, et al., 2020). In one of the studies a specific electrochemical cytosensor was described which was constructed on the Cr-MOF@CoPc nano hybrids for the detection of living CT26 cells (Duan et al., 2020). A Chlorin-Based nanoscale metal-organic framework was used for photodynamic therapy of colon cancers using mouse models and found that these have a great potential for clinical translation (Kuangda et al., 2015). In a recent study, zirconium-based metal-organic framework (MOF), PCN-223, was synthesized and used as a potential vehicle for 5FU for rectal delivery in diagnosis for CRC (Nea et al., 2020).

Gold Nanoparticles

Among the noble metal, gold nanoparticles (AuNPs) are considered the most stable nanomaterial that is used for the preparation of nanostructure with various structures and shapes such as nanocubes, nanospheres, nanorods, nanoflowers, nanobranches, nanowires, nano-bipyramids, nanoshells, and nanocages (Chen et al., 2008; Ramalingam et al., 2014; Li et al., 2015; Xiao et al., 2019). The recent advancement in technology has led to accurate surface coating of Au NPs with specific particle shape and size. These specificities of gold nanomaterials make it safer and specific anticancer and drug delivery agent (Siddique and Chow, 2020). Moreover, Artificial Intelligence and mathematical modeling-based study revealed that gold nanoparticle has the capability to adjust its optical densities, light absorbency, wavelengths. Therefore, adjusting the ideal wavelength with nanoparticle size may allow the higher amount of light absorbance within the nanoparticle itself leading to enhanced efficacy of gold nanoparticle against cancer cells (Moore and Chow, 2021). In a recent study gold NPs increase cisplatin delivery and potentiate chemotherapy by decompressing of CRC vessels (Zhao et al., 2018). Gold NPs along with nucleic acids used as molecular method for cellular uptake (Graczyk et al., 2020).

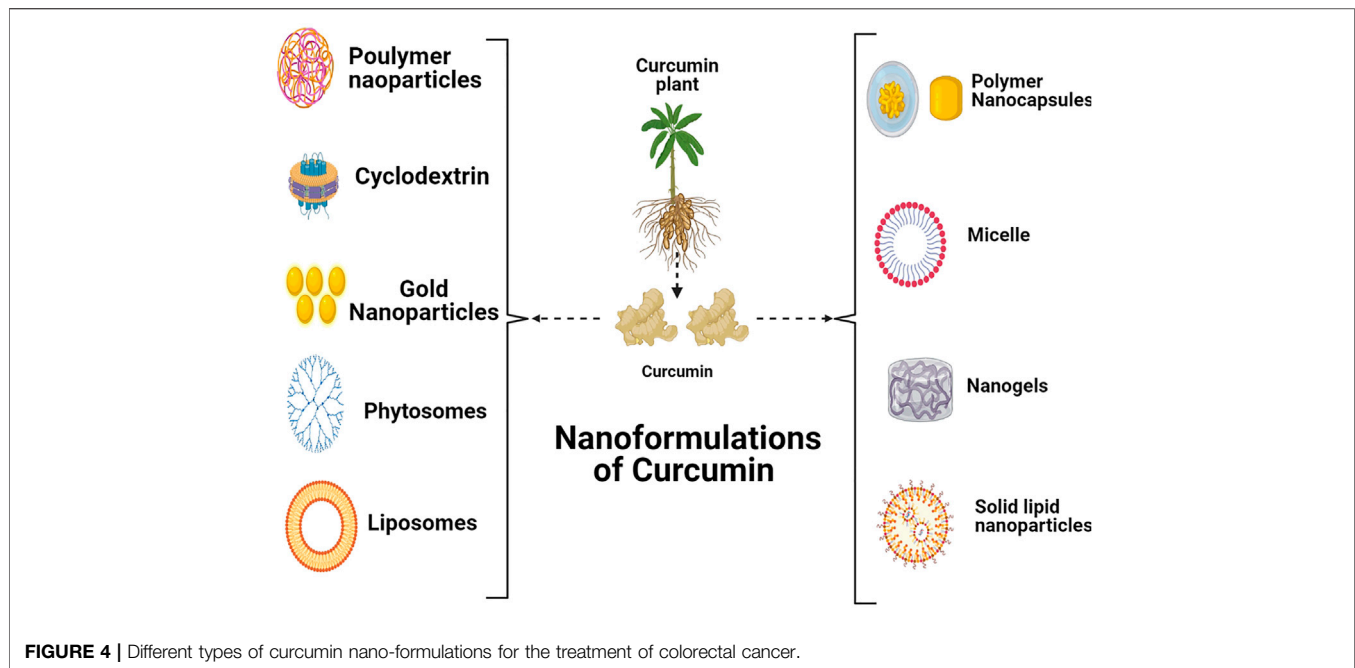
AuNPs have the property of easy to functionalize with biologically active organic molecules along with high physical

and chemical stability leading to their excellent biocompatibility that is an essential feature to be used in the medical field (Pissuwan et al., 2019). Moreover, AuNPs can directly conjugate with several other molecules including antibodies, nucleic acids, proteins, enzymes, fluorescent dyes, and drugs which enhance their applications in medical and biological activities (Ramalingam, 2019). Gold nanoshells or gold nanospheres have also been intensively studied over the past decade because of their specific and localized surface plasmon resonance. Gold nanospheres can also easily be conjugated with several imaging reporters and can carry genes, drug payloads, and other chemotherapeutic agents for theranostic applications. Gold nanoparticles usually passively accumulate in tumors and exert specific pharmaceutical effects with active targeting ligands such as Apts, antibodies, and peptides to the required targets (Singhana et al., 2015).

In one of the studies, cellular prion protein (PrPC) aptamer (Apt) conjugated with AuNPs was used for targeted delivery of doxorubicin (Dox) to CRC as PrPC-Apt-functionalized doxorubicin-oligomer-AuNPs (PrPC-Apt DOA). The result revealed that in comparison with free Dox treatment, the oligomer PrPC-Apt DOA decreased the growth and increased the apoptosis of CRC cells to a significant extent. This indicated the possibility of the application of PrPC-Apt DOA as a therapeutic agent for CRC (Go et al., 2021). The gold nanoparticles (GNPs) may also be used to enhance the anticancer efficacy of 5-FU and reduce its side effects. The 5-FU can be loaded to GNPs using thiol-containing ligands, thioglycolic acid (TGA), and glutathione (GSH) as 5-FU/GSH-GNPs. Further study revealed that the release of 5-FU from GNPs was slow and induced apoptosis in colorectal cancer cells. Overall, 5-FU/GSH-GNPs showed two-fold higher anticancer efficacies than that with free 5-FU (Safwat et al., 2016). Moreover, the electroporation-GNPs technique may also provide the opportunity for colon cancer therapy especially for the highly immunogenic cancers where otherwise tumors are unresectable (Arab-Bafrani et al., 2020). The gold nanoparticle can also be used for the early diagnosis of CRC cells using colonoscopy. The Center of Cancer Nanotechnology at Stanford University developed a technique where gold nanoparticles are specifically allowed to bind the CRC cells. Subsequently, light from a colonoscope is allowed to shine, the gold nanoparticle bounded cancer cells stand out from the normal cells that can be removed easily. This technique is going to begin a clinical trial for safety evaluation in human patients soon (Advances in Colorectal Cancer Research, 2016).

Other Nanoparticles in Colorectal Cancer Detection and Treatment

Nanodrug delivery system can be used to encapsulate the chemotherapeutics agent to targeted drug delivery to cancer cells which can avoid the adverse effect of conventional treatment. In recent years several nano carriers with diverse characteristics have already been tested where polypeptide-based copolymers were found substantial consideration for their biocompatibility, slow biodegradability, and considerably



lower toxicity. One such example assessed is poly(trimethylene carbonate)-block-poly(L-glutamic acid) derived polymersomes, targeted against the EGFR. It was loaded with plitidepsin and tested for efficacy and specificity in LS174T and HT29 cell lines (Goni-de-Cerio et al., 2015). Moreover, a systematic *in vitro* cytotoxicity study was also conducted with the unloaded polymersomes to determine the cell membrane asymmetry, viability, biocompatibility check, etc. which recognized the fine biocompatibility of plitidepsin-unloaded polymersomes. Further study revealed that the cellular uptake and cytotoxic effect exhibited by the EGFR-targeted plitideps in loaded polymersomes in CRC cell lines were found much more sensitive to anti-EGFR drug-loaded in comparison with targeted drug-loaded polymersomes. Moreover, untargeted polymersomes reduce the plitideps in cytotoxicity and cellular uptake which revealed the use of targeted nano-carrier in cell lines may be a line of treatment for CRC without any adverse effect on normal cells (Goni-de-Cerio et al., 2015).

Recently, one of the near-infra-red fluorescent proteinoid-poly(L-lactic acid)(PLLA) NPs preparation was described where a P(EF-PLLA) random copolymer was prepared using thermal copolymerization of L-phenylalanine(F), L-glutamicacid(E), and PLLA (Kolitz-Domb et al., 2014). The study revealed that under optimal conditions, the proteinoid-PLLA copolymer can self-assemble into hollow nano-sized particles that can be used to encapsulate the indocyanine green and NIR dye. Further study revealed that the encapsulation process enhances the photo stability of the dye. The anti-CEA antibodies and tumor-targeting ligands such as peanut agglutinin can be covalently conjugated to the surface of P (EF-PLLA) NPs and increases the detectable fluorescent signal from tumors. The efficacy of P (EF-PLLA) NPs for colon tumor detection has been demonstrated in the chicken embryo (Kolitz-Domb et al., 2014).

Over the years, several nano-formulations were being used to improve curcumin delivery to cancer sites (**Figure 4**). Nano-formulations are primarily used to enhance curcumin water solubility and to present a better consistent delivery for curcumin (Wong et al., 2019). Preferably, nano-formulation of curcumin for tumors should have improved anticancer activity as compared when curcumin alone and also nontoxic to normal cells. For CRC nano-formulation of curcumin has been documented in several investigations comprising micelles (Javadi et al., 2018), nano-gels (Madhusudana Rao et al., 2015), liposomes (Sesarman et al., 2018), polymeric NPs (Xiao et al., 2015), cyclodextrins (Ndong Ntoutuome et al., 2016), phytosomes (Marjaneh et al., 2018), solid lipid nanoparticles (Chirio et al., 2011), and gold NPs (SanojRejinold et al., 2015). Different nanomaterials for CRC detection and treatment have been discussed in **Table 3**. Although several nano formulations are undergoing clinical trials, the number of nano formulations used in clinical trials against the CRC is limited. Some of the nano formulations used for the appropriate clinical trials against the CRC are summarized in **Table 4**.

Combinatorial Nanomedicine and Colorectal Cancer Therapy

Nanomedicine has proved its efficacy in revolutionizing the therapeutics and diagnostics of cancers with the advancement in the development of nano devices. NPs have been used for the delivery of multidrug especially to mediate the drug resistance in relapsing cancers (Maya et al., 2014; Anitha et al., 2016). Recently, a study showing an improved efficacy of 5-FU assisted chemotherapy as a combinatorial strategy for colon cancer treatment was described (Anitha et al., 2014a; Maya et al., 2014). In combinatorial nanomedicine efficacy of both the

TABLE 3 | Summary of nano formulations used in the detection and treatment of CRC.

Nano-formulations	Structure	Characteristics	Applications	References
Iron oxide nano-crystals	1–100 nm diameter iron oxide particles. Methotrexate, superparamagnetic iron oxide nanoparticles (SPIONs), and Wheat germ agglutinin (WGA)	Diverse application in different fields and superparamagnetic properties	Cancer detection	Yang et al. (2014), Lima et al. (2017)
Dendrimers	Synthetic polymer of hyper branched pattern with regular repeating monomer unit	Structurally perfect molecules arranged in a characteristic fashion	Treatment & Detection	Xie et al. (2015)
Quantum dots	Nanocrystals of semiconductor ranging in diameter 2–10 nm	Show best optical properties such as photo bleaching resistant, high brightness, and tuneable wavelength	Treatment and detection	Wang et al. (2016)
Gold nano shells	Gold Surface Plasmon resonant made up of silica nano core shell surrounded by ultra-thin shell of gold	Plasmonic nanoparticles exhibit diverse applications as used in cancer therapy, sensing and used as optical filters	Detection and cancer treatment	Singhana et al. (2015)
Nano cells or PLGA nanoparticles	Different structural variants of PLGA copolymers that are used as efficient drug delivery carrier	Easily biodegradable biopolymer on hydrolysis forms the simple monomers as glycolic acid, lactic acid, etc.	US FDA approved therapy and detection technique	Akl et al. (2016)
Liposomes	Lipid bilayer self-assembled structure closed and colloidal in nature	Artificial vesicles of phospholipid bilayer which can effectively transport hydrophilic substances inside and outside the cell	Treatment and detection	Chibaudel et al. (2016)
CUR-CS-NP	Chitosan nanoparticles covering the curcumin	By muco-adhesion process efficient action of curcumin to cancerous cells. Moreover enhance the cell cycle arrest at G2/M phase and apoptosis in HT29 cells	Detection and treatment	Chuah et al. (2014)
Oxaliplatin/DCK and 5-FU	Hydrophilic 5-FU loaded with nano emulsion and N-deoxycholy-L-lysyl-methylester (DCK) linked with amphiphilic Ox-aliplatin	Reduction in tumor volume and enhanced availability of oxaliplatin	Treatment	Pangeni et al. (2016)
NP SQ-emcitabine/isoCA-4	Precipitates of squalene, gemcitabine, and isocombretastatin A-4 (isoCA-4)	Enhanced anti-proliferative and cytotoxic effect. Moreover regresses the tumor	Treatment	Maksimenko et al. (2014)
PFA@PTX NPs	poly(ferulic acid)(PFA) NPs and paclitaxel (PTX)-loaded PFA NPs	poly(ferulic acid) PFA inhibit tumor growth and good drug carrier along with nanoparticles	Treatment and detection	Zheng et al. (2019)
Nanogel	5-FU acts as a cross linker between beta cyclodextrin and nanoparticles in aqueous solution and forms nanogels. Nanogels are the biocompatible and efficiently released drug	Single-walled carbon nanotubes (SWCNTs) conjugated with a synthetic polyampholyte for delivering paclitaxel in cancerous cells	Treatment	Hosseinifar et al. (2018)
Carbon nanotubes	Single-walled carbon nanotubes (SWCNTs) conjugated with a synthetic polyampholyte for delivering paclitaxel in cancerous cells	Exhibit more effective antitumor effects	Diagnosis and treatment	Lee and Geckeler (2012)
SN-38 liposome	SN38-PA was synthesized by conjugating palmitic acid to SN38 via ester bond at C ₁₀ position and then this prodrug was encapsulated into liposomal carrier using the film dispersion method	Act as most potent antitumor analogues	Used in treating patients having metastatic colorectal cancer CRC	Wu et al. (2019), Canton et al. (2019)

drugs was enhanced due to nano encapsulation where 5-FU was used along with a nontoxic polymeric carrier system thiolated chitosan. In combinatorial use the dosage of 5-FU was decreased which was reflected with enhanced chemotherapeutic efficacy and beneficiary effect on CRC patient cases. The nano-sized drug-encapsulation systems possess specific efficacy in terms of passive retention of more drug-loaded NPs in the vicinity of cancer cells. These strategies have assisted in the development of the next phase of anticancer nanomedicine (Anitha et al., 2014; Malarvizhi et al., 2014; Maya et al., 2014; Linton et al., 2016).

Nanotechnology and Chemoprevention

Given side effects associated with chemotherapy, implementation of the nanotechnology-based formulation is very essential for cancer prevention, diagnosis, and treatment because it allows drug targeting and possible drug reduction which can

furthermore increase the safety of drugs by minimizing nontargeted toxicities.

NSAIDs are most extensively used for colon cancer prevention (Alizadeh et al., 2012; Drew et al., 2016; U.S. Preventative Service Task Force, 2016; Pan et al., 2018). The epidemiological investigations demonstrate that out of all the NSAIDs, the most promising agent reported is aspirin in reducing adenomatous tumor recurrence due to the accessibility consistent result with minimum gastrointestinal toxicity and no cardiovascular risk (Umezawa et al., 2019). Additionally, Grade B recommendation has been provided for aspirin by the U.S. Preventative Service Task Force (2016) for its utilization as chronic prophylaxis means for CRC (Dehmer et al., 2015). Although aspirin alone or in combination has been proved for colon chemo preventive activity, nano encapsulation of aspirin

TABLE 4 | Nanoparticle-based FDA drugs and drugs in clinical trials on CRC patients.

S. No	Nanomaterial/nanosystem used	Drug description	Applications	Clinical trial or FDA approval status	References
1	Liposome	Vincristine	Colorectal cancer, Acute Lymphoblastic Leukemia, Sarcoma, Neuroblastoma, Leukemia, Lymphoma, Brain tumors	FDA approved	Gidding et al. (1999)
2	Liposome	Doxorubicin	Colon cancer with liver metastasis	Phase II trial	Celsion (2016)
3	Liposome	SN-38 liposome	Metastatic colorectal cancer	Phase 2 trial (subsequently terminated)	US National Institute of Health (2016)
4	Liposome	Liposome-encapsulated IRI (Irinotecan) hydrochloride PE	Second-line therapy for metastatic colorectal cancer	Phase 2 trial (subsequently terminated)	US National Institute of Health (2016)
5	Liposome	Liposomal Cisplatin Analog (Aroplatin)	Colorectal cancer	Phase I/II trial	Pillai (2014)
6	Liposome	SN38	Metastatic colorectal cancer	Phase II trial	Bala et al. (2013)
7	Liposome	Irinotecan, PEGylated Liposome (Narekt -102)	Breast and colorectal cancer	Phase III I trial	Pillai (2014)
8	CPX-1 liposome	Liposomal IRI (irinotecan) hydrochloride and floxuridine	Advanced colorectal cancer	Phase II trial	Cabeza et al. (2020)
9	PEP02 liposome	Liposomal IRI hydrochloride + 5-FU and LV (leucovorin)	Metastatic colorectal cancer	Phase II trial	Cabeza et al. (2020)
10	MM-398	Liposomal IRI	Advanced cancer of unresectable nature	Phase Ib trial	Cabeza et al. (2020)
11	PROMITIL	PEGylated liposomal mitomycin C	Metastatic colorectal cancer	Phase I trial	Cabeza et al. (2020)
12	Nal-IRI	Liposomal IRI	Colorectal cancer along with advanced gastrointestinal cancers	Phase I/II trial	Cabeza et al. (2020)
13	Polymer	5-fluorouracil (5-FU) and DAVANAT (carbohydrate polymer)	Treatment of colorectal cancer	Phase I/II trial (subsequently terminated)	Xiao et al. (2015)
14	Regulatory lymphocytes (Tregs): anti-CTLA-4 ipilimumab and anti-PD-L1 atezolizumab	Cytotoxic antibodies expressed on surface of Tregs	colorectal cancer	FDA approved	Rampado et al. (2019)
15	NKTR-102/IRI	Formulation for prolonged release of IRI conjugated with PEG/IRI	Metastatic CRC with KRAS-mutant	II clinical trial	Cabeza et al. (2020)
16	Cyclodextrin nanoparticle	Camptothecin	Solid tumors, rectal cancer, renal cell carcinoma, non-small cell lung cancer	Phase I/II trial	Giglio et al. (2015)
17	PEG-PGA polymeric micelle	SN-38	Colorectal, lung, and ovarian cancers	Phase II trial	Hamaguchi et al. (2018)
18	Carbon NPs	Carbon NPs	Laparoscopic surgery of colorectal cancer	Phase I trial	Cabeza et al. (2020)
19	TKM-080301	Lipid NPs with serine/threonine kinase inhibitor	Colorectal cancer with liver metastases and ovarian, gastric, esophageal, and breast cancer	Phase I trial	Cabeza et al. (2020)
20	PEG-rhG-CSF	PEGylated recombinant human granulocyte colony stimulating factor (CSF)	Solid malignant tumors (colorectal, ovarian, lung, head, and neck cancer)	Phase IV trial	Cabeza et al. (2020)
21	Silica NPs	Fluorescent cRGDY-PEG-Cy5.5-C dots	Breast cancer and colorectal malignancies	Phase I-II trial	Cabeza et al. (2020)
22	Polymeric NPs + cetuximab + somatostatin analogue	Combination of NPs Cetuximab and Somatostatin analogue	Metastatic colorectal cancer	Phase I trial	Cabeza et al. (2020)

can enhance its efficacy with a reduced dose. The chemo preventive effect of the combination of calcium, folic acid, and aspirin has been studied on azoxymethane-treated 7-week-old Sprague-Dawley male rats and found that 1.7 fold higher effective during chemoprevention as compared with their un-modified complement regimen (Prabhu et al., 2007; Chaudhary et al., 2011). Another NSAID is Celecoxib that is being broadly explored in clinical uses for its chemoprevention potential; however, it offers pharmacokinetic variability and cardiotoxicity (Solomon et al., 2005). Celecoxib polymeric

NPs have been prepared using ethyl cellulose with lipid hybrid NPs, sodium casein ate bile salt and micro emulsions enhanced its bioavailability allowing a decrease in dose, crystallization, and related toxicity (Margulis-Goshen et al., 2011; Tan et al., 2011; Morgen et al., 2012). Naturally extracted plant-based chemicals are phytochemicals that are broadly studied as potential chemo preventive sources for their nontoxicity and pleiotropic effects (Thomasset et al., 2007; Zubair et al., 2017; Wong et al., 2019). In colon and intestinal cancer, curcumin has reported competent chemo

protective activity, but it has poor absorption, minimum water solubility, and low bioavailability. To conquer the problem, curcumin whey protein nano capsules were synthesized, which not only found >70% release within 48 h but also showed improved bioavailability and cell internalization (Jayaprakasha et al., 2016). In other research, it was found that curcumin encapsulated in polymeric nano carrier enhanced the solubility of curcumin and showed less structural abnormalities, considerable reduction in tumors and beta-catenin in the treated group with curcumin NPs as compared with the group treated only with curcumin (Alizadeh et al., 2012).

CONCLUSION AND FUTURE PROSPECTIVE

With the advancement in nanotechnologies, there has been increased research on the development of medical devices, targeted therapies, and novel drug delivery systems. Nanotechnology enabled the development of medical products from a single mode of action to multifunctional platforms such as nano theranostics to combine diagnostics and therapeutics. The preclinical study of nanomaterials has proved its efficacy as a therapeutics purpose of cancer. The detailed study of predictive immuno toxicity assays, nanoparticle surface characterization, and quantitative evaluation of encapsulated versus free drug fractions has highlighted the importance of nanotechnology in modern medicine. The advancement in drug delivery systems with the capability to modify the tissue uptake, bio-distribution of drugs, and pharmacokinetics of therapeutic agents has immense significance in biomedical research. Some of the nano carriers are even able to cross the blood-brain barrier (BBB) and may act at the cellular level. Moreover, several nanoparticle-based studies have focused on the development of techniques to customize novel drug conjugates and diagnostics as well as therapeutic devices. Nano carriers can also be programmed to release the therapeutic agents, fluorescent molecules, or even magnetic

materials to the colorectal cancer site to increase the bioavailability, drug solubility, stability, and tumor specificity of therapeutics agent in comparison with free molecular cargo. Moreover, nano carriers-based therapeutics agents may decrease the tumor multidrug resistance resulting in ineffective treatment by reducing the overall drug requirement and potential side effects. In recent years, the nanotechnology applied to CRC has evolved enough to complement the latest advances in tumor diagnosis and therapy far beyond the traditional systems. It can be combined with completely newer concepts of diagnostics and therapeutics synergistically with available methodology. Thus, in the coming future despite the several challenges in the application of nanotechnology, nanomedicine is going to gain the capability to play a critical role in the management of human CRC. The specific research on new methods directed at the understanding of nano-bio interface may reveal some additional relationships between the nanoparticle structure and its biological activity. Such information may be used in devising new strategies for further development of nanotechnology to improve the existing pharmaceuticals and development of novel therapeutics products in the future.

AUTHOR CONTRIBUTIONS

BB first prepare the whole manuscript. BB, KR, AP, MG, RK, and SS edited manuscript. Finally BB and PM critically analyse and finalize the manuscript.

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Hybrid Nanoparticles as an Efficient Porphyrin Delivery System for Cancer Cells to Enhance Photodynamic Therapy

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Photodynamic therapy (PDT) is a potential non-invasive approach for application in oncological diseases, based on the activation of a photosensitizer (PS) by light at a specific wavelength in the presence of molecular oxygen to produce reactive oxygen species (ROS) that trigger the death tumor cells. In this context, porphyrins are interesting PS because they are robust, have high chemical, photo, thermal, and oxidative stability, and can generate singlet oxygen (1O_2). However, porphyrins exhibit low solubility and a strong tendency to aggregate in a biological environment which limits their clinical application. To overcome these challenges, we developed hybrid nanostructures to immobilize 5,10,15,20-tetrakis[4-carboxyphenyl]thio-2,3,5,6-tetrafluorophenyl (**P**), a new third-generation PS. The biological effect of this system was evaluated against bladder cancer (BC) cells with or without light exposition. The nanostructure composed of lipid carriers coated by porphyrin-chitosan (**P-HNP**), presented a size of ca. 130 nm and low polydispersity (ca. 0.25). The presence of the porphyrin-chitosan (**P-chitosan**) on lipid nanoparticle surfaces increased the nanoparticle size, changed the zeta potential to positive, decreased the recrystallization index, and increased the thermal stability of nanoparticles. Furthermore, P-chitosan incorporation on nanoparticles increased the stability and enhanced the self-organization of the system and the formation of spherical structures, as observed by small-angle X-ray scattering (SAXS) analysis. Furthermore, the immobilization process maintained the **P** photoactivity and improved the photophysical properties of PS, minimizing its aggregation in the cell culture medium. In the photoinduction assays, the **P-HNP** displayed high phototoxicity with IC_{50} 3.2-folds lower than free porphyrin. This higher cytotoxic effect can be correlated to the high cellular uptake of porphyrin immobilized, as observed by confocal images. Moreover, the coated nanoparticles showed mucoadhesive properties interesting to its application *in vivo*. Therefore, the physical and chemical properties of nanoparticles may be relevant to improve the porphyrin photodynamic activity in BC cells.

Keywords: hybrid nanoparticles, chitosan, porphyrin, PDT-photodynamic therapy, bladder cancer cells

INTRODUCTION

Photodynamic therapy (PDT) is a non-invasive approach to treat oncological and non-oncological diseases (Figueira et al., 2014; Kwiatkowski et al., 2018; Mesquita et al., 2018a,b; Gazzi et al., 2019; Negri et al., 2019; Castro et al., 2020; Gomes et al., 2020; Lee et al., 2020). PDT is a clinically approved treatment based on the activation of a photosensitizer (PS) by the light of a specific wavelength, in the presence of molecular oxygen to produce reactive oxygen species (ROS), resulting in neoplastic cell death (Agostinis et al., 2011; Bogoeva et al., 2016). When the PS is activated, its molecules absorb photons, transmitting energy to molecular oxygen and triggering the production of singlet oxygen (1O_2) (type II), such as other ROS production by an electron transfer mechanism (type I) (Dougherty, 2002; Castano et al., 2004; Purushothaman et al., 2019). These ROS may activate a complex cascade of biochemical and physiological reactions able to induce tumor cell death by apoptosis, necrosis, or autophagy. The damage of microvessels suppresses the malignant tissue nutrition, oxygenation, and promotes adaptive antitumor immunity activation (Garg et al., 2010; Agostinis et al., 2011; Bacellar et al., 2015; Luiza Andreazza et al., 2016).

Bladder cancer (BC) is the tenth highest occurring malignant disease worldwide and the sixth with more incidence in men (Ferlay et al., 2019). In 2018, about 550,000 new cases were reported worldwide (Ferlay et al., 2019). Moreover, 75% of BC cases are diagnosed as non-muscle invasive bladder cancer (NMIBC) or superficial (Tse et al., 2019; Ramuta et al., 2020). Thus, due to the easy access to the bladder and the high exposition of malignant tissue on the bladder surface in cases of NMIBC, PDT has been suggested to enhance the effectiveness of BC therapy (Agostinis et al., 2011; Gomes et al., 2020).

Several porphyrins and their reduced derivatives (chlorins) have been developed and used in the clinic and clinical trials for cancer treatment (e.g., Photofrin or 5-ALA) (Dougherty, 2002; Master et al., 2013; Li et al., 2015; Zhang et al., 2016; Kou et al., 2017; dos Santos et al., 2019).

Porphyrins are aromatic heterocycle compounds formed by four pyrrole rings linked by four methyl bridges. These compounds are robust and have a high chemical, photo, thermal, and oxidative stability (Ptaszyńska et al., 2018). Porphyrins with varied structures and characteristics can be isolated from nature or synthesized. The ability of these derivatives to generate singlet oxygen enables its use as a photosensitizer in PDT (Plaetzer et al., 2003; Allison and Moghissi, 2013; Kou et al., 2017).

Although the use of porphyrin has been proposed as PS used in PDT, the disadvantages of this compound class have been observed, as it has clinical limitations due to its aggregation process (Nawalany et al., 2009; Liang et al., 2014). It is well established in literature that the photosensitizer must maintain a monomeric form to be photoactive (Ricchelli et al., 1998; Chen et al., 2005; Nawalany et al., 2009; Rabiee et al., 2020). Furthermore, several of these photosensitizers have shown low selectivity by cancer tissues (Purushothaman et al., 2019), resulting in the deactivation or reduction of the photosensitizer action. To overcome these challenges, several nanostructures have been developed as a photosensitizer delivery system to

increase their selectivity (Zhou et al., 2016; Battogtokh and Ko, 2017; Ujiie et al., 2019).

Berndt-Paetz et al. (2019) reported the success of PDT using tetrahydroporphyrin-tetratosylate (THPTS) encapsulated into liposomes. This system was able to inhibit BC cell growth, trigger cells to apoptosis or necrosis, mainly due to their subcellular localization through the cytoplasm and in lysosomes (Berndt-Paetz et al., 2019). Similar results were reported by Gomes et al. (2020) using polyvinylpyrrolidone micelles containing a triazole-porphyrin derivative.

Mucoadhesive nanoparticles and lipid nanostructures have also been used as PS delivery systems to enhance PDT. The porphyrin 5,10,15,20-tetrakis(*m*-hydroxyphenyl) (*m*THPP) was encapsulated in PLGA nanoparticles coated with poly(ethylene glycol) (PEG) or chitosan (Anderski et al., 2018; Mahler et al., 2019). These systems showed an increase in the intracellular accumulation of *m*THPP in HT-29-MTX and Caco-2 cells. Moreover, PEG-PLGA-*m*THPP nanoparticles showed superior cytotoxicity to chitosan-PLGA-*m*THPP nanoparticles. The PS chlorin *e*6 (Ce6) (Lee et al., 2011) and protoporphyrin IX (Lee et al., 2009) conjugated with chitosan nanoparticles and exhibited efficient accumulation in the tumor, exhibiting a superior therapeutic efficacy to free PS in tumor-bearing mice models. The encapsulation of verteporfin in nanostructured lipid carriers (NLCs-verteporfin) increased the uptake of this PS in 2D and 3D models of ovarian cancer cells and triggered a higher phototoxicity effect compared with free verteporfin (Michy et al., 2019). An enhancement of the phototoxic effect of *meso*-(tetra hydroxyphenyl) chlorin (*m*THPC) in breast cancer cells was also obtained when this porphyrin was encapsulated in solid lipid nanoparticles (Navarro et al., 2014). Zhang et al. (2019) developed a NLC with a surface modified with folate to co-delivery Ce6 and paclitaxel. This nanostructured system enhanced the Ce6 and PTX internalization in cancer cells and showed, in an animal model, a significant decrease in the tumor volume compared with groups in dark conditions (Zhang et al., 2019).

Another strategy explored is the use of hybrid nanoparticles. Hybrid nanosystems can be obtained by the combination of different materials (organic-inorganic or organic-organic), combining the attributes of each material to obtain a nanostructure with unique properties (Bochicchio et al., 2018; Mukherjee et al., 2019; Ferreira Soares et al., 2020). Hybrid chitosan-coated gold nanoparticle conjugated with *meso*-tetrakis(4-sulphonatophenyl) porphyrin (TPPS) exhibited high photothermal conversion efficiency, enhancing the cellular uptake of TPPS in HepG2 cells, and showed superior cytotoxicity to free TPPS (Zeng et al., 2018). Thus, the association of PS with nanostructures can improve the photodynamic action, reduce its side effects in healthy tissue (Battogtokh and Ko, 2017; Kwiatkowski et al., 2018; Ujiie et al., 2019), improve its stability and tissue penetration, and enhance the PS uptake in cancer cells (Ujiie et al., 2019).

This study reports on the immobilization of the new 5,10,15,20-tetrakis[(4-carboxyphenyl) thio-2,3,5,6-tetrafluorophenyl] porphyrin (P) (**Supplementary Figure S1**) in a biodegradable hybrid nanoparticle of the NLCs coated with chitosan, aiming to minimize the aggregation process, enhance

cellular uptake, and consequently improve the efficiency of this PS in PDT against BC cells.

EXPERIMENTAL SECTION

Materials

In general, the materials were purchased from Sigma-Aldrich without further purification. The Crodamol SS was obtained from CRODA and the *N,N*-Dimethylformamide (DMF) from Exodo.

Porphyrin Synthesis and Quantum Yields of Singlet Oxygen Determination

The porphyrin derivative was prepared according to the literature, following two steps: briefly. (1) The precursor 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin [**H₂(TPFPP)**] was synthesized by condensation of pyrrole with pentafluorobenzaldehyde in the presence of acetic acid and nitrobenzene under reflux conditions. (2) The tetra-substituted porphyrin 5,10,15,20-tetrakis[(4-carboxyphenyl)thio-2,3,5,6-tetrafluorophenyl] (simplified by **P**) was obtained by structural modification of [**H₂(TPFPP)**] in the presence of the nucleophile 4-mercaptobenzoic acid and pyridine, using DMF as solvent at room temperature for 24 h (Castro et al., 2015). The compound **P** was characterized by ¹H and ¹⁹F NMR (Bruker Avance 300 spectrometer at 300.13 and 282.38 MHz), UV-Vis (Agilent 8453 spectrophotometer), and fluorescence (F4500 – Hitachi spectrofluorometer) spectroscopies. In the fluorescent analysis, the widths of both excitation and emission slits were set at 3.0 nm. Additionally, the quantum yield of singlet oxygen (ϕ_{Δ}) was determined from the rate of decay of the ¹O₂ phosphorescence at 1270 nm using an Edinburgh F900 instrument consisting of a Rainbow OPO (Quantel Laser-France) 10 Hz, 2 mJ/pulse, which was pumped by a Brilliant NdYAG laser (Quantel Laser-France) and using 5,10,15,20-tetraphenylporphyrin (**TPP**) as standard in DMF ($\phi_{\Delta} = 0.65$) (Castro et al., 2020). The absorbance of the sample in DMF was adjusted to 0.1 at the excitation wavelength (420 nm).

Porphyrin Immobilized in Chitosan (P-Chitosan)

Chitosan solution in pH 5.2 at a concentration of 6.6 mg/mL was slowly dripped on a porphyrin solution in acetone (0.83 M) under magnetic stirring. The mixture was stirred (900 rpm) in dark conditions for 72 h at room temperature to acetone evaporation. Then, the homogeneous dispersion of the **P**-chitosan was incorporated into the aqueous phase of the nanoparticle's preparation process (see below).

Hybrid Nanoparticle Preparation

The nanostructured lipid carriers-NLCs (**NP**) coated with chitosan or **P**-chitosan (hybrid nanoparticles, **HNP** or **P-HNP**) were prepared in one step by the emulsification-ultrasonication method, a simple and scalable method (Pivetta et al., 2019). The lipid phase composed of 1.4% (m/v) of Crodamol SS and 0.4% (m/v) of oleic acid was melted at 70°C in a water bath. Thereafter,

the aqueous solution of Tween® 80 (1.25% m/v) containing or not 0.24% (m/v) of chitosan, or **P**-chitosan at 70°C was added to the lipid phase. The hot emulsion was sonicated for 10 min (Sonics VCX 750, probe of 13 mm, 40% of amplitude) and then the dispersion was cooled at 25°C.

Particle Size and Zeta Potential

The diameter by intensity and polydispersity index (PdI) were measured by dynamic light scattering using NanoSize ZS (Malvern®) with a scattering angle of 90°. The zeta potential (ZP) was determined by electrophoretic light scattering (ELS) using NanoSize ZS (Malvern®). Samples (**NP**, **HNP**, and **P-HNP**) were diluted with deionized water (for size) and KCl solution (for ZP) and analyzed at 25°C.

Porphyrin Immobilization Efficiency (IE%)

The efficiency of porphyrin immobilization in the hybrid nanoparticles was calculated based on free porphyrin (**P**) amount in the dispersion, an indirect method (Equation 1). For this, 500 μ L of the **P-HNP** dispersion was centrifugate at 5000 $\times$ g in an Amicon filter system from 10 kDa for 10 min. The filtrate containing the free **P** was diluted in DMSO and the absorbance was assessed using a UV-Vis (Agilent 8453 spectrophotometer). The concentration of non-immobilized **P** into nanoparticles dispersion was quantified using the molar extinction coefficient (ϵ) (127,902 M⁻¹ cm⁻¹) of **P** in DMSO, previously determined using calibration curves. In the equation below, the total amount of **P** added to the nanoparticle was considered 100%.

$$IE\% = \frac{([P]_{total} - [P]_{free})}{[P]_{total}} \times 100 \quad (1)$$

[*P*]_{total}, total concentration of *P*; [*P*]_{free}, free porphyrin concentration.

Atomic Force Microscopy

The morphology of the **HNP** was performed in a Shimadzu Scanning Probe Microscope (SPM-9600 model) operating in tapping mode. A probe of silicon (PPP-NCHR) was used with a length of 125 $\pm$ 10 μ m, a resonance frequency of 204–497 kHz, and a constant force of 10–130 N/m. The **HNP** dispersion was dripped on mica, followed by evaporation for 24 h at room temperature before the analysis.

Cryogenic Transmission Electron Microscopy

The size and the morphology of coated nanoparticles were determined by cryogenic transmission electron microscopy (Cryo-TEM). The **HNP** and **P-HNP** dispersions were dripped in a grid. After 24 h, dry samples were frozen at –184°C and analyzed in a high-resolution transmission electron microscope FEI TECNAI G² F20 (Thermo Fisher Scientific, United States), operating a beam voltage of 200 KeV. TEM images were analyzed in the ImageJ software (NIH, United States) to determine the size distribution of **HNP** and **P-HNP**.

Differential Scanning Calorimetry

The crystallinity of nanoparticles and their compounds were analyzed by differential scanning calorimetry (DSC) (Shimadzu DSC-50). The lyophilized samples were hermetically sealed in an aluminum pan and heated in the temperature range of 15–350°C. The heating rate was 10°C/min under nitrogen gas flow (3 kgf/cm²). The recrystallization index percentage (% IR) was calculated following Equation 2 (Shah and Pathak, 2010).

$$\% \text{ IR} = \frac{\Delta H_{\text{nanoparticle}}}{\Delta H_{\text{bulk lipid}} \times \text{fraction of lipid phase}} \times 100 \quad (2)$$

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was carried out using Shimadzu DSC-50. The previously dried samples were hermetically sealed in an aluminum pan. Then, the samples were heated under an inert atmosphere with a rate of 10°C/min in a temperature range from 30 to 880°C.

Small-Angle X-Ray Scattering

The equipment was adjusted for a sample detector distance of 3.7 m to investigate sizes around 100 nm. The radiation used was Copper K α ($\lambda = 1.54 \text{ \AA}$) and a measurement range of $0.038 < q < 1.03 \text{ (nm}^{-1}\text{)}$. Scattering data were collected using a two-dimensional Pilatus 300K detector. To guarantee the normalization of the data and the correct subtraction of medium contribution, capillary sample holders glued on steel liners were used. The azimuthal integration of the images was performed with the FIT2D program (Hammersley, 2016). Data processing was carried out according to standard procedures (Oliveira, 2011). The data were normalized to an absolute scale using water as a primary standard. Ten frames of 30 min were obtained for each sample. The treated data were compared to evaluate the stability of the sample and, later, experimental data were optimized. For the analysis, the Indirect Fourier Transformation method (Glatter, 1977) was used on a slightly different implementation (Oliveira et al., 2009). As a result, the theoretical fit of the scattering intensity and the corresponding pair distance distribution function [p(r)] are obtained. The overall shape of the p(r) curve provides indications of the particle shape in the system (Oliveira, 2011).

Evaluation of Porphyrin Photophysical Properties

Free porphyrin (P), P-chitosan, and P-HNP at 200 nM were evaluated regarding UV-Vis (Agilent 8453 spectrophotometer) and fluorescence (F4500 – Hitachi spectrofluorometer) spectroscopies. P-HNP and P-chitosan were prepared in deionized water, whereas free porphyrin (P) was prepared in DMSO 1% (v/v). The controls of chitosan, NP, and HNP were prepared in deionized water according to the same volume used for the P samples. All samples were excited at 420 nm. In the fluorescent analysis, the widths of both excitation and emission slits were set at 3.0 nm. The absorption and emission spectra were normalized.

Accelerated Stability

The accelerated stability of the nanoparticles was performed in a Dispersion Analyzer LUMiSizer 6120 centrifuges (L.U.M. GmbH, Berlin, Germany) using the SEPView v.6.4 software. This dispersion analyzer allows simultaneous recording of the intensity of transmitted light (808 nm) in dispersed systems as a function of time and the position of the sample in the cuvette using space and time resolved extinction profiles (STEP-technology) (Caddeo et al., 2013; Tan et al., 2016; Zielinska et al., 2019). NP, HNP, and P-HNP were added in 2 mm disposable polycarbonate sample cells. Measurements were performed at 25°C and the light transmission profile was acquired by the detector every 70 s for 250 min (200 profiles) at a rotation speed of 3801 rpm and light factor of 1. This analysis allows to detect signs of instability such as sedimentation, creaming, coalescence, flocculation, or phase separation (Caddeo et al., 2013; Tan et al., 2016; Zielinska et al., 2019). Instability indexes were calculated by SEPView v.6.4. The parameters used in this experiment mimic the stability or shelf life of 12 months (Caddeo et al., 2013; Tan et al., 2016; Zielinska et al., 2019).

Mucoadhesion Studies *in vitro*

To assess the mucoadhesive property of the coated nanoparticles *in vitro*, a mucin solution (1 mg/mL) was titrated under the nanoparticle dispersions diluted 50 times. This titration was performed in an MPT-2 accessory of the ZetaSizer Nano ZS. The concentration range analyzed was 0–0.05 mg mL⁻¹. For each point of the titration, the ZP of HNP and P-HNP was determined.

T24 Bladder Cancer Cells Culture

The human BC cells T24 were obtained from the Rio de Janeiro Cells Bank (BCRJ). T24 cells were cultured in RPMI medium supplemented with 10% of fetal bovine serum and 1% of a streptomycin-penicillin mixture and kept at 5% of CO₂ and 37°C (MCO-170AIC-PE, PHCbi, Canada).

Cytotoxicity (Dark Condition)

The cytotoxicity of free porphyrin (P), HNP, and P-HNP was evaluated by the resazurin assay. T24 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 24 h, the cells were treated with different concentrations of the samples (12.5–300 nM) prepared in RPMI phenol red free medium supplemented with 2% of fetal bovine serum and 1% of streptomycin-penicillin mixture (RPMI medium with 2% FBS). P stock solutions were prepared in DMSO (1 mM) and then diluted in RPMI medium [(DMSO) <0.5%]. After 6 or 24 h, the cells were washed with 200 μ L PBS and then 200 μ L of resazurin solution (25 μ g/mL) in RPMI medium without phenol red and serum was added to each well. The cells were incubated at 37°C for 4 h. After this period, the fluorescence was then measured with excitation at 530 nm and emission at 590 nm in a microtiter plate reader spectrophotometer (SynergyTM HTX Multi-Mode Microplate Reader, BioTek). DMSO (20% v/v) was used as positive control and untreated cells (cells + RPMI medium) were used as negative control.

Photocytotoxicity

Photodynamic studies were carried out in T24 cells with free porphyrin (**P**), **HNP**, and **P-HNP** using the resazurin assay. Briefly, 2×10^4 cells/well were seeded and then incubated at 37°C for 24 h. After incubation, cells were treated with the samples at the same concentrations used in the cytotoxicity experiment. After 6 or 24 h of incubation, the cells were washed with PBS, and then fresh RPMI medium with 2% FBS was added in each well. Immediately, the cells were irradiated at room temperature using a set of LEDs with the emission band ranging between 385 and 425 nm at an irradiance of 24.5 mW cm^{-2} with a total light dose of 5 or 10 J/cm^2 . The cells were incubated for 20 h at 37°C in a CO₂ incubator. Then, the resazurin solution was added and the cells were incubated for 4 h. After incubation, fluorescence was evaluated under the same conditions described previously. In the assay with a treatment time of 6 h, the cells were irradiated with only the fluence of 10 J/cm^2 .

The phototoxicity was also evaluated in T24 cells using Cell-titer Glo 2.0 Cell Viability Assay Kit in the same condition described above. After incubation, cells were treated with the samples at the range concentrations (12.5–300 nM) and then incubated. After 6 h, the cells were washed with PBS, and then fresh RPMI medium with 2% FBS was added in each well. The cells were exposed to 10 J/cm^2 at room temperature. Then, the cells were incubated at 37°C in a CO₂ incubator for 20 h. After incubation, Cell-titer Glo 2.0 reagent was added to each well according to Promega Protocol, and cells were incubated at room temperature. After 10 min, samples were transferred to 96 wells opaque plates, and luminescence was measured in a microtiter plate reader spectrophotometer (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). For those studies, the positive and negative controls were the same as those used for cytotoxicity assays. The IC₅₀ values were calculated using Prism 5.01 (GraphPad Software Inc., San Diego, CA, United States) software.

Evaluation of Porphyrin Uptake by Laser Scanning Confocal Microscopy

For the laser scanning confocal microscopy studies, 80,000 cells were placed in each well of a glass-bottom plate (four divisions/wells, CELL view dish-Greiner Bio-One, Brazil). After 24 h, the cells were treated with **P** or **P-HNP** at 10 μM for 30 min. Then, the cells were washed twice with PBS and marked using Fluoroshield with DAPI (nucleus probe, Sigma-Aldrich). After 2 h of incubation, the cells were analyzed by a laser scanning confocal microscope (LEICA-TCS SP2)

using a magnification of 64× a diode and HeNe laser with blue filter for DAPI and **P** ($\lambda_{\text{excitation}} = 405 \text{ nm}$). Untreated cells (cells + RPMI medium) marked with DAPI were used as a negative control. The bandwidth established for emission detection ranged from 411 to 540 nm for DAPI and from 630 to 710 nm for **P**.

Statistical Analysis

The results of size, Pdl, and ZP were analyzed by Prism 5 (GraphPad Software Inc., San Diego, CA, United States) software, using one-way analyses of variance (ANOVA) followed by Tukey. Phototoxicity results were analyzed using two-way analyses of variance followed by Bonferroni post-tests, with a significance level of 5% (0.05), 1% (0.001), and 0.1% (0.001). Data were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Physicochemical Characterization of Nanostructures

This study explored 5,10,15,20-tetrakis[(4-carboxyphenyl) thio-2,3,5,6-tetrafluorophenyl] porphyrin (**P**) as a photosensitizer. It was chosen due to its interesting photophysical properties and encouraging results when immobilized in chitosan film, against *Listeria monocytogenes* biofilm after the light exposition (Castro et al., 2017). This porphyrin has never been applied as a photosensitizer against cancer cells.

Porphyrins have been immobilized or encapsulated in nanostructures as a strategy to avoid the potential problems intrinsic to these compounds, such as aggregation (Zhou et al., 2016). Furthermore, this strategy may increase the delivery of these compounds within the target cells, leading to an interesting intracellular localization to potentialize the effect of this PS on PDT (Lavado et al., 2015; Zhao et al., 2016; Kwiatkowski et al., 2018). Among the nanostructures, chitosan nanoparticles, and lipid nanoparticles have been explored as PS delivery systems (Anderski et al., 2018; Mahler et al., 2019; Michy et al., 2019; Zhang et al., 2019). However, our group developed solid lipid-polymer hybrid nanoparticles to immobilize porphyrin on its surface, a new particle that has not to date been explored in the PDT area. Lipid-polymer hybrid nanoparticles are a powerful approach to mitigate disadvantages to lipid and polymeric nanoparticles, achieving a nanostructure with superior features (Wong et al., 2006; Wang et al., 2018; Khan et al., 2019).

TABLE 1 | Values of size, polydispersity index (Pdl), zeta potential (ZP), pH of the formulations **NP**, **HNP**, and percentage of immobilization efficiency (%IE) of **P-HNP**.

Samples	Size (nm)	Size SD	Pdl	Pdl SD	ZP (mV)	ZP SD	pH	IE%	SD IE%
NP	71.3	3.2	0.122	0.07	−11.3	0.7	3.7	–	–
HNP	100.9	11.1	0.207	0.009	22.5	0.3	5.29	–	–
P-HNP	127.4	4.5	0.249	0.018	27.1	1.2	5.22	95.9	1.2

IE%, percentage of immobilization efficiency; SD, standard deviation, $n = 3$.

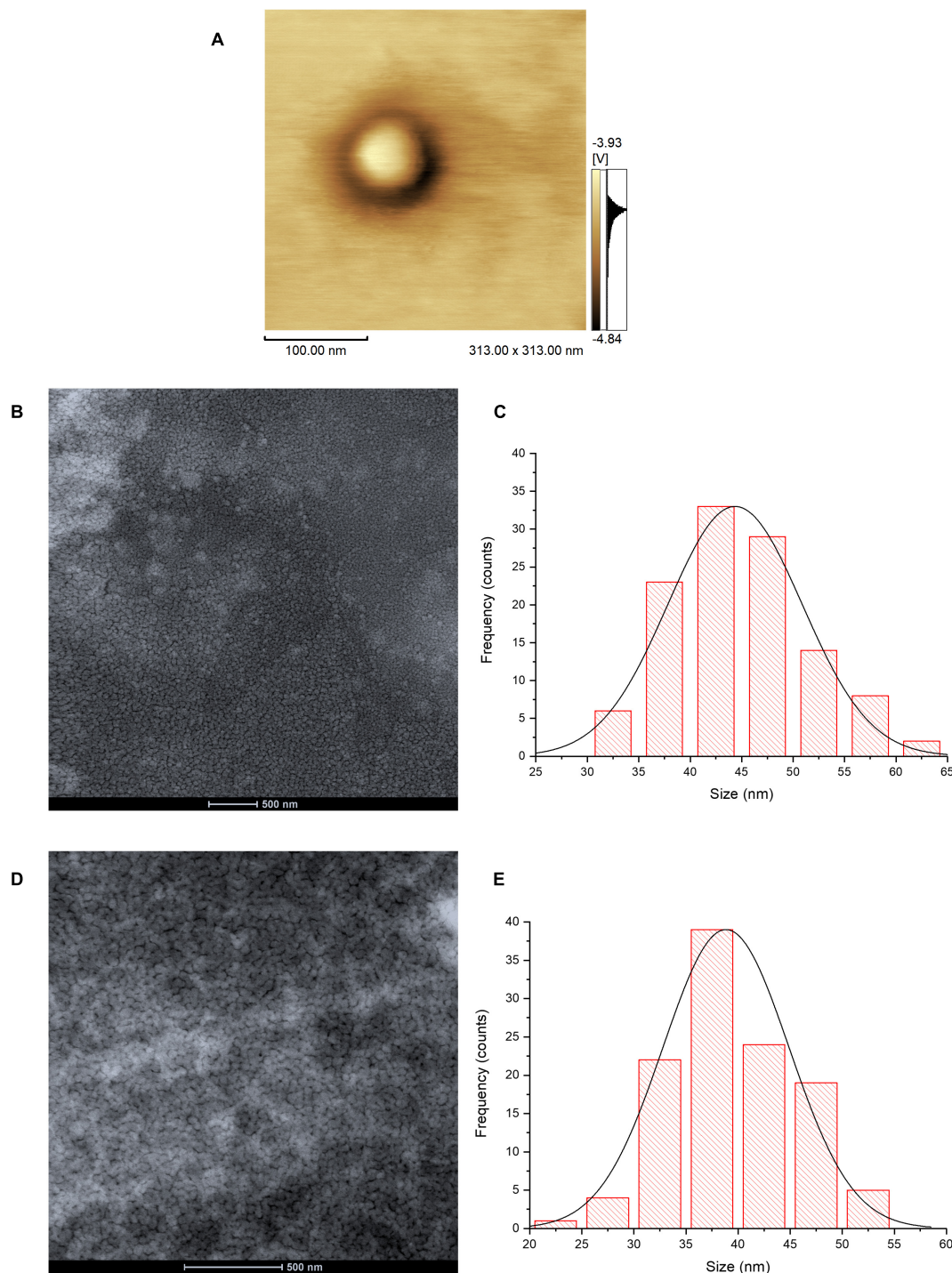


FIGURE 1 | Atomic force microscopy image of **HNP** in phase mode **(A)**; Cryo-TEM images of **HNP** **(B)** and **P-HNP** **(D)**; size distribution histograms of **HNP** **(C)** and **P-HNP** **(E)**.

The polymer chitosan is a non-toxic and biodegradable polysaccharide that plays a special role in porphyrin immobilization due to its ability to form stable complexes with negative charge compounds and substances (Knorr, 1984; Muzzarelli, 1996; Yang et al., 2009; Ferreira et al., 2016;

Castro et al., 2017). In the pH of chitosan solution and the hybrid nanoparticles dispersions (pH *ca.* 5.2), the chitosan amino groups ($-\text{NH}_2$) are protonated ($-\text{NH}_3^+$), with high positive density, and may interact with carboxylate *meso*-substituents groups from porphyrin (Knorr, 1984; Muzzarelli, 1996;

TABLE 2 | Enthalpy, melting point, and recrystallization index (RI) of solid lipid (Crodamol SS), **NP**, **HNP**, and **P-HNP**.

Samples	Enthalpy (J/g)	Melting point (°C)	RI (%)
Crodamol SS	156.3	48.2	100
NP	60.0	47.0	27.4
HNP	55.3	46.1	25.3
P-HNP	62.0	48.6	28.3

Khunawattanakul et al., 2008; Yang et al., 2009; Mohammed et al., 2017). This interaction was promoted in two steps: (1) A solution of porphyrin in acetone was mixed with a homogeneous chitosan solution (pH = 5.2) under stirring to immobilize **P** on the chitosan surface by electrostatic interaction; (2) The homogeneous dispersion of **P**-chitosan acetone-free was mixed with an aqueous surfactant solution that then, after heating, was added to the melted lipid phase to obtain NLC coating with porphyrin-chitosan (**P-HNP**). The **NP** showed negative ZP while the hybrid nanoparticle (**HNP** and **P-HNP**) exhibited a positive value (Table 1). This result confirmed the success of the carrier's coating process with the hypothesis of the chitosan adsorption in **NP** negative surface since this natural polymer is well known for its positive charge (Cheung et al., 2015; Liu et al., 2016a). Furthermore, values of ZP > |20 mV| could prevent aggregates formation by electrostatic repulsion forces (Feng and Huang, 2001).

The nanoparticle surface modification with chitosan and **P**-chitosan significantly increased the hydrodynamic size and changed the ZP of **HNP** and **P-HNP** ($p < 0.05$, one-way ANOVA) (Table 1). Several studies have described the increase of lipid nanoparticles after the chitosan coating process (Liu et al., 2016a; Vieira et al., 2018; Malgarim Cordenonsi et al., 2019). However, the PDI was only significantly influenced by the incorporation of **P**-chitosan ($p < 0.05$, one-way ANOVA) (Table 1). Additionally, PDI values were below 0.3 for all nanoparticles, which is a characteristic of nanoparticles with low polydispersity and a narrow range of size distribution (Üner et al., 2004; Tomasina et al., 2013).

The percentage of immobilization efficiency (IE%) of porphyrin on **HNP** was measured through an indirect method and was high (ca. 96%).

The accelerated stability study showed that **NP** presented a low instability index of 0.065 and kept its transmittance profile

practically unchanged over the 1-year, being considered stable in this period at a storage temperature of 25°C (Supplementary Figure S2). The hybrid nanoparticles **HNP** and **P-HNP**, over 1 year at 25°C, exhibited a tendency to instability phenomena, with signs of creaming (Supplementary Figure S2), exhibiting instability indexes of 0.818 and 0.700 (Pereira et al., 2018). However, over 31 days, the **P-HNP** was considered stable with instability indexes of 0.105, showing a stability time 1.6-fold higher than **HNP**. Thus, the porphyrin incorporation increased the **HNP**'s long-term stability.

Atomic Force Microscopy and Transmission Electron Cryo-Microscopy

The image of atomic force microscopy (AFM) in the phase mode shows a color variation on the particle, suggesting the structure core-shell of **HNP** due to the difference of materials in the surface (Figures 1A,B). The histograms from the Cryo-TEM analysis showed that both **HNP** and **P-HNP** exhibited a homogeneous distribution with well-defined nanoparticles size (Figures 1D,E). The **HNP** size ranged from 30 to 65 nm, whereas the size of **P-HNP** varied between 20 and 55 nm (Figures 1D,E).

Thermal Analysis: Differential Scanning Calorimetry and Thermogravimetry

The chitosan addition decreased the recrystallization index (RI) of nanoparticles from 27.4 to 25.3%, probably because of the enhancement of lattice defects in the lipid matrix of **NP** triggered by the interaction among chitosan, lipids, and surfactants (Table 2; Vieira et al., 2018; Malgarim Cordenonsi et al., 2019).

Furthermore, the porphyrin incorporation increased the melting point (°C) of the nanoparticles from 46.1 to 48.6°C and the RI from 25.3 to 28.3% when compared with nanoparticles without porphyrin (Table 2).

Both chitosan and **P**-chitosan coating increased the thermal stability of nanoparticles as shown in TGA (Table 3). **HNP** and **P-HNP** showed a remaining weight percentage (RW %) of 37.5 and 40.5%, respectively, for the second maximum degradation temperature (T_{dmax2}), whereas **NP** had only 20.7% of remaining weight for a lower temperature of 415°C (Table 3). Furthermore, the **HNP** presented a lower weight percentage (ca. 4.4% less) than

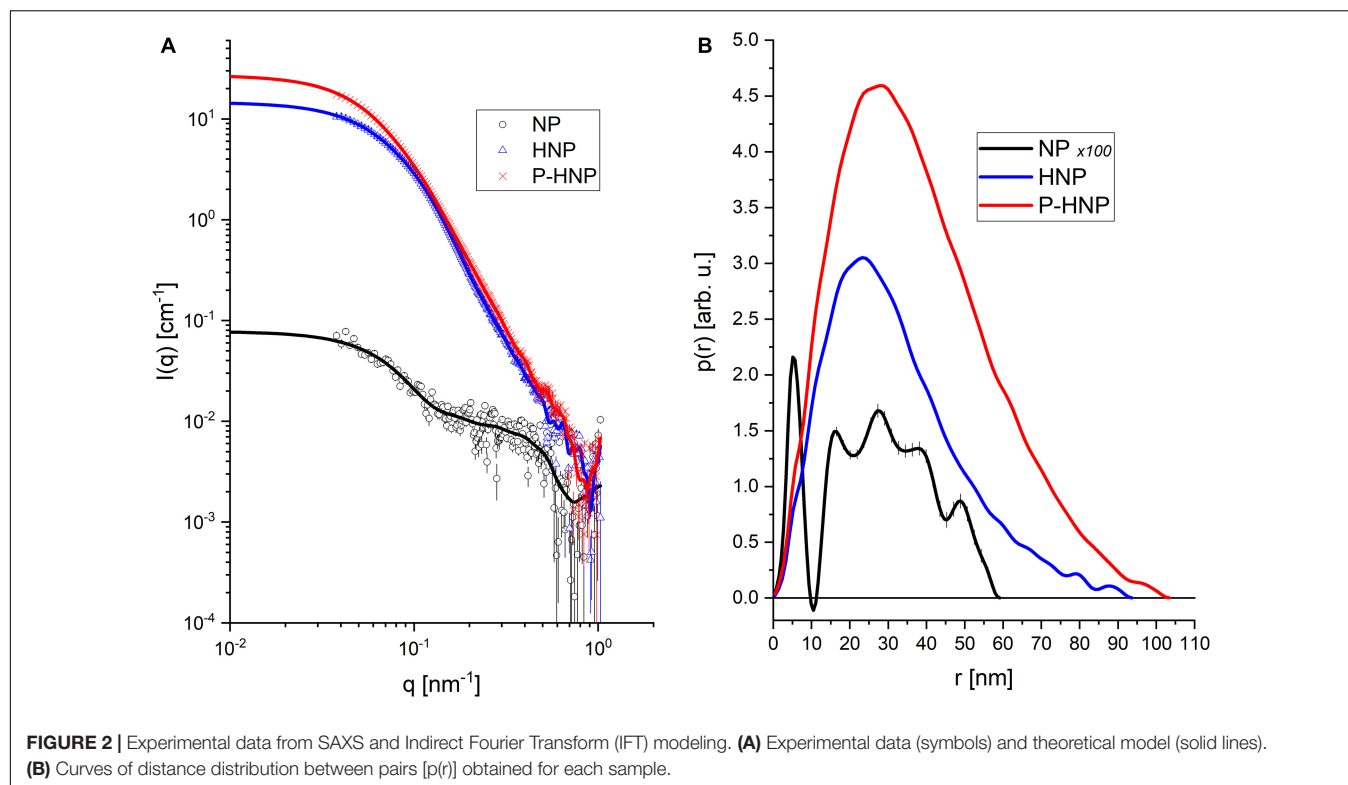
TABLE 3 | Thermogravimetric data of the porphyrin (**P**), chitosan (**Ch**), nanostructured particles (**NP**), hybrid nanoparticles (**HNP**), and hybrid nanoparticles with porphyrin (**P-HNP**).

Sample	T_{di} ^a (°C)	RW (%)	T_{dmax1} ^b (°C)	RW ^c (%)	T_{dmax2} (°C)	RW (%)	T_{dmax3} (°C)	RW (%)	T_{dmax4} (°C)	°RW (%)
P	290	84.7	336	80.3	400	70.4	456	62.8	622	44.9
Ch	260	87.3	315	73.6	–	–	–	–	–	–
NP	188	96.6	369	47.2	415	20.7	–	–	–	–
HNP	246	96.8	360	68.8	422	37.5	511	10.8	–	–
P-HNP	252	90.5	350	71.0	424	40.5	507	15.2	–	–

^a T_{di} , initial degradation temperature.

^b T_{dmax} , maximum degradation temperature.

^cRW%, remained weight percentage.



the values obtained for **P-HNP** at $T_{\text{dmax}3}$, which suggests that the incorporation of **P** improves the **P-HNP** heating resistance.

Small-Angle X-Ray Scattering

For a better understanding of the changes in the structure of nanoparticles after chitosan and **P**-chitosan incorporation, X-ray scattering analysis was carried out. The X-ray scattering intensity in the origin, $I(0)$, and radius of gyration (R_g) of **NP**, **HNP**, and **P-HNP** are shown in **Figure 2** and **Table 4**, respectively. Small-angle X-ray scattering (SAXS) intensity varies according to the concentration and size of nanoparticles (Li et al., 2016). Thus, the results suggested that the addition of coatings modulated the size and concentration of the nanoparticles. **HNP** and **P-HNP** exhibited well-defined size and higher concentrations of nanostructures when compared to nanoparticles without chitosan, which can be correlated with the increase in X-ray scattering (**Figure 2A**). Consequently, nanoparticles prepared with chitosan showed greater efficiency in the self-organization

of components. Furthermore, the $p(r)$ function for (**HNP**) or (**P-HNP**) with bell-shape suggests the interaction of the chitosan with other formulation components to form globular (spherical) nanostructures, while non-defined size and morphology were verified for **NP** (**Figure 2B**). These results agree with the AFM and Cryo-TEM images (**Figure 1**).

Photophysical Properties

The absorption and emission spectra of the porphyrin are shown in **Figure 3**. The non-immobilized porphyrin (**P**) showed the typical Soret band at 421 nm attributed to the $S_0 \rightarrow S_2$ transitions, and four weak Q-bands between 506 and 584 attributed to the $S_0 \rightarrow S_1$ transitions (Gouterman, 1961). The value of singlet oxygen quantum yield (ϕ_Δ) found to **P** was 0.69 (**Supplementary Figure S3**). The UV-Vis spectra of the **P**-chitosan and **P-HNP** materials showed the typical porphyrin absorption profile. Additionally, the spectra of the nanoparticles (**HNP**) and raw chitosan did not present bands in the region of 400 nm (**Figure 3A**), confirming the successful immobilization.

Castro et al. (2017) and Ferreira et al. (2016) immobilized porphyrins in a chitosan film and observed the broadening and slightly red-shifted in the solid state. The same behavior was observed for **P**-chitosan in solution when compared to the **P** in an aqueous solution of DMSO (1%) at a concentration of 10 μM , red-shifted of Soret (*ca.* 4–5 nm) and Q-bands (*ca.* 4–5 nm). For **P-HNP**, the Soret and Q-bands are blue-shifted (*ca.* 5–11 nm). The emission spectra of **P**-chitosan and **P-HNP** also exhibited

TABLE 4 | Values of X-ray scattering intensity in the origin $[I(0)]$ and radius of gyration (R_g) of **NP**, **HNP**, and **P-HNP**.

Sample	$I(0)$ (cm^{-1})	R_g (nm)
NP	0.076	21.8
HNP	14.64	25.7
P-HNP	27.19	29.9

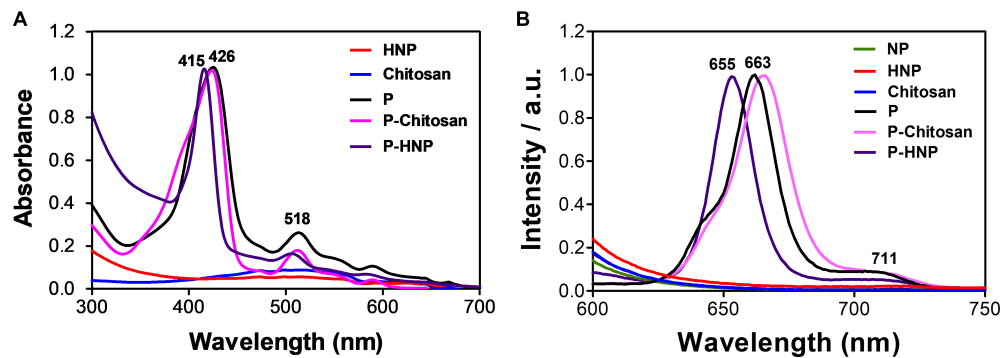


FIGURE 3 | Normalized absorption and emission spectra of free porphyrin (**P**), immobilized porphyrin (**P-chitosan** and **P-HNP**), and controls (**NP**, chitosan, and **HNP**). **(A)** Absorption spectra; **(B)** emission spectra, $\lambda_{exc} = 420$ nm.

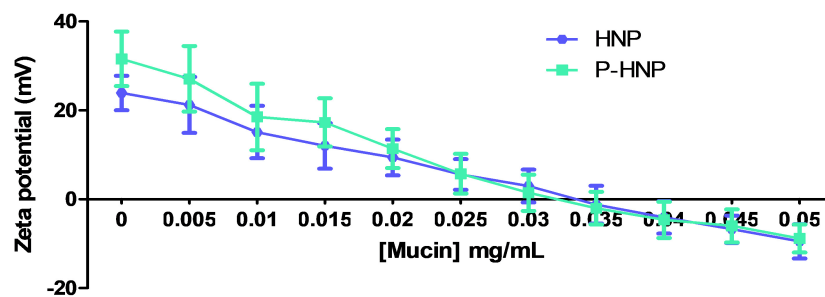


FIGURE 4 | Zeta potential values of **HNP** and **P-HNP** as function of mucin concentration in the mucoadhesion assays.

two emission peaks with the first fluorescence vibrational mode more remarkable than the second one, slightly red-shifted or blue-shifted effects were observed, respectively, compared to the non-immobilized porphyrin **P** according to the absorption spectra. These shifts are attributed to the small alterations in the porphyrin molecular environment to maximize the interaction with the support. Non-immobilized porphyrin (**P**) displayed two emission peaks at 663 and 711 nm, whereas **P-HNP** exhibited peaks at 655 and 710 nm, upon excitation at 420 nm (**Figure 3B**). Typical emission features of free or immobilized porphyrin allow cellular uptake studies and subcellular localization. As expected, both the nanoparticles and chitosan in absence of porphyrin **P** are non-luminescent. Furthermore, these results confirm that the process of nanoparticle preparation did not interfere with the photophysical properties of **P** to act as PS.

Mucoadhesion Evaluation *in vitro*

In general, when the drugs are administered in the bladder in an intravesical way, it shows a short residence time with easy elimination by the periodic emptying bladder, which decreases the therapeutic effect (Weintraub et al., 2014). A strong interaction of drugs or nanoparticles with the mucous bladder layer (the most superficial layer) may increase the residence time of the drug. Thus, the dose and number of administrations might be lower, and the biological effect improved (Weintraub et al., 2014). Chitosan can extend the residence time of nanoparticles in mucous membranes (Fonte et al., 2011; Liu et al., 2016b; Anderski

et al., 2018). Additionally, this natural polymer was described as absorption-promoting due to its capacity of open tight junctions (Artursson et al., 1994; Sonaje et al., 2012). Hence, nanoparticles coated with chitosan are an interesting strategy to improve the permanence of nanoparticles in the bladder (Erdoğan et al., 2012).

The main expected mechanism accountable for the mucoadhesive property of nanocarriers is the electrostatic interaction (Yoncheva et al., 2011; Bhatta et al., 2012). The *in vitro* mucoadhesion studies employed by us explored the ionic interaction between the positive charge surface of hybrid nanoparticles and the negative sialic groups of mucin. This ionic interaction can be observed by the change in the ZP value of the nanoparticle as shown in **Figure 4**. A decrease in the positive charge of **HNP** and **P-HNP** by mucin concentration increase can be observed, indicating a mucoadhesive property of these particles (**Figure 4**). At 0.4 mg/mL of mucin, the **HNP** and **P-HNP** exhibited a negative ZP. Rençber et al. (2016) showed that polymeric nanoparticles coated with chitosan also displayed a reduction in the ZP after incubation with 0.1% of mucin.

Cytotoxicity and Phototoxicity Assays

The study in the dark condition revealed that **HNP**, **P-HNP**, and free **P** were considered no cytotoxic for T24 cells once the results showed cell viability above 76% for all concentrations evaluated (**Figure 5A**; ISO, 2009). These data are relevant because the property of PS to be non-toxic under dark is crucial to its application in clinical trials (Nawalany et al., 2009).

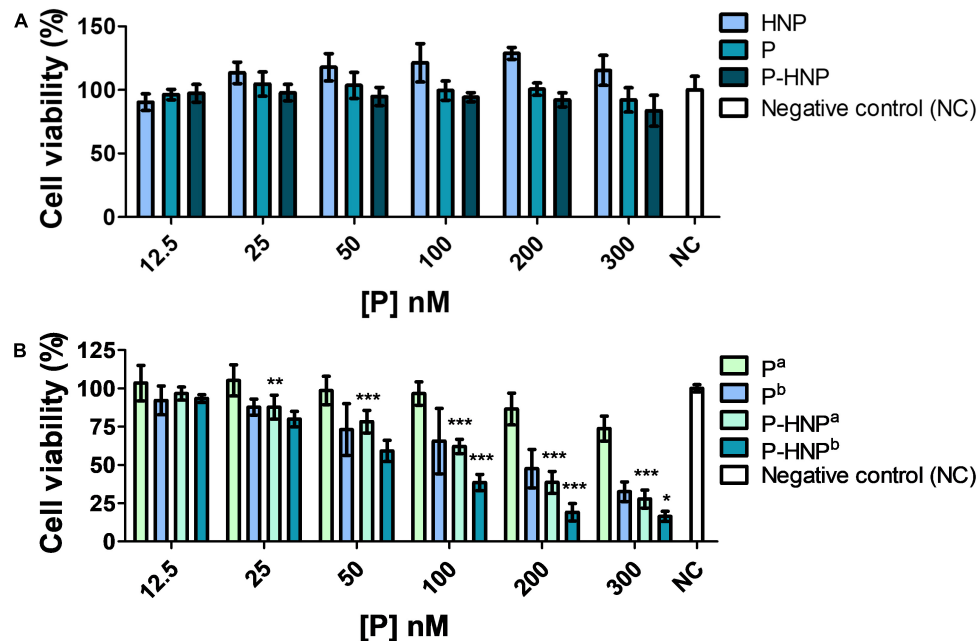


FIGURE 5 | Cell viability of T24 assessed by resazurin. **(A)** Cytotoxicity of hybrid nanoparticles (HNP), free porphyrin (P), and immobilized porphyrin on hybrid nanoparticles (P-HNP) after 24 h of treatment in the dark conditions. **(B)** Phototoxicity of free porphyrin (P) and porphyrin immobilized on hybrid nanoparticles (P-HNP) after 24 h of treatment followed by laser irradiation. ^a5 and ^b10 J/cm² dose of light. Negative control (NC)-untreated cells (cells + RPMI medium). The results are presented as mean $\pm$ SD of three independent experiments performed in triplicate. Significant differences relative to P and P-HNP, in each photoinduction dose, are indicated with an asterisk. Statistical significance: *** p < 0.001, ** p < 0.01, * p < 0.05. Data obtained by two-way ANOVA analysis followed by Bonferroni post-test.

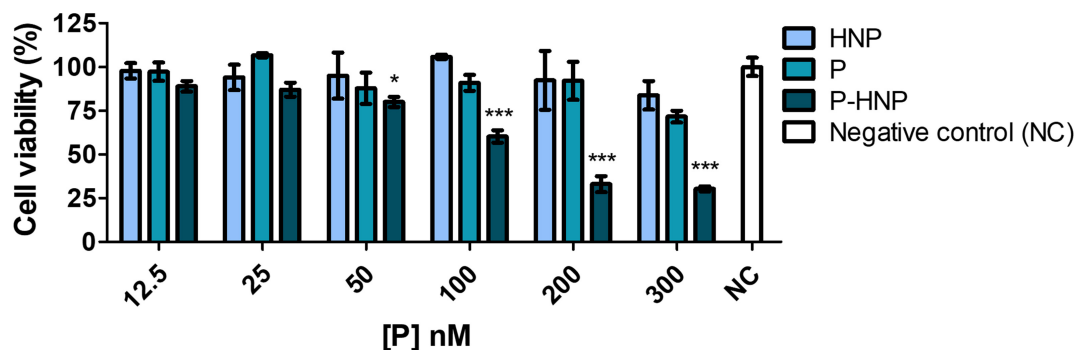


FIGURE 6 | Phototoxicity of free porphyrin (P) and porphyrin immobilized on hybrid nanoparticles (P-HNP) in T24 cells evaluated by resazurin after 6 h of treatment followed by photoinduction with a dose of 10 J/cm². Negative control (NC)-untreated cells (cells + RPMI medium). The results are presented as mean $\pm$ SD of three independent experiments performed in triplicate. Significant differences relative to P and P-HNP, in each photoinduction dose, are indicated with an asterisk. Statistical significance: *** p < 0.001, * p < 0.05. Data obtained by two-way ANOVA analysis followed by Bonferroni post-test.

The experiments of PDT using 24 h of treatment showed that P-HNP and P displayed a light dose-dependency phototoxicity (Figure 5B), while HNP did not show phototoxicity against T24 cells for both fluences assessed (Supplementary Figure S4). P, as well as P-HNP, were phototoxic to T24 cells for concentrations above 50 nM. In the concentration of 300 nM, P reduced the cell viability to 32.5%, whereas P-HNP was reduced to 16.3%. The IC₅₀ for P-HNP was 2.2-fold smaller for cells exposed to 10 J/cm² (IC₅₀ = 66.5 nM) than for the cells exposed to 5 J/cm² (IC₅₀ = 149.5). Comparing

the fluences of 5 and 10 J/cm², the IC₅₀ of P-HNP were, respectively, 3.2- and 2.5-fold lower than free P (IC₅₀ = 483.7 and 165.8 nM). Thus, P-HNP exhibited superior phototoxicity than P.

A phototoxicity assay using reduced treatment time (6 h) at a fluence of 10 J/cm² was also performed. This study suggested that 6 h of treatment is enough for P-HNP delivery of P into cells and improves their phototoxicity. For the concentrations of 300, 200, and 100 nM, P-HNP significantly reduced the cell viability when compared to free P (Figure 6). The P-HNP IC₅₀ was 130.6 nM,

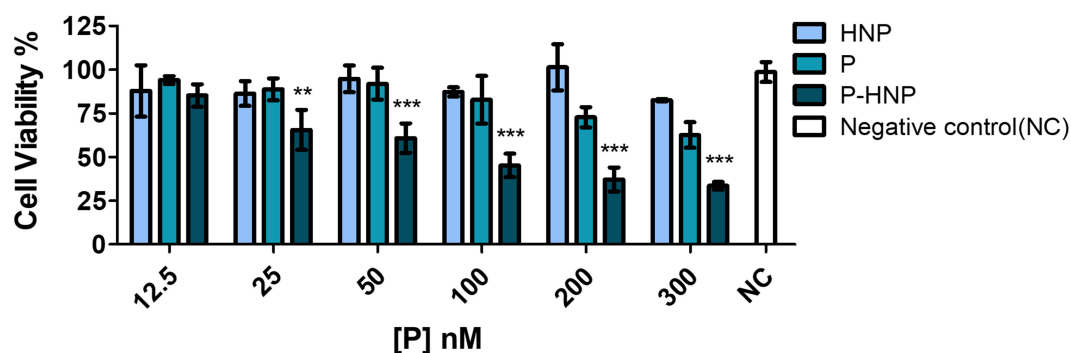


FIGURE 7 | Phototoxicity of free porphyrin (**P**) and porphyrin immobilized on hybrid nanoparticles (**P-HNP**) in T24 cells using ATP bioluminescent assay after treatment of 6 h followed by PDT with 10 J/cm² of fluence. Negative control (NC)-untreated cells (cells + RPMI medium). The results are presented as mean $\pm$ SD ($n = 3$). Significant differences relative to free **P** and **P-HNP** are indicated with an asterisk. Statistical significance: *** $p < 0.001$, ** $p < 0.01$. Data obtained by two-way ANOVA analysis with post-tests of Bonferroni.

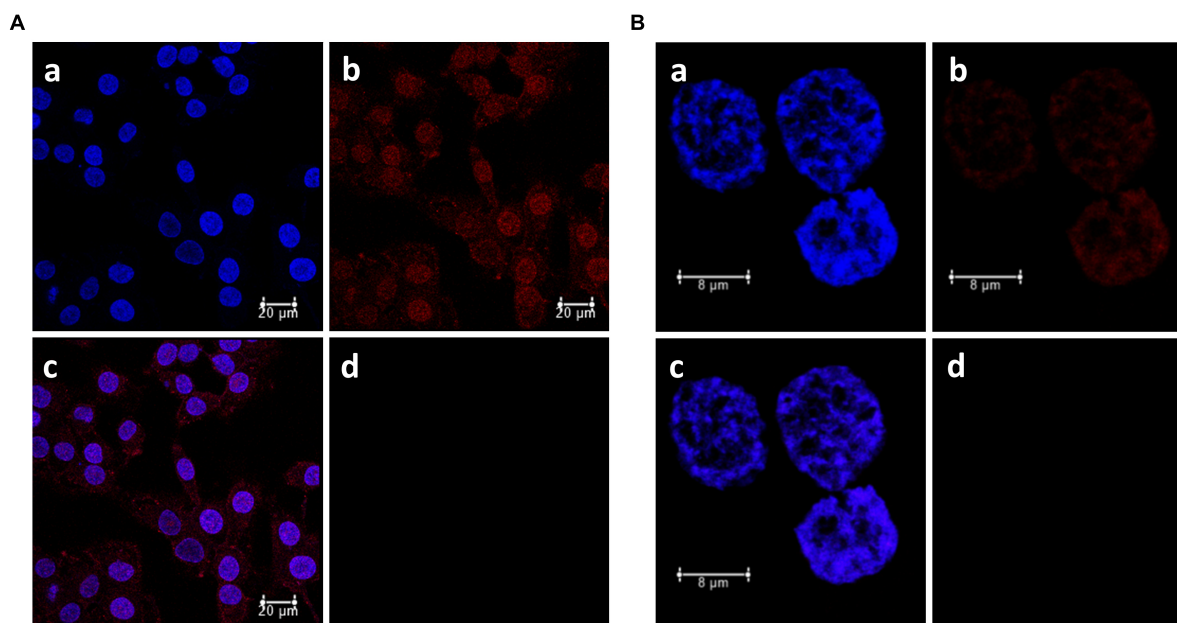


FIGURE 8 | Subcellular localization of porphyrin (**P**) in bladder cancer cells T24 determined by confocal laser scanning microscopy. (A) **P-HNP** uptake. (B) Free **P** uptake. **a:** DAPI (blue fluorescence), **b:** porphyrin (red fluorescence), **c:** merged, **d:** control (untreated cells + DAPI, emission 630–710 nm). T24 cells were incubated sequentially with **P** or **P-HNP** for 30 min and marked with DAPI (nucleus probe).

whereas the IC₅₀ of free **P** could not be estimated because it was non-toxic for cells.

Furthermore, a bioluminescence ATP assay was carried out to evaluate the free **P** and **P-HNP** phototoxicity against T24 BC cells. This assay was chosen because it is considered more sensitive, robust, and precise than colorimetric and fluorescence assays. It also has a high correlation between ATP detection and cell viability values, since ATP production is immediately interrupted during cell death (Maehara et al., 1987; Zumpe et al., 2010).

The same phototoxicity profile observed in resazurin assay for **P** and **P-HNP** were also verified in the ATP experiment, confirming the significant phototoxic effect of **P-HNP** (Figure 7).

As result, **P-HNP** exhibited significantly higher phototoxic effects when compared with free **P** for all concentrations except to 12.5 nM. The IC₅₀ of **P-HNP** was 86.12 nM, while the free **P** was non-toxic for cells in the concentration range evaluated.

The superior activity of **P-HNP** compared to free **P** can be attributed to the favorable physical and chemical properties of hybrid nanoparticles, which promoted a quick and efficient **P** delivery into cells, avoiding **P** aggregation. In this context, we evaluated the photophysical properties of **PS** in the cell culture medium under dark conditions. The free porphyrin (**P**) exhibited a broad Soret band when compared to free **P** in DMSO 1% (Supplementary Figure S5A). **P** self-aggregation changed drastically its absorption spectral profile, once the bands

enlarged and decreased the absorption intensity as shown in **Supplementary Figure S4**. Consequently, the emission intensity decreased, and new bands appeared. Such changes may be due to the mixture of monomeric and aggregates species of porphyrin that trigger fluorescence quenching (**Supplementary Figure S5B**). However, when assessed in cell culture medium, **P** immobilized in nanoparticles (**P-HNP**) was stable, and its aggregation was minimized since the absorption and emission spectra of the PS were similar to those shown in **Figure 3**.

Evaluation of Porphyrin Uptake by Laser Scanning Confocal Microscopy

Figure 8A showed the high uptake of immobilized **P** (**P-HNP**) into T24 cells for only 30 min of incubation (red fluorescence). This high and fast porphyrin internalization explains the enhancement of the phototoxicity of **P-HNP** previously described (see **Figures 5–7**). The use of nanoparticles as a PS delivery system increased the accumulation of **P** at strategic points in the cancer cell. In contrast, the free **P**, in the same conditions of incubation, exhibited a weaker fluorescence when compared with **P-HNP** that may be due to the free aggregation phenomena in cell culture medium (**Figure 8B**). According to Nawalany et al. (2009) and Brezanióva et al. (2016), the high internalization and accumulation of PS are responsible for the great phototoxic effect of porphyrin.

Photosensitizer is supposed to be in specific organelles from cells to trigger the mechanism involved in cellular death by the production of ROS including singlet oxygen (Nawalany et al., 2009; Bacellar et al., 2015; Gazzi et al., 2019). The merged fluorescence images showed that **P-HNP** is distributed throughout the cell, including the nucleus (**Figures 8A,B**). Nevertheless, free **P** was localized only in the nucleus. Some studies describe that the DNA can also be oxidized in PDT treatments (Bacellar et al., 2015). This is a strategy explored in cancer treatment (Zhu et al., 2017; Kadhimi et al., 2019). Porphyrin and metalloporphyrins can break DNA single-strand by photoinduction mechanisms with singlet oxygen action or by cleavage of the sugar moieties from nucleic acids (Defedericis et al., 2006). Anti-tumor drugs into nuclei can inhibit the replication and transcription of DNA, wrecking genetic material and inducing cells to apoptosis (Wang et al., 2011). Therefore, targeting-nuclei drugs are considered interesting and efficient to kill cancer cells (Zhu et al., 2017). However, nucleus and organelle dual-targeting drugs have been applied successfully in cancer therapy once the drugs can act trigger two different mechanisms, increasing drug efficiency on tumors as reported by Zhu et al. (2017). Thus, those results suggest that the dual-targeting and the reduced aggregation showed by the immobilized **P** (**P-HNP**) can explain the higher phototoxic effect of this PS compared to free **P** in BC cells.

CONCLUSION

The unique properties of the hybrid nanoparticles developed in this study increased the stability, improved the photophysical properties of the porphyrin in the cell culture medium, and

quickly and efficiently delivered it within T24 cells. The application of **P-HNP** triggered a potent phototoxic effect (low $IC_{50} = 66.5$ nM) in PDT against T24 BC cells. Thus, this study represents an advance to the development of macrocycle incorporation in biodegradable hybrid nanoparticles as a possibility to potentialize their PDT action. Furthermore, the hybrid nanoparticles presented mucoadhesive properties in studies *in vitro*. This property is interesting for BC treatment since it might improve the permanence time of nanoparticles in the bladder after its intravesical administration. When these results are together, the porphyrin delivery system developed in this study has the potential to be applied in other cancer models and should be explored in animal models with the aim of enhancing PDT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LS: porphyrin synthesis, development of nanoparticles, materials preparation, and biological studies, and writing—original draft. KC: porphyrin synthesis and characterization, and writing—review and editing. CB: biological studies, review, and editing. RS: writing—review and editing and supervision. CO: writing, review, and editing. PM: validation, writing—review and editing, and supervision. All authors contributed to write the manuscript and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbioe.2021.679128/full#supplementary-material>

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