



# Lighting up Individual Organelles With Fluorescent Carbon Dots

Haifang Liu<sup>1</sup>, Jiancheng Guo<sup>1</sup>, Aaron Albert Aryee<sup>2</sup>, Linlin Hua<sup>1</sup>, Yuanqiang Sun<sup>2</sup>, Zhaohui Li<sup>2</sup>, Jianbo Liu<sup>1\*</sup> and Wenxue Tang<sup>1\*</sup>

<sup>1</sup>Precision Medicine Center of the Second Affiliated Hospital of Zhengzhou University, Zhengzhou, China, <sup>2</sup>College of Chemistry of Zhengzhou University, Zhengzhou, China

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### \*Correspondence:

Jianbo Liu  
jbluuzz@zzu.edu.cn  
Wenxue Tang  
twx@zzu.edu.cn

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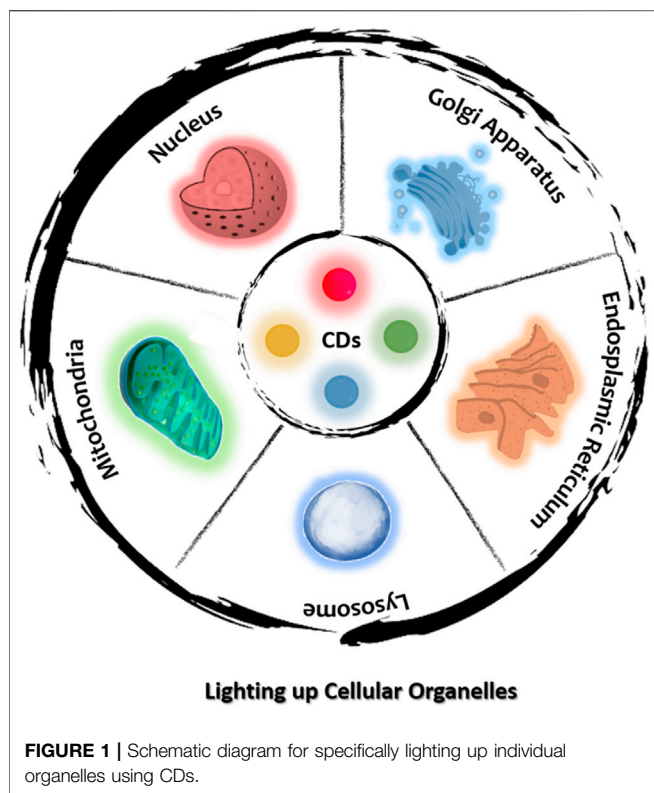
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Cell organelles play crucial roles in the normal functioning of an organism, therefore the disruption of their operation is associated with diseases and in some cases death. Thus, the detection and monitoring of the activities within these organelles are of great importance. Several probes based on graphene oxide, small molecules, and other nanomaterials have been developed for targeting specific organelles. Among these materials, organelle-targeted fluorescent probes based on carbon dots have attracted substantial attention in recent years owing to their superior characteristics, which include facile synthesis, good photostability, low cytotoxicity, and high selectivity. The ability of these probes to target specific organelles enables researchers to obtain valuable information for understanding the processes involved in their functions and/or malfunctions and may also aid in effective targeted drug delivery. This review highlights recently reported organelle-specific fluorescent probes based on carbon dots. The precursors of these carbon dots are also discussed because studies have shown that many of the intrinsic properties of these probes originate from the precursor used. An overview of the functions of the discussed organelles, the types of probes used, and their advantages and limitations are also provided. Organelles such as the mitochondria, nucleus, lysosomes, and endoplasmic reticulum have been the central focus of research to date, whereas the Golgi body, centrosome, vesicles, and others have received comparatively little attention. It is therefore the hope of the authors that further studies will be conducted in an effort to design probes with the ability to localize within these less studied organelles so as to fully elucidate the mechanisms underlying their function.

**Keywords:** carbon dots, organelle targeted, cell imaging, fluorescence, precursors

## INTRODUCTION

The eukaryotic cell contains numerous organelles, such as the nucleus, lysosomes, Golgi body, and mitochondria, which all play critical roles in the normal functioning of biological systems. For instance, mitochondria are often regarded as the powerhouse of the cell and support a diverse range of cellular processes mediated by adenosine triphosphate (ATP), including biomolecule synthesis, cell division, and so on (Newmeyer and Ferguson Miller, 2003; Lodish et al., 2008). The nucleus, which can be considered the heart of the cell, stores the hereditary material (DNA) as well as coordinating certain cellular processes such as growth, protein synthesis, and reproduction (Dean and Hinshelwood, 1963). The biomolecules present in cell organelles have also been observed to play



key roles in their overall function. Furthermore, the difference in the signal of these biomolecules has been found to be influenced by their location in the cell; thus, the ability to track their activities within specific organelles is very important as it provides the opportunity to understand the basic function of the organelle as well as lead to improvements in the treatment of the associated diseases (Alamudi et al., 2016).

In recent years, several techniques have been developed for monitoring various activities within the cell, among which fluorescent labelling has received the most attention. Fluorescent labelling is a powerful tool for examining the localization and movement of biomolecules in cellular processes owing to its ability to afford non-invasive real-time signals with high spatial resolution and sensitivity, alongside additional advantages such as facile preparation, low cost, and high selectivity (D'Angelis do Barbosa et al., 2015; Alamudi et al., 2016; Zhu et al., 2016; Gao et al., 2019; Liu and Gao et al., 2020; Chen et al., 2020; Liu et al., 2019a; Yang et al., 2020; Zhu et al., 2019; Zlitni et al., 2020). In this regard, numerous fluorescent probes, such as small molecules (D'Angelis do Barbosa et al., 2015; Liu et al., 2020), quantum dots (Derfus et al., 2004; Lovrić et al., 2005), nanodots (Mao et al., 2016; Hua et al., 2018a), and carbon dots (CDs) (D'Angelis do Barbosa et al., 2015; Chen et al., 2019; Liu et al., 2019a), have been designed and synthesized for the monitoring of subcellular activities.

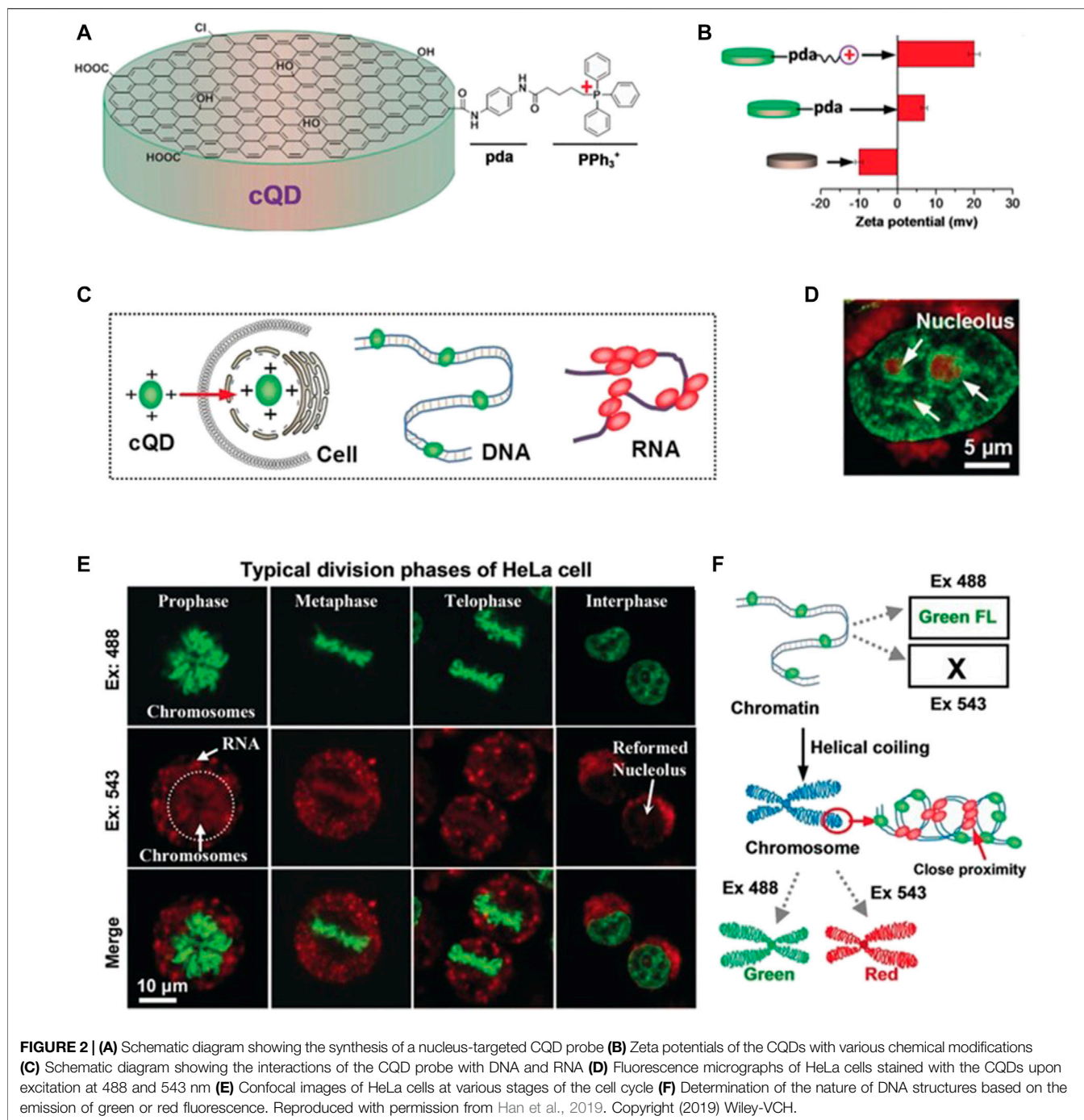
As a new kind of 0D carbon nanomaterial, CDs have emerged as a novel type of photo luminescence nanomaterial, which has attracted significant and growing interest in both scientific and technical area. CDs are made from carbon, which are the basic

building block of life itself, that is their important distinction. Based on this simple fact, CDs have demonstrated better biocompatibility in many tested cell lines, mice and zebrafish compared with most inorganic nanoparticles and organic small molecule probes. The most striking property of CDs is the excitation-dependent photoluminescence, which arises from the quantum confinement effects, the large rigid *p*-conjugated structure and the presence of surface defects. (Yuan et al., 2016). Currently, the fluorescence emission wavelength of CDs can be adjusted from blue to far red by different synthesis methods (Zheng et al., 2016) and via the use of modification agents, (Ding et al., 2016). This distinct property makes CDs favourable in bioimaging applications (Wang et al., 2011). Compared with commercially available organic dyes, CDs-based probes have been reported to exhibit some unique properties, such as high photostability, facile design and synthesis methods, low cytotoxicity, and good aqueous solubility. Based on these excellent features, the application of CDs have thus emerged as a powerful tool for organelle monitoring (Liu et al., 2019a; Chen et al., 2019).

This review aims to provide an up-to-date summary of CDs-based organelle-specific probes, which are grouped by their target organelle so as to highlight the underlying design strategies and recent progress made in this field (Figure 1). The precursors of CDs are first highlighted in this review because the ability of some CDs to exhibit intrinsic organelle targeting has been observed to originate from its precursors. Herein, we present some information on the latest probes based on CDs and their application in imaging specific organelle, such as nucleus, mitochondria, lysosomes, and endoplasmic reticulum, Golgi body. For each organelle, a brief overview of its composition and functions is first provided, followed by a discussion of the properties of the corresponding probes, their advantages and limitations, and opportunities for future research. Some applications of these CDs-based organelle-specific probes are also presented. Next, we discuss the CDs which can migrate between subcellular organelles. These CDs could enrich our understanding of how CDs interact with cells, and shed new lights on the design of novel CDs for biomedical applications. Finally, we discuss the challenges in this field and share some possible future directions.

## NUCLEUS

The nucleus, which is enclosed within a double membrane referred to as the nuclear envelope, can be regarded as the control center of the cell owing to its function of regulating gene expression (Liu et al., 2019a). The nucleus is known to contain DNA, RNA and various proteins. The phospholipid bilayer nature of the nuclear membrane prevents the movement of large molecules into the nucleus but allows for the transmembrane diffusion of small nonpolar molecules (Dean and Hinshelwood, 1963; Lodish et al., 2019). Abnormalities in the nuclear morphology and envelope have been linked to many diseases and conditions, including ageing (Stephens et al., 2018), heart disease (Butin Israeli et al., 2012), skeletal myopathies



(Burke and Stewart, 2002), cancer (Reddy and Feinberg, 2013), progeria (Papanicolaou and Traut, 1997), Alzheimer's disease (Su et al., 1994), and so on. Therefore, targeted imaging of the nucleus is essential for the early diagnosis of these conditions. CDs can be classified as nanomaterials with dimensions of 1–10 nm. This relatively small size of CDs serves as an additional advantage for their application as nucleus-specific probes as they can penetrate the nuclear membrane (Liu et al., 2019a; Wu et al., 2021). Also,

the surface charge on CDs has been observed to be a crucial factor in nucleus-targeted imaging (Wang et al., 2018).

### Modified CDs for Nucleus-Targeted Imaging

In the past, nucleus-targeted imaging was predominantly accomplished by the attachment of nuclear localization signal

(NLS) peptides to the positively charged surface of CDs (Zhu et al., 2019). NLS peptides are composed of functional amino acid sequences whose functions are dependent on their sequence and structure (Roy et al., 2017). The ability of NLS peptides to target the nucleus has been demonstrated to originate from their interactions with importin within the nuclear pore complex. Several studies have been conducted based on the use of NLS peptides for nucleus-targeted imaging and related applications. For instance, Yang and Wang et al. synthesized blue-emitting CDs (average diameter = 5.6 nm,  $\lambda_{\text{abs}} = 350$  nm,  $\lambda_{\text{em}} = 450$  nm) using citric acid, PEG5000, and ethylenediamine as precursors via hydrothermal treatment (Yang et al., 2016). The CDs were then coupled with an NLS peptide (PKKKRKVG) via EDC/NHS conjugation to realize the nucleus-targeted imaging of MCF7 and A549 cells. The authors observed that in the absence of the NLS peptide the CDs were mainly localized in the cytoplasm. In contrast, the conjugation of the NLS peptide enabled nucleus-targeted imaging, which was also influenced by the small size of the CDs (<10 nm).

Another study by Gao and Yang et al. utilized an NLS peptide to achieve nuclear targeting by carbon quantum dots (CQDs; average diameter = 3.75 nm,  $\lambda_{\text{abs}} = 479$  nm,  $\lambda_{\text{em}} = 546$  nm) functionalized with azido molecular beacon DNA by a copper(I)-catalysed alkyne-azide cycloaddition reaction (Gao M. X. et al., 2017). The CQDs were prepared from 3,4,9,10-perylenetetracarboxylic dianhydride, which endowed the as-synthesized CQDs with carboxyl functional groups. The authors observed that the functionalization of the CQDs with gold nanoparticles (AuNPs) and DNA resulted in a higher migration rate with respect to AuNPs functionalized with DNA. They concluded that this was attributable to the difference in charge-to-mass ratio resulting from the different conjugation strategies. Confocal microscopy images confirmed the ability of the NLS-CQDs to localize within the nucleus of cells, which was ascribed to the conjugation with the NLS peptide as without it the alkynylated CQDs did not exhibit nuclear staining.

In 2019, Zhang et al. developed a novel nuclear targeting probe based on 4-carboxybutyltriphenylphosphonium ( $\text{PPh}_3^+$ ) bromide and CQDs (Figure 2) (Han et al., 2019). In addition to the excellent properties displayed by this probe, it could also distinguish between RNA and DNA, thus providing valuable and specific information concerning the nuclear activities. The zeta potential, diameter, and absorption and emission wavelengths of this nanoprobe were reported to be +20 mV, ~3 nm, 265 nm, and 510 nm, respectively. Confocal images revealed that the interactions of this nanoprobe with dsDNA and ssRNA resulted in the emission of green fluorescence and red fluorescence, respectively, upon incubation with HeLa cells, thus providing a basis for their differentiation.

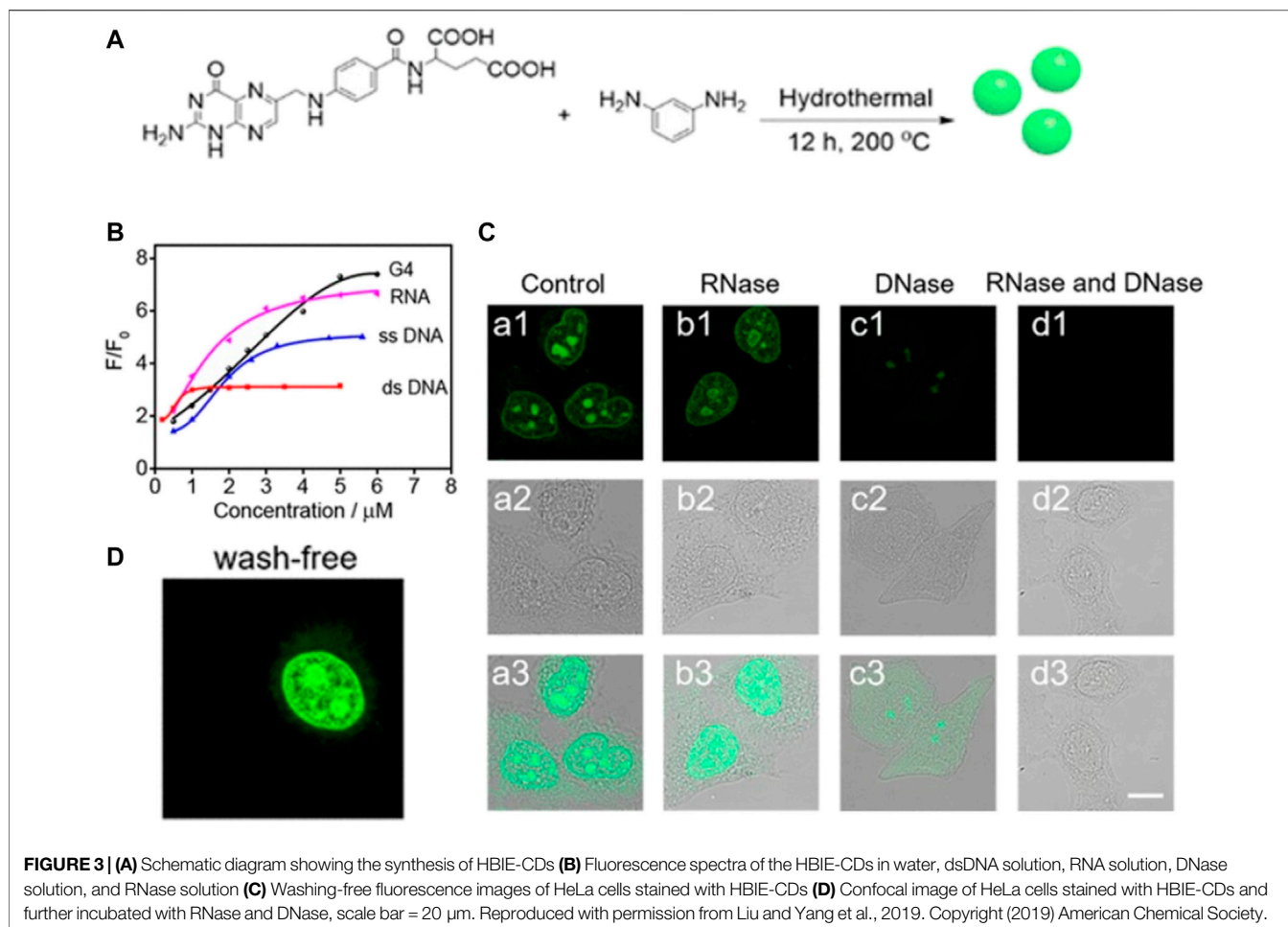
## CDs With Intrinsic Nuclear Targeting Properties

In recent years, CDs with intrinsic nuclear targeting properties have also been synthesized, thus expanding the horizon for CDs-based probes for such purposes. These probes are typically based on the use of substances possessing hydrophobic properties. For

example, Zhu et al. developed nucleoli-specific CDs-based probes with tunable surface charge properties that could be varied by altering the molar ratio of the precursors, i.e. citric acid (CA) and ethanediamine (EDA) (Zhu et al., 2019). The authors observed that the surface charge increased (from -17.9 to -2.84 mV) as the molar ratio of CA:EDA was decreased (from 1:0.5 to 1:3) with a concomitant enhancement in the staining of nucleoli. This enhancement was ascribed to the higher cellular uptake of the CDs with higher zeta potential.

Recently, novel negatively charged CDs with intrinsic nuclear targeting properties were synthesized by Hua et al. and applied to the delivery of protoporphyrin into the nucleus (Hua et al., 2018b). The CDs were prepared from *m*-phenylenediamine and L-cysteine through one-pot hydrothermal treatment. Characterization of the CDs revealed an average diameter of  $3.8 \pm 0.5$  nm, two absorption peaks at 269 and 405 nm, and an emission peak at 520 nm. Confocal images showed that the probe was able to stain the nuclei of two cancer cell lines (HepG2 and A549) and three normal cell lines (RAW264.7, AT II, and L02) to afford green fluorescence. However, owing to the structure of nucleic acids, which are rich in negatively charged phosphate units, it is generally difficult for neutral and negatively charged molecules to pass through the nuclear membrane (Xu et al., 2007; Cai et al., 2017). In contrast, positively charged CDs have been observed to circumvent this challenge as a result of their ability to evade the endo/lysosomes through the so-called sponge effect (Mohammadinejad et al., 2019). Therefore, CDs possessing a positive charge (zeta potential) have been applied extensively for nuclear targeting. The design strategy for forming these cationic CDs usually involves passivation with substances bearing amino groups (Li et al., 2018). However, factors such as their facile removal from the cytoplasm and unfavourable interactions with negatively charged intracellular proteins may result in severe serum inhibition, which greatly limits *in vivo* applications (Vankayala et al., 2015).

For the purpose of bioimaging, the fluorescence quantum yield of CDs is an important factor; hence, fluorophores with longer wavelengths, brighter fluorescence, and high quantum yield are generally preferred (He et al., 2016). Hydrogen bonding has been observed to play significant roles in biological fluorophores as it can inhibit nonradiative processes to afford a high fluorescence quantum yield. On the basis of this principle, Liu and Yang et al. synthesized novel DNA- and RNA-sensitive hydrogen-bond-induced emission CDs (HBIE-CDs) (Liu et al., 2019a). The CDs were fabricated by a hydrothermal method using *m*-phenylenediamine and folic acid as precursors, which have the ability to bind nucleobases at multiple sites. This HBIE-based sensing mechanism can potentially be generalized for the fabrication of sensors displaying fluorescence turn-on detection. The average size, absorption wavelength, and emission wavelength of the HBIE-CDs were observed to be 2.6, 394, and 535 nm, respectively. The as-synthesized HBIE-CDs displayed several excellent characteristics, such as longer-wavelength emission upon hydrogen bond formation and the



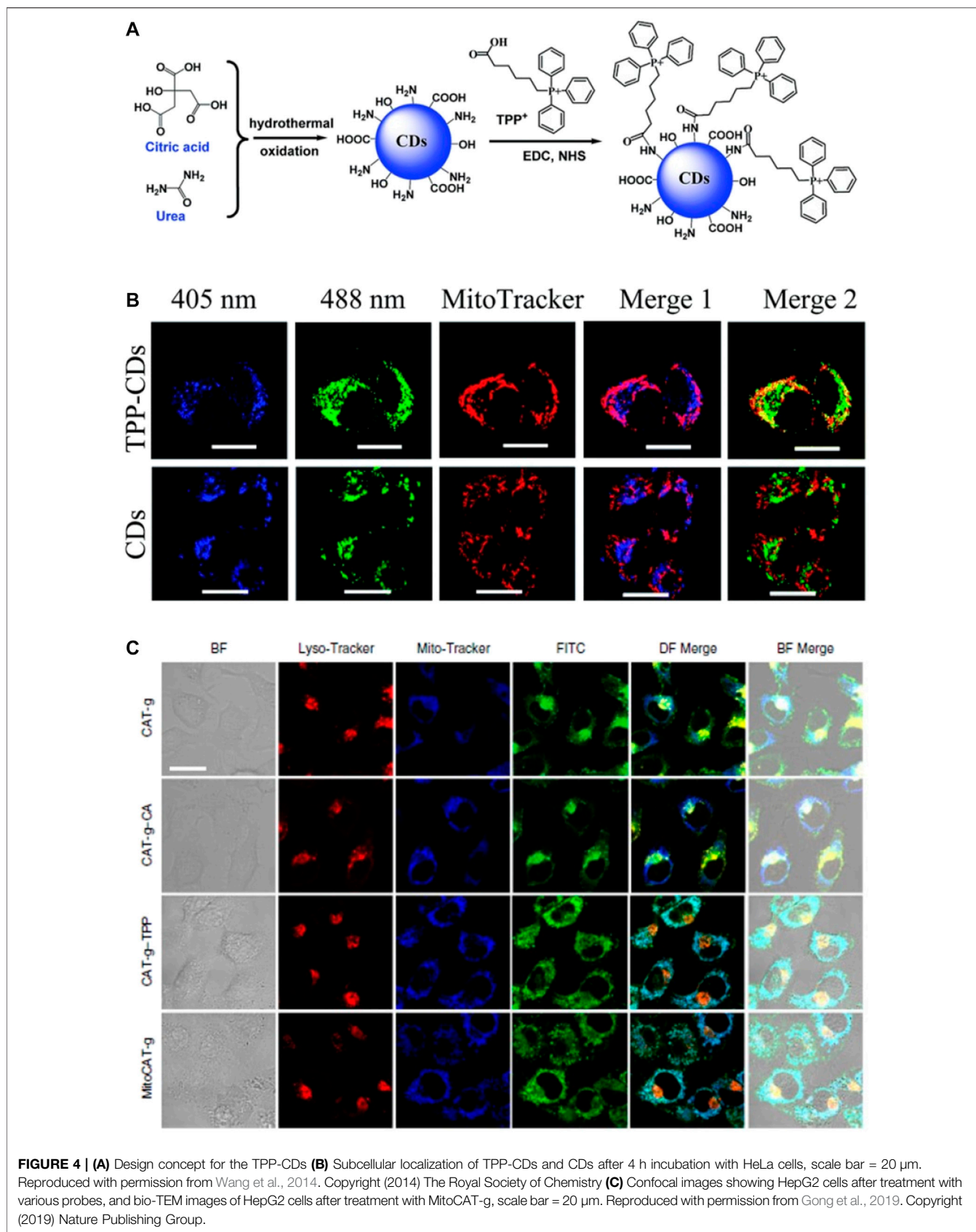
ability to mediate washing-free subcellular labelling and imaging of the nucleus in living cells (Figure 3). These CDs have huge potential in clinical diagnosis for imaging of the nuclear morphology.

Red-emitting CDs capable of nuclear targeting were synthesized by Hua et al. using *p*-phenylenediamine and doped with Ni by a hydrothermal method (Hua et al., 2019). This probe was observed to exhibit a quantum yield of 45.6% (in DMSO) as well as good photostability. The size, absorption wavelength, and emission wavelength of these CDs were reported to be approximately 2.9, 510, and 605 nm, respectively. The authors proposed that  $\text{Ni}^{2+}$  ions could be beneficial for promoting the formation of CDs and realizing higher surface oxidation. The CDs were also observed to be sensitive towards RNA and DNA, enabling them to be used to monitor these components within the nucleolus.

Malfunctioning of the cell nucleus has also been associated with disorders such as Down syndrome, cystic fibrosis, and leukaemia (Xiang et al., 2012; McKiernan et al., 2013). Thus, there is substantial demand for probes that are able to specifically locate the nucleus and can be applied as biosensors and theranostic agents for drug delivery. In recent years, various probes capable of serving as carriers for drug delivery into the

nucleus have been developed, with doxorubicin (Dox) frequently employed as the model drug (Wang et al., 2013; Jung et al., 2015; Jia et al., 2016; Yang et al., 2016; Yao et al., 2016; Wang et al., 2017; Yao et al., 2017; Hua et al., 2018a; Pei et al., 2018; Li et al., 2019). The conjugation of Dox to CDs is usually accomplished through  $\pi$ - $\pi^*$  stacking and electrostatic interactions (Yang et al., 2016).

As an alternative method, Yang and Wang et al. achieved the nuclear staining of A549 cells within 4 h using NLS-modified CDs covalently conjugated with Dox, as mentioned in Section 2.1 (Yang et al., 2016). Similar results have been reported by Yao et al. and Yuan et al. The former group synthesized Gd (III)-doped CDs using citric acid and  $\text{GdCl}_3$  prior to encapsulating a high concentration of Dox (Yao et al., 2016). Characterization of the CDs revealed an average diameter of 5 nm, an absorption peak at 360 nm, and an emission peak at 485 nm. The CDs were applied as both an MRI contrast agent as well as a carrier agent for the delivery of Dox into the nucleus of MCF-7 cells. This conjugated system was able to localize in the nucleus within 2 h. Meanwhile, Yuan et al. synthesized CDs from milk and subsequently conjugated them with Dox (Li et al., 2019). Characterization of the CDs revealed an average diameter of 20 nm, an absorption peak at 290 nm, and an emission peak at 444 nm. This system was reported to exhibit enhanced antitumour behaviour towards



ACC-2 cells, with confocal images confirming localization in the nucleus within 4 h.

In summary, previous nucleolus-targeted results based on CDs could be mainly attributed to the accumulation of the CDs in the cell nucleus through various noncovalent interactions between the nanoparticles and nucleic acid, such as  $\pi$ - $\pi$  stacking, electrostatic interaction, and hydrogen bonding. Nucleus staining CDs have great potential to reveal the biological studies in cellular metabolism, growth, differentiation and heredity, and also clinical diagnosis which are associated with the changes of nuclear morphology or microenvironment.

## MITOCHONDRIA

Mitochondria are often regarded as the powerhouse of cells. The primary function of these rod-shaped organelles is converting oxygen and nutrients into ATP. In addition, mitochondria are also involved in a diverse variety of cellular processes, such as metabolism, cell division, signal transduction, and so on (Ahn et al., 2018; Yang et al., 2018). The dysfunction of these processes is associated with neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease (Chan, 2006; Lin and Beal, 2006). The generation of ATP in the mitochondria during cellular respiration results in the mitochondrial membrane possessing a negative potential (Modica-Napolitano and Aprile., 2001; Modica-Napolitano and Singh, 2004; Walker and Dickson., 2006; Lin et al., 2018). Hence, one strategy for targeting the mitochondria is the use of fluorophores bearing positively charged lipophilic moieties, such as methylpyridinium cations, triphenylphosphonium (TPP) cations, and indolium cations, which are attracted by the negative membrane potential (Ross et al., 2004).

### TPP Based Mitochondria Targeted Imaging

Ligands bearing TPP cations have been widely used for targeting mitochondria owing to the delocalized nature and high lipophilicity of these cations (Wang et al., 2014; Zhang et al., 2015; Dong et al., 2016; Hua et al., 2017; Wu X. et al., 2017; Zhang et al., 2017; Gong et al., 2019; Zhou et al., 2019). In 2014, Wang et al. synthesized fluorescent TPP-conjugated CDs (TPP-CDs) by a hydrothermal method that could target mitochondria (**Figure 4A**) (Wang et al., 2014). Characterization of the TPP-CDs revealed an average diameter of 8.5 nm, an absorption peak at 340 nm, and an emission peak at 425 nm. The TPP-CDs exhibited low cytotoxicity and were biocompatible at incubation times of 8 and 6 h, respectively. The CDs were synthesized from urea and citric acid using a one-pot hydrothermal method. These precursors endowed the obtained CDs with various functional moieties including  $\text{-NH}_2$ ,  $\text{-COOH}$ , and  $\text{-OH}$  groups, enabling the conjugation of TPP via amidation between TPP-COOH and the  $\text{NH}_2$ -bearing CDs. After conjugation with TPP, the zeta potential of the CDs increased remarkably. Two-photon confocal microscopy analysis demonstrated the selective accumulation of the TPP-CDs in the mitochondria and a high two-photon fluorescence signal around the nucleus.

In a recent study, Zhou et al. developed a ratiometric nanosensor suitable for tracking  $\bullet\text{OH}$  radicals in the mitochondria (Zhou et al., 2019). This nanosensor was based on CDs synthesized via the microwave-assisted hydrothermal treatment of 1,2,4-triaminobenzene and formamide followed by conjugation with coumarin-3-carboxylic acid (CCA) and (4-carboxybutyl) triphenylphosphonium bromide. In this system, the CCA moiety served as the recognition site for  $\bullet\text{OH}$  radicals, switching from non-fluorescent to strong blue fluorescence upon reaction with  $\bullet\text{OH}$ . The zeta potential of the nanosensor was observed to be more positive (+19.6 mV) compared to that of the CDs (+3.2 mV), thus making it more suitable for mitochondrial targeting. The blue fluorescence emission of the sensor increased upon the addition of  $\bullet\text{OH}$  within the concentration range from 0.1 to 160  $\mu\text{M}$ , and the detection limit of the sensor was reported to be 70 nM. Confocal images of RAW 264.7 cells stained with the nanosensor revealed successful mitochondrial localization within 4 h. Further treatment with the CDs via different systems showed changes in the fluorescence signal based on the concentration of  $\bullet\text{OH}$ .

The mitochondria are the major cellular source of reactive oxygen species (ROS) (Dickinson and Chang., 2011). These species may originate as normal byproducts of mitochondrial processes and have also been implicated in certain pathological processes (James et al., 2007). Hence, the detection of these ROS and determination of their specific sites of origin within the cells are crucial. In this regard, Wu et al. prepared CDs for the detection of  $\text{ONOO}^-$  in the mitochondria (Wu L. et al., 2017). The CDs were synthesized from *o*-phenylenediamine and TPP and exhibited two absorption bands characteristic of CDs prepared from amine precursors. In the presence of  $\text{ONOO}^-$ , the fluorescence of the CDs was quenched by photoinduced electron transfer, and benzotriazole structures were formed on the surface of the CDs by the reaction between the surface *o*-diaminobenzene moieties and  $\bullet\text{NO}_2$  produced by the decomposition of  $\text{ONOO}^-$ . Cell imaging experiments revealed that the fluorescence of the CDs incubated with MCF-7 cells decreased after treatment with lipopolysaccharide and interferon- $\gamma$ , indicating the suitability of the probe for intracellular peroxynitrite sensing. By virtue of their sensing mechanism, ratiometric probes have been found to possess some superior qualities, such as improved sensitivity and quantification.

Remarkable applications of mitochondria-targeted CDs in theranostics have emerged recently (Ge et al., 2014; Wang et al., 2014; Matai et al., 2015; Yang et al., 2015; Xiang et al., 2016a; Xiang et al., 2016b; Zhang et al., 2017; Geng et al., 2018; Hua et al., 2018b; Yang et al., 2018). Gong et al. reported the use of a mitochondria-targeted probe (MitoCAT-g) as a mitochondrial oxidative stress amplifier and a promising anticancer agent (**Figure 4B**) (Gong et al., 2019). The probe was prepared via a three-step approach, in which CDs were first synthesized from citric acid and polyene polyamine then loaded with atomically dispersed gold and conjugated with both cinnamaldehyde for ROS generation and TPP for mitochondrial targeting. The design of the probe was based on the depletion of mitochondrial glutathione, which leads to an increase in ROS and ultimately apoptosis. MitoCAT-g was also labelled with fluorescein





## LYSOSOMES

Lysosomes are specialized spherical vesicles inside cells that degrade large molecules through the action of hydrolytic enzymes, thus playing various roles in autophagy, homeostasis, plasma membrane repair, and the removal of waste substances from the cell (Weissmann, 1967; Yu et al., 2016). These enzymes are most active at the acidic pH (4–5.0) of the lysosome interior (Mindell, 2012). Thus, one reported strategy for lysosome targeting is the use of probes containing lipophilic amine moieties, which become protonated after diffusion through the lysosomal membrane, thus trapping the probe inside the lysosome and leading to its enrichment (Xu et al., 2016). Lysosomes are known to contain approximately 50 acid hydrolases that are responsible for hydrolysing various biomacromolecules, such as lipids, nucleic acids, proteins, and polysaccharides. Lysosomal malfunction or abnormal lysosome activities have been linked to various disorders, including lysosomal storage diseases, inflammation, neurodegenerative disease, and cancer (Tong et al., 2020; Li et al., 2021; Strzyz, 2021).

CDs-based probes have been shown to enter cells via the process of endocytosis, while the lysosomes playing a key role in this process via its pH value is less than that of the cytoplasm (Esteves da Silva and Gonçalves, 2011; Zhou et al., 2014). The main principle underlying this design requires probes to have a low  $pK_a$  value (Xu et al., 2016). One way of achieving lysosome targeted is the use of bearing lipophilic amines on the CDs. In this regard, the application of amino acids as passivating agents or carbon sources in the synthesis of CDs has been shown to be effective (Liu et al., 2019b; Ronzani et al., 2019; Qin et al., 2020; Sun et al., 2020).

### Morpholine-Modified CDs for Lysosome Targeting

Studies on the use of morpholine as a functional group for lysosome-targeting probes have been reported (Wan et al., 2014; Wu X. et al., 2017; He et al., 2017; Biswas et al., 2021; Mukherjee et al., 2021). For example, Wu et al. synthesized CDs functionalized with a morpholine derivative (CDs-PEI-ML) to serve as a lysosomal probe (Wu L. et al., 2017). In this study, the CDs-PEI-ML probe was prepared from citric acid and PEI via a hydrothermal method and the morpholine was then covalently attached to the CDs using EDC and NHS. Probes with morpholine groups modified typically display  $pK_a$  values of five to six, thus enabling them to be used to target lysosomes with their internal pH of 4–5. The zeta potential of the CDs was observed to change from  $-21.0$  to  $+28.0$  mV upon passivation with PEI, indicating the presence of positive charges on the CDs surface. Characterization of the CDs-PEI-ML probe revealed an average size of 3.15 nm, an absorption peak at 359 nm, an emission maximum at 441 nm, and a quantum yield of 37.7%. Confocal images demonstrated that the probe exhibited high photostability against bleaching, low cytotoxicity in HeLa cells, and effective lysosomal localization.

Lysosomal pH is an important factor in its normal functioning, therefore probes capable of revealing pH

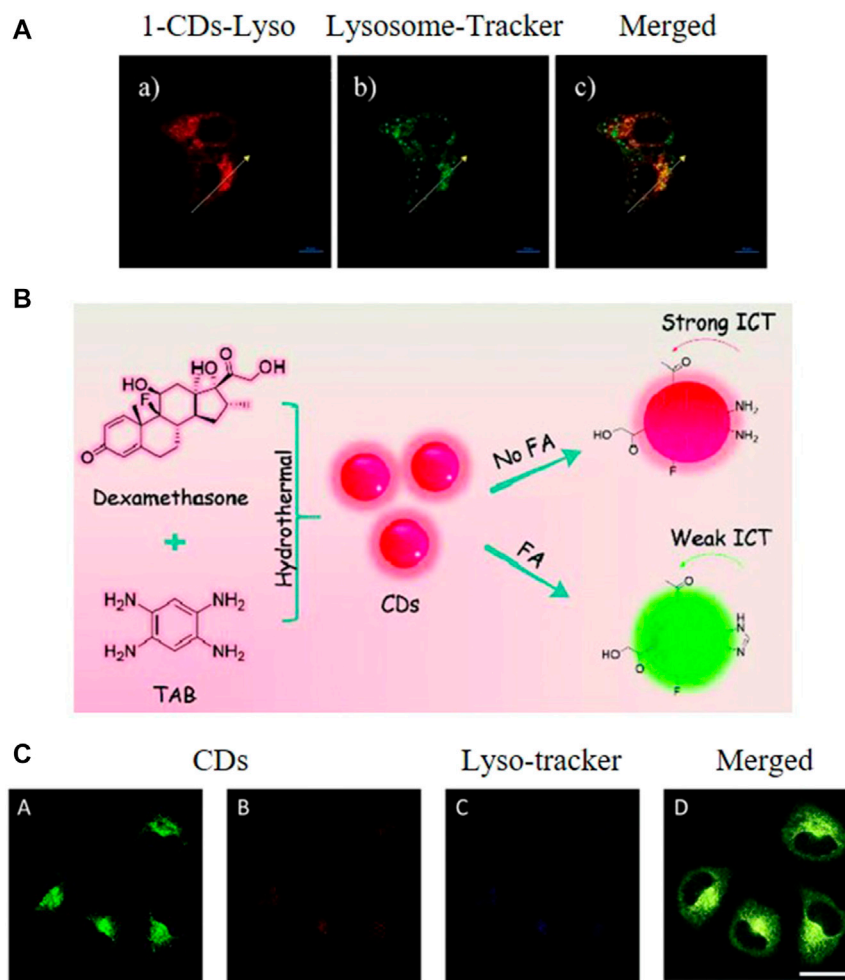
fluctuations within the lysosomes offer profound benefits. In this regard, He et al. developed a ratiometric nanosensor capable of monitoring lysosomal pH (He et al., 2017). This nanosensor was based on 1,8-naphthalimide derivative, morpholine, and CDs synthesized using CA and EDA as precursors by a simple hydrothermal method. In acidic medium, the nanosensor was observed to exhibit green fluorescence. However, a gradual blue shift occurred with increasing pH, resulting in the nanosensor emitting blue fluorescence (Figure 6A). The nanosensor was reported to display a linear range for pH sensing of 5.6–7.4. In addition, confocal images displayed a pH-dependent signal when the nanosensor was applied to HeLa cells.

### Lipophilic-Amine-Functionalized CDs for Lysosome Targeting

Owing to the acidotropic behaviour of weakly basic amines, probes bearing such groups can selectively accumulate inside lysosomes with their acidic interior pH (Abeywickrama et al., 2019; Fan et al., 2021). Even though probes equipped with functional groups have proved effective for lysosome targeting, their complex modification process limit their broader application. Recently, another type of CDs bearing weakly basic amino groups has been applied to lysosome targeting (Chen et al., 2018; Zhang et al., 2018; Geng et al., 2020; Gao et al., 2020; Guo et al., 2020b). These CDs combine the good biocompatibility and fluorescence properties of CDs and the lysosome targeting properties of lipophilic amines. For example, Zhang et al. synthesized highly photoluminescent emerald CDs from ethanediamine and *p*-benzoquinone and reported their suitability for lysosome targeting (Singh et al., 2019). The CDs exhibited an average size of 2.02 nm, absorption peaks at 234 and 410 nm, an emission maximum at 530 nm, a quantum yield of 15%, good aqueous solubility, pH sensitivity, and high photostability. The CDs possessed abundant amino groups, and the confocal images after incubation with A549 cells and HepG2 cells revealed a time-dependent decrease in the fluorescence emission when the probe was treated with dexamethasone to induce morphological changes within the cells.

Hydrophobic CDs capable of targeting the lysosomes were synthesized by Mao et al. using 1-ethyl-3-methylimidazolium bromide via a hydrothermal method (Mao et al., 2016). Characterization of the CDs revealed an average size of 8.52 nm, concentration-dependent absorbance and photoluminescence where the emission wavelength shifted from 450 to 540 nm, and a quantum yield of 2.5–4.8%. Furthermore, these hydrophobic CDs were found to have a rapid penetration rate as they emitted fluorescence within 1 min of entering HeLa cells. These properties make this probe very promising for bioimaging, which was further confirmed using A549, MCF-7, and HeLa cells.

CDs with intrinsic lysosome targeting and cancer targeting abilities were first synthesized by Liu and Sun et al. from 1,2,4,5-tetraaminobenzene and dexamethasone using a microwave-assisted hydrothermal method (Figure 6B) (Liu et al., 2019b). The as-synthesized CDs



**FIGURE 6 | (A)** Fluorescence images of HeLa cells stained with the synthesized CDs and a lysosome tracker, scale bar = 10  $\mu\text{m}$ . Reproduced with permission from He et al., 2019. Copyright (2017) Elsevier **(B)** Synthesis of the CDs **(C)** Localization of the CDs in the lysosomes of HeLa cells, scale bar = 25  $\mu\text{m}$ . Reproduced with permission from Liu et al., 2019b. Copyright (2019) The Royal Society of Chemistry.

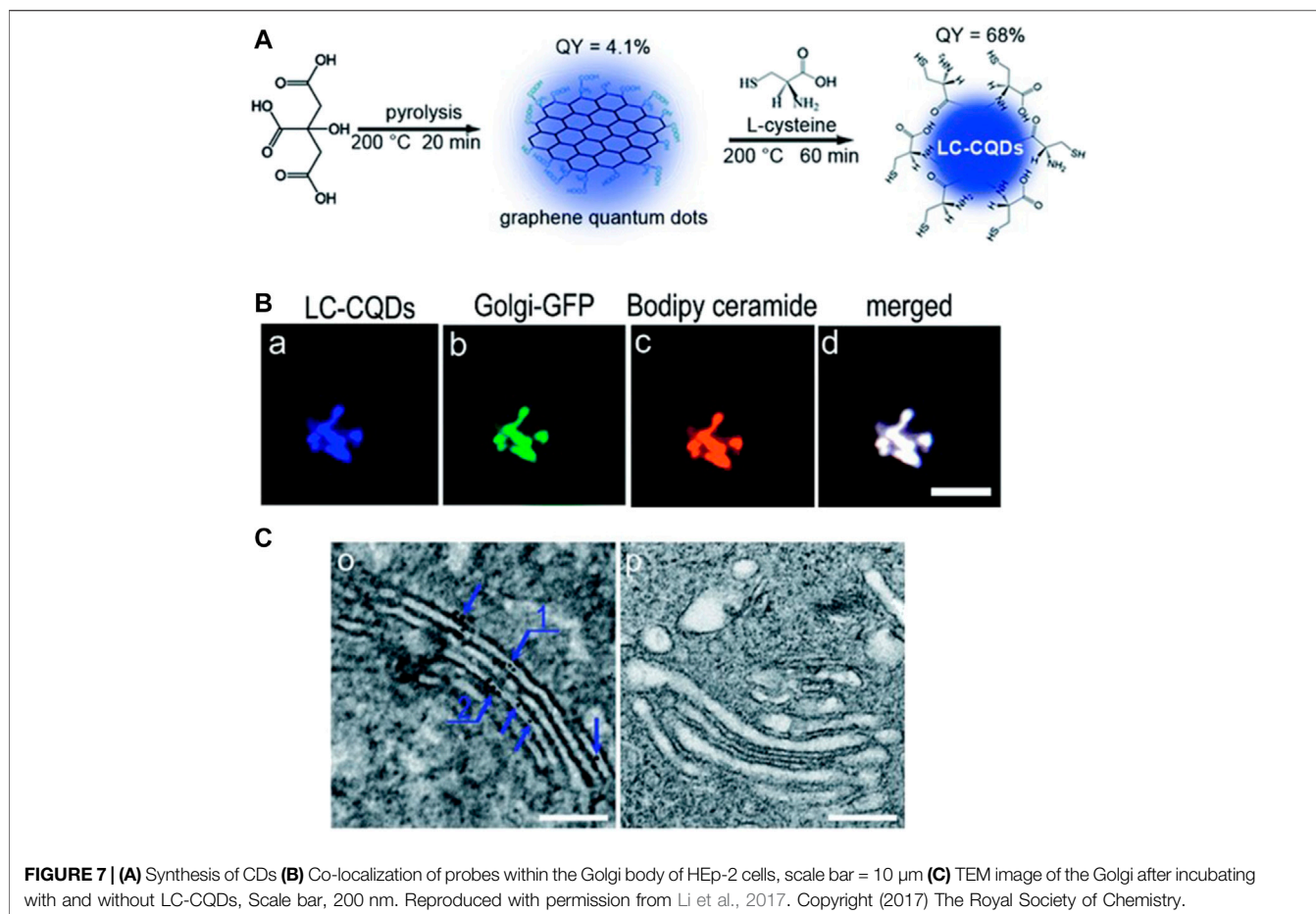
were reported to exhibit some excellent properties, such as longer-wavelength emission (absorption peak at 496 nm and emission peak at 585 nm), good aqueous solubility and biocompatibility, and high photostability. The *o*-diamino moieties on the CDs were demonstrated to react with formaldehyde and target lysosomes. As an abnormal formaldehyde concentration in lysosomes is closely related to Alzheimer's disease, thus the detection of formaldehyde in lysosomes is of great significance. This CDs also exhibited a high selectivity for formaldehyde with a fast response and great changes of ratio values. The developed system was thus used for the detection of formaldehyde fluctuation in the lysosomes of living cells.

In addition, the lysosome dysfunction is associated with cell apoptosis and oncogenic transformation, which also might relate to various diseases, such as cancer and neurodegenerative diseases. Thus, tracking the behavior and status of lysosome, also a detailed investigation of the environment in lysosome is crucial to enrich our knowledge of related diseases.

## ENDOSPLASMIC RETICULUM

The endoplasmic reticulum (ER) is an organelle in eukaryotic cells that is involved in the production, processing, and transport of proteins and lipids. The ER is composed of two compartments, namely, the rough ER and the smooth ER, with each playing different roles. In particular, the rough ER is involved in the secretion of proteins, whereas the smooth ER is involved in the synthesis of carbohydrates and lipids (Baiceanu et al., 2016). As described by Zhang et al., the accumulation of unfolded or misfolded proteins can disturb the normal functions of the ER, which induces ER stress or the cellular stress response (Zhang and Kaufman, 2008). One strategy for developing ER-specific probes involves targeting the ER-associated proteins.

Bao et al. synthesized a novel probe that targeted the ER (Bao et al., 2019). This probe, which the authors referred to as trace metal (M)-, N-, and O-doped carbon-dominated nanoparticles (MNOCNPs), was synthesized from *p*-phenylenediamine and selected trace metals via a one-pot hydrothermal approach

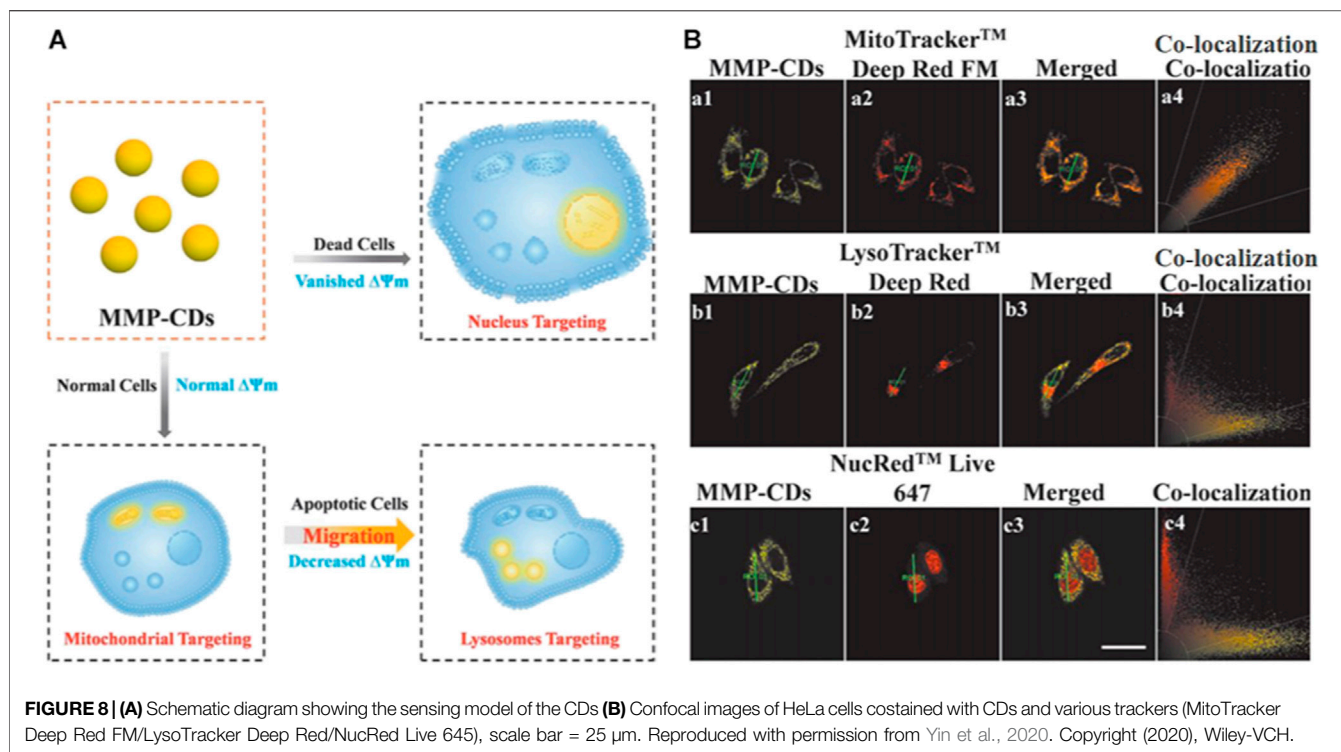


followed by conjugation with PEG. The zeta potentials of these MNOCNPs were reported to be +52.7, +34.8, and +40.3 mV for NiNOCNPs, PdNOCNPs, and CuNOCNPs, respectively. Subsequent modification of the NiNOCNPs with PEG (PEG5k-NiNOCNPs) led to a change in the zeta potential from +52.7 to 31.2 mV and improved the dispersibility of the system in various solvents. This conjugated system was found to possess good photostability, low cytotoxicity, and high biocompatibility. Conjugation with FITC confirmed the co-localization of the PEG5k-NiNOCNPs in the ER region of the cell.

The pH is known to be a critical factor regulating the activity of the ER. For instance, Dong et al. observed that an acidic intracellular pH caused by the overproduction of protons could stimulate ER stress through the activation of GPR4, leading to adverse effects such as apoptosis and inflammation thus confirming their positive surface charge (Dong et al., 2017). Thus, probes that exhibit the dual functions of ER targeting and pH responsiveness are in high demand. Shuang et al. synthesized novel CDs with the ability to target ER as well as reflect fluctuations in its pH (Shuang et al., 2018). The CDs were prepared from urea and citric acid (these amine groups functionalized CDs referred to as ACDs) and then functionalized with laurylamine to realize ER targeting. The laurylamine functionalized CDs (LCDs)

possessed an average size of approximately 3 nm and displayed a red shift in their  $n-\pi^*$  transition compared to the ACDs (i.e. from 336 to 360 nm), an emission maximum of 440 nm, and a fluorescence quantum yield of 1.3%. The LCDs were sensitive to pH in the range of 6.2–7.2 with a correlation coefficient of 0.9944. This pH sensitivity of the LCDs was ascribed to the amount of laurylamine in the system. This led the authors to conclude that by regulating the surface functional groups on CDs, there exists the possibility of forming pH-sensitive probes. The results of FTIR and XPS analysis confirmed the successful functionalization. Laurylamine was also observed to endow the CDs with hydrophobic properties that enhanced their interaction with the cell membrane, resulting in endocytosis and subsequent localization in the ER, which was confirmed using endocytosis inhibitors. Furthermore, confocal images confirmed the pH sensitivity and ER-targeting properties of the probes in MCF-7 cells in the presence of chloroquine and dexamethasone.

In summary, CDs which are functionalized with amine groups are found to be effective for ER targeting; similar with lysosome-targeted CDs. However, the aggregation of the CDs inside ER and lysosome might be different because of the difference in their environment. Thus, the CDs' fluorescence properties must be studied in detail to enhance their application for monitoring and imaging of ER.



## GOLGI BODY

The Golgi body (also referred to as the Golgi apparatus or Golgi complex) is the organelle responsible for processing proteins received from the ER for further transportation to other organelles such as the lysosomes, endosomes, and plasma membrane (Xu et al., 2016). During this process, most of the proteins undergo significant changes; hence, changes in the environment of the Golgi body are likely to affect its function and subsequently result in diseases (Bexiga and Simpson, 2013). Owing to the role of the Golgi body in binding proteins, it has been observed that biomolecules with abundant thiol groups can identify protein retention within the Golgi body by anchoring on the surfaces of the Golgi body, hence serving as a means for detection (Gauthier et al., 2009).

Cysteine has been found to be a useful precursor for synthesizing probes capable of staining the Golgi body due to the anchoring of galactosyltransferase and protein kinase D in the Golgi body. Inspired by this, Li et al. synthesized novel probes via pyrolysis using citric acid and L-cysteine as precursors for the imaging of the Golgi body (Li et al., 2017). The as-synthesized CDs were observed to have chiral properties, good fluorescence characteristics, and abundant cysteine residues, which has proven to be essential for Golgi targeting. Confocal images revealed that the as-synthesized probe stained the Golgi body of HEp-2 cells after 4 h of incubation and also did not affect the function of the Golgi body (Figure 7). In a further study conducted by the same authors (Li et al., 2019), these CDs were conjugated with the ricin A-chain of (CD-RTA) and found to be effective carriers for the

delivery of ricin A protein into the Golgi body, thus demonstrating the potential of such CDs for selective drug delivery.

Yuan et al. synthesized optically active light-emitting carbon nanoparticles that were intrinsically capable of localizing within the Golgi body (Yuan M. et al., 2017). The CDs were synthesized from CA and d/L-penicillamine via pyrolysis. Characterization of the CDs revealed a typical size of 1–4 nm, absorption peaks at 247 and 344 nm, and the emission of blue fluorescence upon excitation at 363 nm. Further studies demonstrated that the CDs had excellent cellular uptake properties, high photoluminescence quantum yield, and low cytotoxicity as well as effectively staining the Golgi body. The authors concluded that the vicinal effect resulting from the achiral core of citric acid with the chiral penicillamine ligands in an achiral adsorption pattern induced chirality in the as-synthesized CDs via optically active surface passivation, while the abundance of thiol groups on the CDs surface enabled detection of the Golgi body. Although various CDs synthesised from precursors containing L-stereo functional groups were used for Golgi targeted imaging, a clear mechanism for aggregation in Golgi body is lacking.

## TRANSLOCATION BETWEEN ORGANELLES

The enclosed environment or surface which acts as a partitioning provided by subcellular organelles could create space for the tailored chemical reactions of the organelles to perform specific functions. The subcellular migration of CDs

enriches our understanding of how CDs interact with cells, shedding new light on the design of novel CDs for biomedical applications. Recently, Yin et al. synthesized CDs from 2,4-dihydroxybenzaldehyde and 1,2,3,3-tetramethyl-3H-indolium that were sensitive to the mitochondrial membrane potential (Figure 8) (Yin et al., 2020). The authors also found that the CDs displayed distinct targeting capabilities (mitochondria/lysosome/nucleus) at different mitochondrial membrane potential levels (normal/decreased/negligible) corresponding to three different degrees of cell viability (healthy/damaged/dead).

## CONCLUSION

This review presented a summary on recent advances in CD-based probes that can target specific organelles. These probes have been demonstrated to exhibit a variety of unique characteristics, such as high selectivity and sensitivity alongside low cytotoxicity, thus enabling their use as biological probes. In recent years, numerous probes have been designed with the ability to target key organelles, such as the nucleus, mitochondria, and lysosomes, thus providing valuable information about their activities. Such probes may offer several additional benefits as efficient drug delivery systems or nanocarriers. However, organelles such as the Golgi body and other secondary organelles have received little attention. By referring to the design of small-molecule fluorescent probes, a diverse range of organelle-targeting functional groups can be conjugated to CDs to enable their localization in specific organelles, and would help to develop a theragnostic nanomedicine for drug delivery and treatment applications for various diseases.

Although the field of CDs is still in its infancy, great advances can be anticipated in the future. For instance, CDs with NIR-I and even NIR-II emissions or up-conversion fluorescence property are still highly desired thus the need for further investigations. Moreover, there still exists some challenges with respect to subcellular imaging with CDs, such as realizing the visualization of numerous subcellular structures (microtubules, centrosome, actin filaments, and others) that still cannot

be imaged with fluorescent CDs at present, the determination of the functions of proteins at the subcellular level, the correlations between fluctuations in the reactive species in organelles and the generation or progression of disease. Although low cytotoxicity of CDs has been reported from different research teams, standardized toxicity assessments methodologies are absent. Based on this situation, the comparison on the safety of CDs is still difficult. Therefore, there is the need for a systematic assessment of potential toxicity risks associated with the application of CDs so as to suitability for clinical applications such as in diagnosis and treatments. For better practical application in animal and clinical studies, CDs with high QYs and NIR-I and NIR-II emission are needed. It is expected that more high-performance CDs for subcellular targeted imaging and laboratory medicine on the subcellular scale will be developed in the near future. Bioimaging at the subcellular level would greatly increase our understanding of human biology and disease, and the development of CDs as a versatile tool for the prevention and treatment of disease shows enormous promise.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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