



# Tailoring Scaffolds for Orthopedic Application With Anti-Microbial Properties: Current Scenario and Future Prospects

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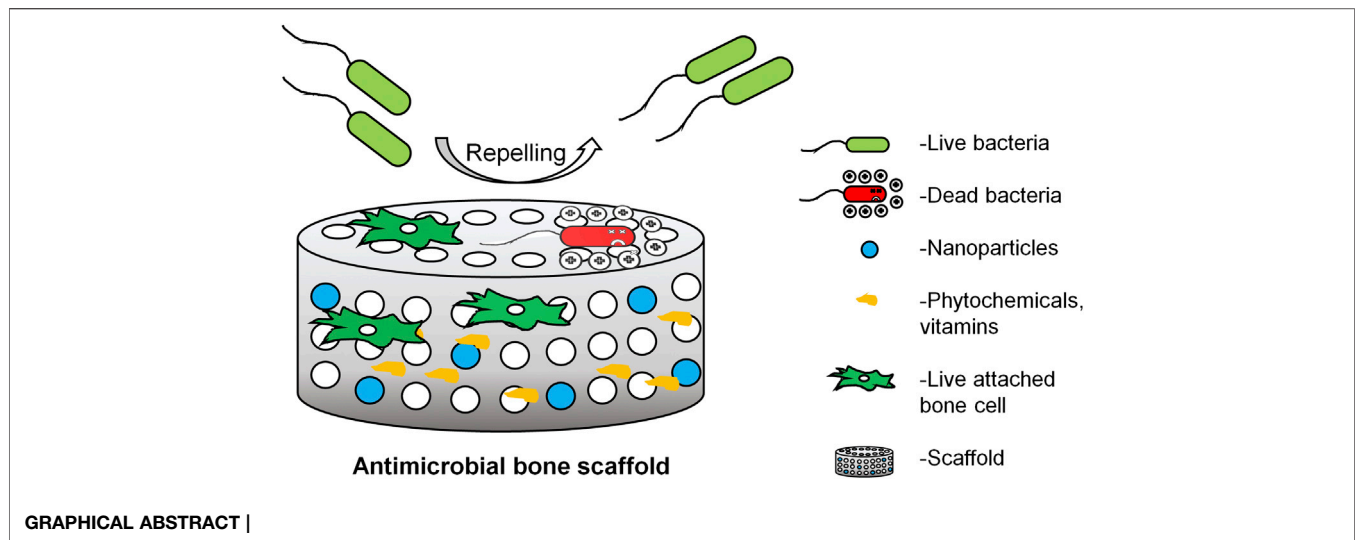
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Research in the orthopedic application has attracted the scientific community to design and develop advanced, engineered synthetic scaffolds that possess all the essential cues properties necessary for successful regeneration and restoration of tissue function. Despite having the best surgical practices, nosocomial infections like surgical site infections can negatively impact the outcome of the scaffolds that are intended only for regenerative purposes. Therefore, there is a need to include anti-microbial properties as one of the parameters while designing a scaffold. There are various methods implemented to tackle microbial colonization such as modifying the scaffold surface that deflects microbes from surface adhesion and modifying the scaffold surface by inactivating microbes upon contact while maintaining the biocompatibility of the scaffold and also maintaining the scaffold viable for new bone formation. This review lists various anti-microbial polymers and compounds available in nature and methods to incorporate them into the scaffold with polymer and ceramics as the base material, without compromising bioactivity. We have reviewed various anti-microbial compounds, biomaterials and have also identified various biomolecules (vitamins, phytochemicals and anti-microbial peptides) that can be utilized for orthopedic application.

**Keywords:** anti-microbial property, biocompatible, biomaterial, resorbable, scaffolds, orthopedic, tissue engineering

## HIGHLIGHTS

1. Antimicrobial action can be from added nanoparticles, chemicals or chemical modifications on the polymer.
2. Metal, ceramics and carbon based nanoparticles and their modification in the scaffold are identified.
3. Phytochemicals, vitamins and anti-microbial peptide with anti-microbial properties are reviewed.
4. Possible chemical modification of polymer with innate anti-microbial properties are discussed.



## INTRODUCTION

With 2.2 million bone graft procedures performed per year, bone becomes the second most transplanted tissue followed by blood (Kashte et al., 2017). Even though autografts were considered the “Gold standard”, their availability, donor site morbidity, and increased risk of infection has made researchers to seek for alternative materials that fulfill the advantages autografts possess. Over the years, the design aspects of synthetic grafts have been modified to incorporate the requirements of the grafts that they are intended for. 1) Initially, the synthetic graft was meant to serve the mechanical needs of the host tissue but not to interact with them. Metals like titanium and its alloy, polymers like PMMA, PEEK, and ceramics like alumina and zirconia were used. 2) Then, the material design of synthetic graft was further modified by incorporating bioactive compounds like calcium phosphates, calcium sulfates, along with polymers that degrade with time *in vivo* that includes synthetic polymers like PCL, PLGA, PGA, PLA, etc., and natural compounds like gelatin, collagen, and bioactive glasses were used. 3) Later, it was further modified by incorporating growth factors like compounds and compounds that modify according to external stimuli (Qu et al., 2019). Based on the evolution of synthetic graft’s design requirement the scaffold can be defined as a three dimensional template that provides biophysical, chemical and mechanical stimuli essential for successful tissue regeneration, restoration and function.

United States faces approximately 500,000 surgical site infections per year. 25% of open fractures have a risk of infection based on severity of fracture (Schmitt, 2017). This may result in complications like non-unions or delayed unions of bones (Lee et al., 2015). Osteomyelitis (bone marrow inflammation) is predominantly caused by *S. aureus* in bone. Other microorganisms frequently involved are *P. aeruginosa* and methicillin resistant *S. aureus* (MRSA), *S. epidermidis*, *E. coli*, *E. faecalis*, *E. faecium*. 30–60% percent of infection is caused by *Staphylococcus aureus* (Hofstee et al., 2020). Osteomyelitis can be caused through 1) infection spreading through blood stream, 2) infection spreading through adjacent

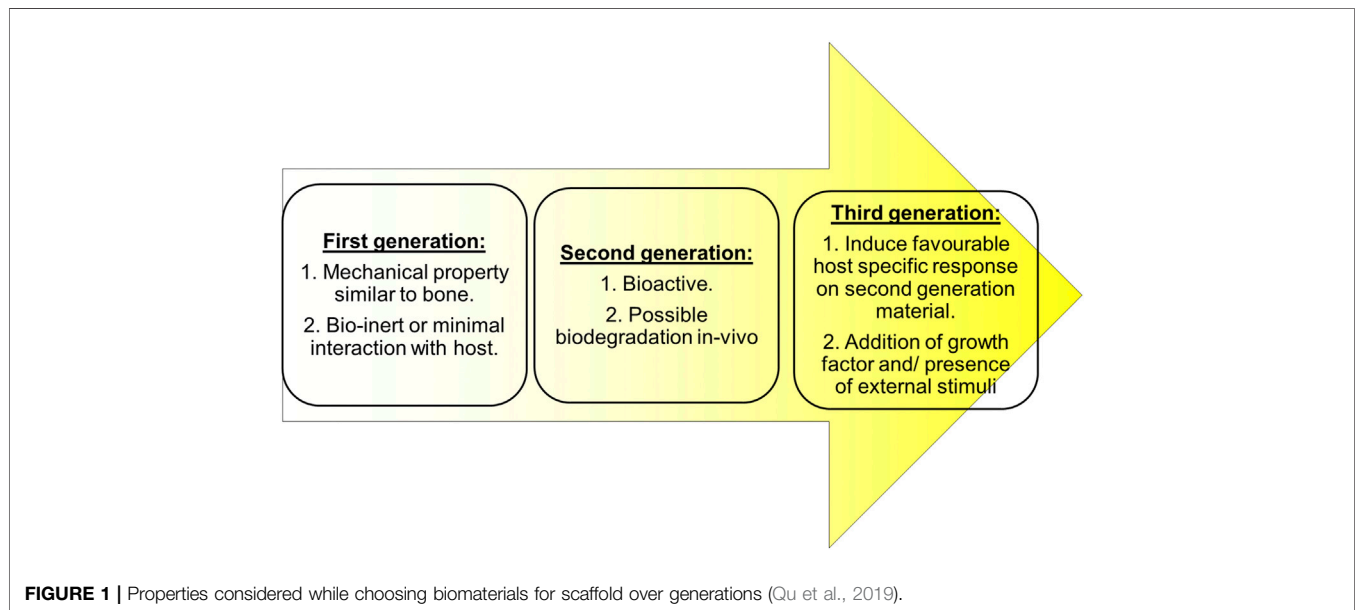
focus of infection and 3) diabetic foot infection (Brit et al., 2017; Schmitt, 2017). The general treatment involves surgery i.e., aggressive debridement of necrotic tissue followed by antibiotic therapy. Antibiotic therapy involves vancomycin, ciprofloxacin, cloxacillin, gentamycin, clindamycin, and/or their combination through the intravenous route (del Pozo et al., 2018). In most cases, nosocomial infection, an infection caused by organisms from skin flora as a result of fracture, diabetics, are more prevalent in causing osteomyelitis (Schmitt, 2017). 25% of open fractures have a risk of infection based on how severe the fracture is (Schmitt, 2017).

Even with the usage of appropriate surgical techniques, pre and post-operative procedures, all the orthopedic and related surgeries are at risk of bone infection. There is about 20–30% relapse of osteomyelitis, even after surgical debridement and/or anti-microbial therapy for 4–6 weeks (Bhattacharya et al., 2013). Due to insufficient blood supply, the required amount of antibiotics does not reach the target site, also, biofilms are growing resistant towards antibiotics (Bhattacharya et al., 2013). Therefore, one section of research is focused on incorporating antibiotics or their cocktail into the scaffold that locally deliver antibiotics to the infected site. Some of the antibiotics incorporated biodegradable antimicrobial orthopedic scaffold are listed in **Table 1**. The diagnosis, classification, treatment for osteomyelitis are discussed elsewhere (Scherping and Aaron, 2007; Witso, 2014; Winkler, 2017).

Currently, the antibiotic loaded scaffold available in the market are OSTEASET<sup>®</sup> T (Humm et al., 2014) containing tobramycin sulfate, STIMULAN<sup>®</sup> (Kallala et al., 2018) containing antibiotics like vancomycin, gentamicin or tobramycin and injectable bone substitute like CERAMENT|G eluting gentamicin (Drampalos et al., 2018). There are various scaffolds, designed to release anti-microbial agents on demand on the site of infection. Even if the microbe is dead, the presence of a dead microbe can still be a site for antibiotic resistant biofilm formation (Johnson and Garcia, 2015). But also, there is very limited studies on cytotoxic effects, long term effects of antibiotics eluting scaffold and its involvement in bone’s regeneration

**TABLE 1** | Effect of antibiotics used in scaffold towards osteoblasts and microbes function.

S. no	Antibiotics	Polymer(s)/ ceramic	Effect on biological function	References
1	Roxithomycin	PCL/PEG	↑Anti-bacterial activity against <i>S. aureus</i> than <i>E. coli</i> . ↑Viability of MG63	Bai et al. (2020)
2	Minocycline hydrochloride	PLA/collagen/HA	↑Anti-bacterial activity against <i>S. aureus</i> . ↑P, ↑OD of hMSC.	Martin et al. (2019)
3	Levofloxacin	PU/HA/silica	↑OD of BMSC. ↑Anti-bacterial activity against <i>S. aureus</i> and <i>E. coli</i> co-culture	Kuang et al. (2019)
4	Amoxicillin	Bredigite	↑Anti-bacterial activity against <i>S. aureus</i> and <i>E. coli</i> . ↓MG63 adhesion ↑amoxicillin concentration	Bakhsheshi-Rad et al. (2018)
5	Vancomycin	PLGA/Bioactive glass	↑P, ↑OD of hBMSC. Anti-bacterial activity against <i>S. aureus</i>	Cheng et al. (2018)
6	Enoxacin	PLGA/Mg scaffold	↓P of <i>S. aureus</i> and <i>S. epidermidis</i> and ↑P of NIH-3T3 and ↓osteoclast and activity	Li et al. (2016a), Li et al. (2016b)



capacity **Figure 1**. Therefore there is a need to design a fourth generation orthopedic scaffold that, also has anti-microbial property so that it not only prevents microbial adhesion, inactivates microbes but also reduces the need for anti-microbial therapy for long term use without compromising bone's regeneration capability. This review describes favorable factors of microbial and osteoblast interaction on the scaffold and the host, essential to describe a suitable biodegradable resorbable antimicrobial orthopedic scaffold design.

## SCAFFOLD DESIGN PARAMETERS THAT OPPOSE MICROBIAL ADHESION AND ITS MATURATION

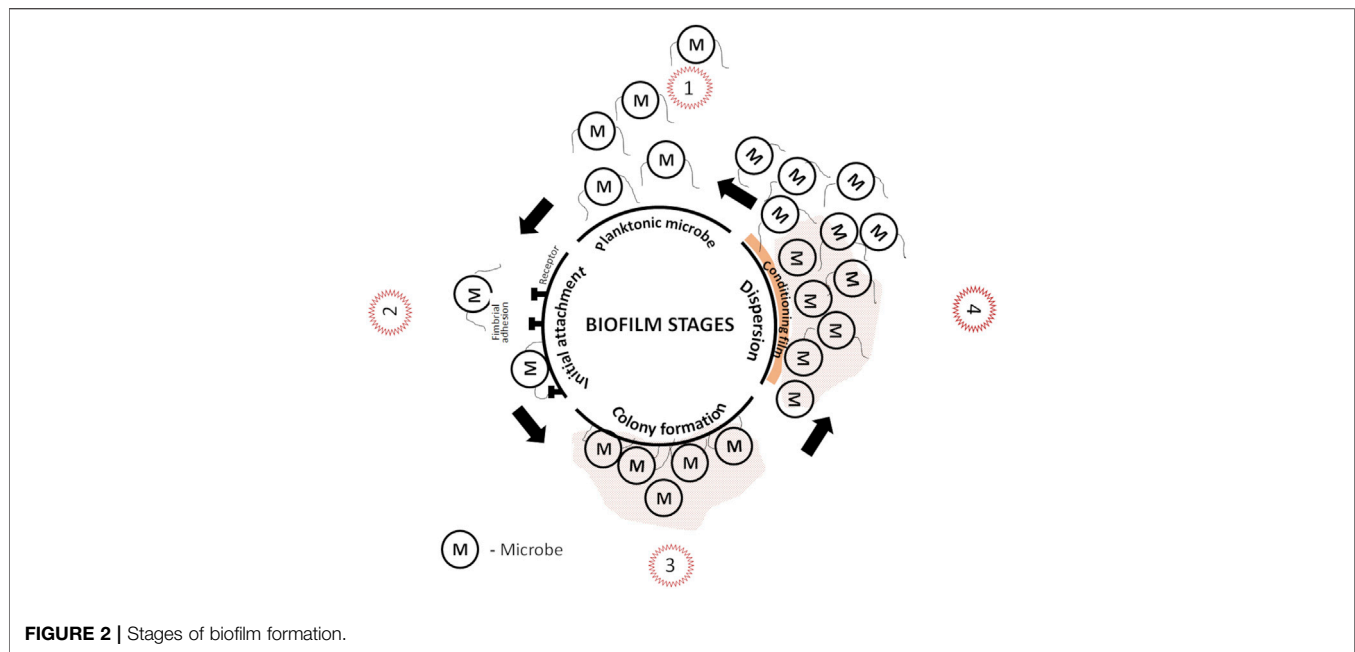
Design on degradable anti-microbial scaffold was aimed 1) to avoid external microbial ambush before implanting and 2) to eliminate established network of microbe in the host tissue.

Microbial adhesion on the scaffold surface is one of the complex multifactorial problems. External microbial ambush

can be avoided by devising strategies that prevent microbial adhesion before it forms permanent bond with the substratum as in **Figure 2**. Microbial adhesion and biofilm formation on the surface has four stages: 1) transport of micro-organism to the surface, 2) initial but reversible binding to the surface, 3) permanent binding, and 4) colonization. The details of biofilm formation and cell to cell communication for both Gram-negative and positive species is discussed elsewhere (Arciola et al., 2018; Lu et al., 2019). Factors that determine microbial adhesion on biomaterials are 1) roughness and topography, 2) surface energy or charge, 3) hydrophilicity or hydrophobicity, and 4) chemical composition of the surface **Figure 3**. The following subsections will discuss the surface factors that can be tuned against initial microbial adhesion.

## Roughness and Topography on the Biomaterial Surface

One way to reduce bacterial colonization is by providing less contact surface area for adherence. Many of the naturally



available surfaces like Wings of cicada (*Psaltoda claripennis*), dragonfly (*Diplacodes bipunctata*), damselfly (*Calopteryx hemorrhoidalis*), taro leaves (*Colocasia esculenta*), and gecko skin (*Lucasium steindachneri*) has high aspect ratio nanostructures on their surface making them bactericidal on contact. Many studies have tried to mimic, evaluate and analyze the bactericidal efficiency of nanostructures arrangement and geometry. Such nanostructures were successfully implemented on the surface of various metallic and polymeric non-degradable implants. Bactericidal efficiency differs between Gram-positive and Gram-negative bacteria. Their difference in shape, cell membrane thickness, motility and plane of cell division, ruptures the cell membrane of bacteria that differentiates the nanopography's bactericidal efficiency (Diu et al., 2014; Bhadra et al., 2015; Kelleher et al., 2016; Tsimbouri et al., 2016; Wu et al., 2018; Linklater et al., 2019). Studies on wrinkled patterns on polymer surface provide combinations of nano and microstructures that prevents microbial colonization (Nguyen et al., 2020). Scaffolds prepared through electrospinning provide roughness and topography essential for reduced bacterial contact.

### Surface Charge, Surface Energy and Wettability of the Biomaterial Surface

The second mode of making an anti-bacterial surface is by utilizing materials with surface charge. Bacteria at biological pH have a negative surface charge. Gram-negative bacteria have a highly negative charge and hydrophilic surface in comparison to Gram-positive bacteria. The lipopolysaccharide renders Gram-negative bacteria a more negative charge than Gram-positive bacteria. When positively charged nanoparticles

from metal and metal oxide are used, they get attached to the cell wall due to electrostatic interaction and they get pulled into the bacteria. These nanoparticles not only alters cell membrane's structure and permeability but also causes ion homeostasis, catalyzes proteins to inactive and produces reactive oxygen species (ROS) (Raghunath and Perumal, 2017). Similar to nanoparticles, polycations adhere to the membrane surface leading to membrane integrity disruption. In quaternary ammonium chitosan salts, an increase in the alkyl group rendered a strong anti-bacterial effect for both Gram-negative and Gram-positive bacteria (Rabea et al., 2003). The hydrophobic alkyl group got inserted or penetrated into phospholipid bilayer causing cytoplasmic leakage and cell death in *E. coli* and *S. aureus*. The presence of a cationic group in the main polymer chain has more anti-bacterial activity in comparison with side chain cationic polymers and small molecule cationic compounds (Guo et al., 2018). These mechanisms do actively kill bacteria.

Other mechanism is to passively drive away the bacteria from the surface. One way is to repel the bacteria by using negatives charged surface instead of killing them (Abbaszadegan et al., 2015). The second way is to delay bacterial adhesion is by using hydrophilic polymer brushes on the biomaterial surface and also by using a hydrophilic surface (Nejadnik et al., 2008). Pidhatika et al., 2010 used cationic hydrophilic polymer brushes to repel *E. coli* by providing both electrostatic repulsion and steric hindrance. Additional, factors that affect bacterial adhesion are temperature, time of exposure, bacterial concentration, the presence of antibiotics, environment electrolyte concentration and surface chemistry (Filipović et al., 2020). Similar concept applies to other microbes as well.

The above discussions focusses on the anti-adhesive properties of scaffold towards microbes. But the presence of fimbriae and flagella on the microbes plays a key role in overcoming all the

**TABLE 2 |** Effect of vitamins towards osteoblast's function.

S. no	Vitamin	Polymer(s)/ceramic	Encapsulation strategy	Other compounds (if present)	Effect on biological function	References
1	D3	PCL	Layered double hydroxide	—	↑P and no significant increase in ALP activity of MG63	Belgheisi et al. (2020)
2		HA	PCL/PEG	—	↓Osteoclastic activity	Vu and Bose (2019)
3		PCL/gelatin	—	HA	↑P, ↑ALP, ↑early OD, ↑M of hADSCs	Sattary et al. (2019)
4		Gelatin	Layered double hydroxide	HA	↑P, ↑ALP, ↑M of G-292	Fayyazbaksh et al. (2017)
5	E	Poly(hydroxy butyrate)	—	Bioactive glass	↑P of MG63	Misra et al. (2009)
6		β-Tricalcium phosphate	PCL	—	No coating on β-Tricalcium phosphate lead to ↑ALP of hFOB	Bose et al. (2019)
7		Titanium	—	HA	↑P of hFOB and ↓P of MG63	Sarkar et al. (2020)
8		Poly urethane grafted vitamin C	—	—	↑P, ↑ALP, ↑collagen-I of osteoblast progenitor cell	Zhang et al. (2003)
9	K	HA	Poly xylitol sebacic adibate	—	↑ALP and ↑M on day 10 in MSC	Dai et al. (2019)
10	K2	PCL, gelatin, poloxamer 188	—	—	Vitamin coated fiber has ↑P, ↑ALP of SaOS-2	Alam et al. (2019)

initial barriers between the cell and substratum (scaffold surface and/or animal tissue) and irreversibly attach. In host, microbes or it's colonies enter through iatrogenic or hematogenous routes and attaches to extra cellular matrix of bone by identifying adhesive molecules thereby forming biofilm (Hofstee et al., 2020). Similarly when a scaffold is inserted into host, the conditioning provided by the host can also be suitable for microbial attachment to the surface (De Cesare et al., 2019). Biofilms are established complex network of irreversibly attached microbes on substratum encapsulated by extracellular polymeric substances (EPS). Depending on the environment they are developed, biofilm may also contain non-cellular substances. The extensive details on EPS properties is discussed elsewhere (Donlan, 2002).

Bacteria's presence in host activates innate immune responses. Planktonic microbes are usually eliminated by phagocytosis, oxidative bursts and by production of antimicrobial peptides, proinflammatory cytokines and chemokines. But microbial colonies has protective network that protects them from immune cells attack. Once a biofilm is established, they become impenetrable by immune cells and they start evolving, bone matrix becomes their nutrient source, osteoclastogenesis is promoted, inhibits bone formation thereby bone tissue loss (Hofstee et al., 2020).

For an established biofilm, changes in the physical properties like roughness and topographies of the scaffold doesnot affect EPS of biofilm. But EPS can be polycationic in case of Gram-positive bacteria and polyanionic in case of Gram-negative bacteria and EPS can be hydrophilic or hydrophobic (Donlan, 2002). Therefore, the scaffold have to be designed based on the organism that caused osteomyelitis. Usually, higher dose of antibiotic supplement on site using scaffold, but, biofilm tends to evolve. Therefore, these scaffold should be redesigned to possess all chemical elements that can penetrate and eliminate biofilm and also support osteoblastogenesis.

## SCAFFOLD DESIGN PARAMETERS THAT SUPPORT ORTHOPEDIC TISSUE ENGINEERING

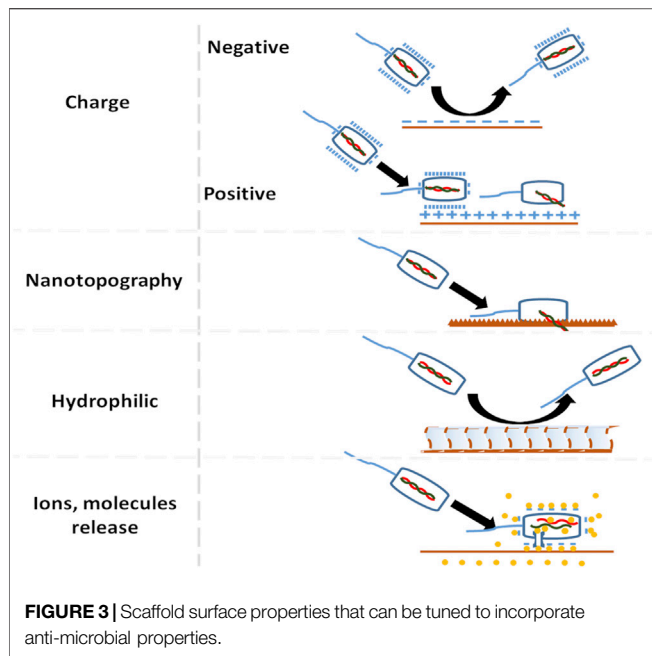
Tissue engineering on scaffold usually deals with mimicking the environment necessary for the successful restoration of tissue and its function. Scaffold for bone regeneration should be biocompatible, bioresorbable, biodegradable, should not create immunogenic responses, should have interconnected pores, it should support angiogenesis along with osteogenesis, should have strength similar to the original tissue along with the characteristics discussed in physical cues, so that it is anti-microbial.

Bone is a complex matrix and vascularized connective tissue that aids in locomotion. The chemical composition of bone can be classified into mineral phase and organic phase. The mineral phase of bone consists of hydroxyapatite and other trace levels of impurities like carbonates, sodium, magnesium, chlorine, fluorine, potassium, strontium, silicon, zinc, chromium, cobalt, and manganese. The organic phase consists of collagen I and non-collagenous proteins like osteocalcin, osteopontin, and alkaline phosphatase. Even though collagen I is present in abundance there are other types III, V and VI are present too (Neto and Ferreria, 2018).

Fabricating ideal scaffold deals with providing adequate, favorable biophysical, chemical and mechanical properties to manipulate complex interplay of various factors in the system towards the regeneration process. Physical and chemical properties play an important role in determining stem cell fate. Physical property that induce stem cells to differentiate towards osteogenic lineage and increase the activity of osteoblast that can be incorporated while designing scaffold are discussed here.

### Roughness and Topography

Roughness and topography has a greater influence on directing stem cell towards osteogenic lineage and modulate immune cell



response. They are directly linked to the number of focal adhesion point which increases stem cell's stiffness and thereby increasing osteogenic expression (Yang et al., 2020). Micro/nanotopography that supports higher spreading area and giving rise to polygon like cell morphology supports osteogenic lineage commitment of stem cells. Similarly, a study on honeycomb like microstructure promoted osteogenic differentiation of hADSC (Zhang et al., 2020a; Zhang et al., 2020b). Additionally, nanotopographies regulate immunological response. Macrophages grown on the surface with grooves showed cytoskeletal structure reorganization and change in phenotype from M1 to M2 (Luu et al., 2015). Surfaces like nanotubes and nanofibers have also shown reduced inflammatory response by switching the phenotype of macrophages from M1 to M2 (Chen et al., 2017). Topography induced change in stem cell's stiffness showed biphasic behavior with respect to the degree of osteogenesis. hMSC cultured on the topography that supported osteogenic differentiation presented with higher focal adhesion area, higher cell stiffness triggered YAP translocation to the nucleus (Yang et al., 2020).

### Pore Size and Porosity

Pore size, porosity and pore interconnection are one of the essential factors involved in scaffold construction. They are necessary for cellular infiltration, nutrient exchange, cellular waste removal and gas transport. Pore structure and size affects osteogenesis and inflammation suppression (Zhu et al., 2020). Very small pores about 100  $\mu\text{m}$  supports cell adhesion and proliferation. Pores above 325  $\mu\text{m}$  supports cell migration. Pores in the range of 290–310  $\mu\text{m}$  exhibit faster bone formation (Dave and Gomes, 2019). Therefore, scaffold having hierarchial pore structure will cell adhesion, proliferation, differentiation and migration of stem cells.

### Degradation

The term degradation in our context can be defined as gradual breakdown of a material mediated by specific biological activity by host. Similarly, the term resorption can be defined as elimination or completely absorption of degraded scaffold (Liu et al., 2017). Tuning scaffold degradation with bone remodeling is one important aspect in designing biodegradable and resorbable scaffold. Scaffolds undergo degradation when they are exposed to body fluids and the scaffolds properties changes as a result of chemical, physical, mechanical and biological interaction between scaffold and the surrounding environment. Degradation can be classified into oxidation and hydrolysis. These reactions may be catalyzed by acids, bases, salts or enzymes (Azevedo et al., 2005). Degradable scaffolds increase the pore size of scaffold with time for oxygen, water and cellular permeation (Dave and Gomes, 2019). Degradation also provides dynamic surface topography and roughness and regulates cell response.

### Surface Charge and Wettability

When the scaffold is placed in the site of interest for bone regeneration, proteins get adsorbed to the surface from the blood within seconds. The surface on which the protein is adsorbed gives appropriate signals to recruit cells and perform a healing cascade. Adhesion of monocyte promotes local inflammation. Hydrophobic materials enhance whereas hydrophilic or neutral surface decreases monocyte adhesion (Jones et al., 2007; Hezi-Yamit et al., 2009; Chen et al., 2016a; Chen et al., 2016b). When it comes to surface charge, rat bone marrow mesenchymal stem cells cultured in basal medium on  $\text{LiNO}_3$ , showed higher cell spreading and directed its fate towards osteogenic lineage on a positively charged substrate compared to a negative and neutral substrate (Li et al., 2015). When osteoblast cell line MC3T3-E1 cultured on charged poly(ethylene glycol)-diacrylate hydrogel, proliferation and differentiation increased with increased for both positive and negative charge with higher surface charge density (Tan et al., 2018). This was because the mammalian cell membrane is neutrally charged and contains zwitterionic phospholipids, lipopolysaccharides, and lipoteichoic acid in its structure (Glukhov et al., 2005). Regarding apatite formation, initially, the usage of a negatively charged surface was preferred over neutral and positively charged surface when the study was conducted on polarized hydroxyapatite surface. The main reason being negative charge can attract  $\text{Ca}^{2+}$  ions and other proteins responsible for apatite formation and cell growth (Ohgaki et al., 2001). When a similar study was conducted on charged titanium surfaces, positively charged surface allowed apatite formation like their negative counterpart (Pattanayak et al., 2012) and bone has grown on the surface of positively charged titanium (Kokubo et al., 2010). But on the surface with no charge thin calcium phosphate layer did form but not as effective as charged surface (Pattanayak et al., 2012). Therefore, hydrophilic surface with high charge density supports cell adhesion and mineralization.

## KEY QUESTION: HOW TO MODULATE THE DESIGN PARAMETERS OF A DEGRADABLE SCAFFOLD THAT HAS A PREFERENCE FOR BONE GROWTH OVER MICROBIAL ADHESION?

Based on the discussion from “Scaffold Design Parameters That Oppose Microbial Adhesion and its Maturation and Scaffold Design Parameters That Support Orthopedic Tissue Engineering” the following conclusion on the desired properties of a biomaterial suitable to support osteoblast adhesion along with antibacterial property, has been listed below.

- (1) The roughness of the scaffold should be in the range of 0.2–2  $\mu\text{m}$  (Hasan et al., 2017).
- (2) Hydrophilic scaffold is preferable as it offers passive resistance towards microbes and better mammalian cell adhesion (Jones et al., 2007; Nejadnik et al., 2008; Hezi-Yamit et al., 2009; Chen et al., 2016a; Chen et al., 2016b).
- (3) Charges on the surface are preferred. The positive charge will give active bactericidal activity, negative charge will offer passive electrostatic repulsion. If the surface is zwitterionic (has both the charges but resulting in net zero charge), they produce a hydration layer that prevents bacterial adhesion by creating a physical and energetic layer (Vallet-Regi et al., 2020) and better osteoblast adhesion.

Generally, biodegradable and bioresorbable scaffold materials for bone tissue engineering scaffold can be classified into ceramics and polymers based on the base mater. To make scaffold with anti-microbial property, the following approaches can be followed:

- (1) To use a degradable and resorbable base material of scaffold that possess anti-microbial property,
- (2) By modifying the surface with a suitable charge/functional group that aides bone formation and repels microbes,
- (3) By incorporating suitable nanofiller that enhances osteogenesis and/or bactericidal or adding filler that possesses all the desired property by surface or chemical modification of filler with ions or molecules and
- (4) By blending or grafting scaffold with anti-microbial adhesive polymers or
- (5) By using the combination of the above-mentioned strategies.

The following sections discuss about nanofillers, biomolecules, plant based extracts and biodegradable polymers that has both antimicrobial and osteogenic property.

### Scaffold With Metal Based Substitutions/ Fillers

Other than the ionic and chemical makeup of bone, bone remodeling is a complex interplay of growth factors, signaling molecules, and cells. Growth factors included in the scaffold activates cascades of active bone repair, but they are associated

with disadvantages like immunogenicity, instability, and high cost. To counteract the disadvantages, metallic ion or its oxide incorporation with or without incorporation of other ions and small molecules are investigated to make scaffold osteoinductive along with anti-microbial properties. Even though new anti-microbial drugs are researched, microbes can eventually develop resistance towards it. The optimal approach is to design scaffold that releases ions whose dosage and release kinetics support osteogenesis and eliminate microbes. Some of the ions are discussed in the section below.

### Ceramic Scaffolds

Hydroxyapatite comprises 65% of bone mass comprises. They provide bone with high strength and toughness. The presence of ionic substituents in biological hydroxyapatites and their nano size makes them highly reactive and has the potential in maintaining ionic equilibrium in the body fluids (Ginebra et al., 2018). While designing ceramics like calcium phosphates, calcium silicates, and hydroxyapatites they are usually doped with ions that are responsible for increased osteoblast activity, angiogenesis, and anti-bacterial activity. Some of them are discussed in the table. Hydroxyapatites though osteoconductive they are not osteoinductive. Therefore the ratio of calcium to phosphate is modified accordingly to get better solubility. If the ratio is less than 1.5, the calcium phosphate has a higher solubility than hydroxyapatite. This was because, the presence of more phosphate ion makes the surrounding acidic, which eventually dissolves the ceramic e.g. the solubility of monocalcium phosphate monohydrate is highest with 18 g/L in water at 25 °C (Ginebra et al., 2018). Initially, the usage of hydroxyapatite in the scaffold was because of their presence in bone. But as research progressed, it was observed that this ceramic can house various element in their interstices and their ions can be substituted to give rise to a range of modified ceramic that can not only enhance the attachment, proliferation, and differentiation of osteoblast like cells but can also induce angiogenesis along with anti-microbial property. Other than the conventional substitutions like zinc, copper, silver, a new class of elements like molybdenum, gallium, selenium, bismuth, tellurium, samarium and cerium also found to possess anti-microbial property against both Gram-negative and positive bacteria and certain fungal strains too (The detailed explanation of anti-microbial activity will be discussed in the following section). Increasing metal ions attributed to microbial death, increased the anti-microbial activity but at the cost of mammalian cell cytotoxicity. Cytotoxicity of metal ion substitution depends on their extent to metal ion substitution, dosage and their release kinetics. The effect of different ionic substituents on the biological functions of ceramics towards osteoblasts and microbes is given in **Supplementary Table S1**.

### Scaffolds With Metal and/or Metal Oxides Nanoparticles

As microbes grow resistant against antibiotics, the demand for alternative antimicrobial therapy that can replace the limited

antibiotic options increased. Over the years it has been established that metal and metal oxide nanoparticles possess antimicrobial activity by the following mechanisms: 1) damaging the outer membrane by increasing permeability, disrupting electron transport chain leading to cellular content's leakage, 2) producing ROS to inactivate respiratory chain activity, and 3) DNA damage, thus disturbing gene replication of bacteria (Nostro et al., 2012; Pasquet et al., 2014; Thukkaram et al., 2014; Sirelkhatim et al., 2015; Li et al., 2016a; Li et al., 2016b; Mohan and Mala, 2019; Li et al., 2020a; Li et al., 2020b; Li et al., 2020c; Li et al., 2020d; Sánchez-López et al., 2020). They have diverse application in dentistry. For example, silver nanoparticles used in dental resin has good antibacterial activity against *S. mutans* and *S. sobrinus* (Foong et al., 2020).

Along with antimicrobial activity, nanoparticles serve multiple functions. They can be used to deliver bioactive agents, increase the mechanical strength of scaffold and with their controlled release from scaffold, nanoparticle can enhance biocompatibility, cell adhesion and proliferation.

Nanoparticles like, silver and silver oxide, copper and copper oxide, gold, zinc and zinc oxide, aluminum and alumina, magnesium and magnesium oxide, Titanium and titania, zirconium and zirconia and iron oxide possess both osteogenic and antimicrobial property. Among them certain metals like zinc and magnesium are responsible for increased synthesis of collagen, ALP, osteocalcin and matrix mineralization (O'Neill et al., 2018). Zinc is present in bone up to 39 ppm and up to  $10^{-4}$  M in blood. Zinc is essential for bone metabolism, the paucity of zinc in the diet leads to abnormalities in bone growth, reduced bone mineral density, and might lead to osteoporosis in the later stage (Yamaguchi, 1988; Yamaguchi, 2010; O'Neill et al., 2018). The activity of zinc on osteoblast is dose-dependent. Zinc positively influences osteoblast in the narrow concentration of 1–50  $\mu$ M, above this concentration they do not inhibit osteogenic activity but they become cytotoxic at a very high concentration of 600–900  $\mu$ M (O'Connor et al., 2020). Magnesium is present at about 0.6–0.72 wt% in bones. Dietary intake of magnesium increases bone mineral density and tougher bones. This was because the incorporation of magnesium in hydroxyapatites made the crystal size smaller but stronger (Orchard et al., 2014). Low magnesium reduces the function of osteoblast while increasing the number of osteoclast. It also creates resistance towards parathyroid hormone and 1,25(OH)<sub>2</sub>-vitamin D. Magnesium deficiency reduces antioxidant defenses, making bone brittle, fragile, impairs mechanical property leading to hypomagnesemia. But high magnesium content inhibits hydroxyapatite content by competing with calcium contributing to osteomalacic renal osteodystrophy (Castiglioni et al., 2013).

Metal and metal oxide nanoparticles at higher concentrations possess toxicity. Because of their small size, shape, charge and surface functional group these nanoparticles can diffuse across the cell or nuclear membrane, can be engulfed, disturb mitochondria and interfere in nucleus function thereby causing cytotoxicity and genotoxicity (Baptista et al., 2018; Lee et al., 2019; Wang et al., 2020). They do not undergo

biodegradation, hence, their retention time is longer (e.g. some nanoparticle like copper do not get degraded by the body. They get accumulated in body causing Wilson's disease) (Varier et al., 2019).

These nanoparticle's antimicrobial and osteogenic activity can further be enhanced or the cytotoxicity of the nanoparticle can be reduced by doping it with other metal ions (Rao et al., 2013; Baptista et al., 2018; Saxena et al., 2018; Saxena and Pandey, 2020) that are present in bone and grafting biomolecules/polymer on its surface (Hasan et al., 2018). The extent of cytotoxicity can also be reduced by tuning the concentration, release kinetics of nanoparticle from the scaffold and their degradation. The effect of commonly used metal and metal oxide nanoparticles on scaffold towards osteoblasts and microbes function is given in **Supplementary Table S2**.

## Scaffolds With Carbon Based Fillers

Carbon based nanomaterials has a versatile nature and has profound use in biomedical application. Their high surface to volume ratio, smaller size, modifiable surface charge, functional groups, wettability, their stability, and strength makes them suitable for bio sensors, drug delivery, tissue engineering and exhibit good anti-microbial properties. The following sections discuss about the anti-microbial and osteogenic potential of allotropes of carbon namely 1) carbon dot, 2) nanodiamond, 3) carbon nanotube, and 4) graphene.

### Carbon Dot (CD)

Carbon dots are zero-dimensional material whose lateral size is below 10 nm. CDs have carbon core and functional groups on the surface by surface passivation. Carbon dots can be easily and economically produced from any carbon source, either by breaking down large carbon molecules and polymerizing (top-down approach) or by carbonizing small molecules (bottom-up approach). The surface functional group can be introduced by using a carbon source that has the desired functional group and/or by modifying the CD through hydrogen bond, covalent bonds and, electrostatic interaction. The common reactive functional groups are hydroxyl, carbonyl, and amine (Du et al., 2014; Liu et al., 2016).

*In vitro* CDs exhibited mild cytotoxicity above 80  $\mu$ g/ml for preosteoblast and supported osteogenesis of preosteoblasts and faster bone formation with blood vessel formation *in vivo* through PERK-eLF2 $\alpha$ -ATF4 pathway (Jin et al., 2020). Similarly, CDs exhibited mild cytotoxicity above 50  $\mu$ g/ml for rBMSC and supported osteogenesis using ROS-mediated MAPK pathway (Shao et al., 2017). CDs also exhibit anti-microbial activity electrostatically interacting with the cell wall (Verma et al., 2019; Li et al., 2020a; Li et al., 2020b; Li et al., 2020c; Li et al., 2020d) and ruptures them, generates ROS (Verma et al., 2019) and also helps in eliminating mature biofilm. Their performance is on par with vancomycin (Wang et al., 2019a; Wang et al., 2019b; Wang et al., 2019c; Wang et al., 2019d). CDs antimicrobial activity was improved by coating with polymers like methyl polyethylene glycol/polyethyleneimine (Liu et al., 2020a; Liu et al., 2020b).



## Nanodiamond (ND)

Nanodiamonds have truncated octahedral architecture whose size is in the range of 2–8 nm. They are inert, biocompatible, and can be functionalized, which makes them an ideal candidate to be used in delivering therapeutics and bone tissue engineering. NDs have shown good biocompatibility, osteogenic potential (Ibrahim et al., 2018) and anti-microbial property. Details of NDs osteogenic potential in various scaffolds is listed in **Supplementary Table S3**. NDs supports osteoblast adhesion over *S. aureus* (Rifai et al., 2019). NDs with negative or positive charge has shown anti-bacterial against *E. coli* and *B. subtilis*. This was because negative charged NDs penetrated bacterial cell, agglomerated and generated ROS because of the presence of high oxygen functional groups. Whereas positive surface charged NDs, attached to the surface of bacteria due to electrostatic force and caused little cell wall deformation making them less bactericidal (Wehling et al., 2014). Similarly, hydroxyl functionalized ND and its modification with menthol (Turcheniuk et al., 2015) and ND grafted with hydrophilic copolymer also showed anti-bacterial against *E. coli* and *B. subtilis* (Cao et al., 2018).

## Carbon Nanotube (CNT)

Carbon nanotubes are one-dimensional allotrope, whose diameter ranges from 10 to 200 nm. CNT can be single-walled or multi-walled. The ability to surface functionalize, excellent tensile property, and mimicking nanostructure makes them suitable for bone tissue engineering. Even though unmodified CNTs are relatively toxic, surface functionalizing and grafting molecules like HA (Song et al., 2019) or a polymer like gelatin (Yoon et al., 2014) that covers the surface to reduce its toxicity. In terms of anti-microbial property, surface functionalizing the MWCNT with -COOH by acid oxidation can delay the growth of *V. parahaemolyticus* but not completely

deactivate it (Ding et al., 2020). But ethanol amine grafted MWCNT causes significant microbial toxicity. The order of toxicity was triethanolamine > diethanolamine > monoethanolamine along with it the triethanolamine grafted MWCNT caused higher toxicity with lower minimum inhibitory concentration (MIC) (Zardini et al., 2014). To further enhance the anti-microbial property, CNTs can be decorated with metal (Jatoi et al., 2020) or metal oxides nanoparticles or both (Mohammed et al., 2019). Similarly, anti-bacterial activity can be enhanced by grafting amino acids like arginine and lysine (Zardini et al., 2012).

## Graphene

Graphene is a two-dimensional allotrope of carbon that has honeycomb lattice with an atomic bond length of 0.142 nm. Apart from possessing good mechanical, electrical, and thermal property, their high surface area and their effective surface modifications can be used as a drug carrier and also in various tissue engineering fields. Graphene exhibits high anti-bacterial property by damaging the surface of bacteria through its sharp edges, inducing oxidative stress within the bacteria, and stabilizing surface through electrostatic interaction due to wrapping. This property was further enhanced by coating nanoparticles on the surface of graphene (Mohammed et al., 2020).

Several scaffolds prepared using graphene as filler has shown that the presence of graphene has supported and improved the commitment of stem cells towards osteogenic lineage *in vitro* (**Table 3**). Presence of graphene activated RhoA/Rho-associated protein kinase that increased the formation of F-actin stress fiber and also for Smad signaling activation for osteogenesis (Xie et al., 2019). Graphene oxide, a derivative of graphene showed toxicity towards MSC above 1 µg/ml concentration by generating high intracellular ROS. At low concentration of 0.1 µg/ml graphene

**TABLE 3** | Effect of phytochemicals on scaffold towards osteoblasts and microbes.

S. no	Plant extract or phytochemical	Polymer(s)/ceramic	Encapsulation strategy	Effect on biological function	References
1	<i>C. quadrangularis</i> extract	Chitosan, collagen and HA	PCL, PVA nanoparticles	Slow release of extract. Proliferation of MC3T3-E1 is not affected	Thongtham et al. (2020)
2		PLA	—	↑P, ↑ALP of rMSCs	Parvathy et al. (2018)
3		Chitosan and sodium carboxymethyl cellulose	—	Early ALP activity and ↑M of SaOS-2	Tamburaci et al. (2018)
4		O-carboxy methyl chitosan and alginate	—	↑P, ↑ALP and ↑M of hMSCs	Soumya et al. (2012)
5	<i>M. tenuiflora</i> extract	Chitosan	—	↑P and ↑ALP of primary calvaria osteoblast	Martel-Estrada et al. (2015)
6	<i>C. quadrangularis</i> and <i>B. monosperma</i> extract	Sulfonated poly aryl ether ketone	—	↑ALP and ↑M of SaOS-2 in the order of <i>B. monosperma</i> coated > <i>C. quadrangularis</i> coated than uncoated Sulfonated poly aryl ether ketone	Raghavan et al. (2013)
7	Veratic acid	PCL, PVP	Chitosan nanoparticle	↑M of mMSCs at 200 µM	Sruthi et al. (2020)
8	Mucic acid	PLA, gelatin	—	↑ALP, ↑M of mMSC at 10 µM	Ashwin et al. (2020)
9	Taurine	PLA, PCL	Gelatin nanofiber	Supports bone cell proliferation <i>in vitro</i> and bone regeneration <i>in vivo</i>	Samadian et al. (2020)
10	Sinapic acid	PCL	Chitosan nanoparticle	↑ALP, ↑Col-I, ↑osteocalcin of mMSCs. Possible activation of TGF-β1/BMP/Smads/Runx2 signalling pathway at 50 µM	Balagangadharan et al. (2019)
11	Silibinin	Alginate, Gelatin	Chitosan nanoparticle	↑M of mMSC at 50 µM	Leena et al. (2017)

oxide enhanced osteogenesis through NJK and FoxO1 signaling pathway activation (Halim et al., 2019).

Eventhough the carbon based nanofillers prove to posses all the physical cues essential for osteogenic and anti-microbial activity, their major limitation lies in ease of manufacture, their nondegradability property therby carbon nanofillers get accumulated and generate excesses ROS *in vivo*. The effect of commonly used metal and metal oxide nanoparticles on scaffold towards osteoblasts and microbes function is given in **Supplementary Table S3**.

## Scaffolds With Vitamins

Ionic balance is essential for active bone remodeling. Also, Vitamins do help in active absorption of ions for bone. Vitamins like A, B complex, C, D, E, and K are essential for maintaining bone in optimal health (Ahmadiesh and Arabi et al., 2011). Among them, some possess anti-microbial property as well.

Vitamin D is a lipophilic protein responsible for bone formation, resorption, and regeneration. This vitamin helps in the absorption of ions like calcium and phosphorous essential for bone metabolism, absence of which causes osteoporosis in old age. Higher levels can cause calcification along with heart, blood vessels, and kidney damage. Vitamin D gets activated to calcidiol or calcitriol by UV irradiation of cholesterol in the skin. This vitamin losses it functionality upon exposure to light heat and oxidation. Vitamin D promotes anti-microbial activity by stimulating anti-microbial peptide production of LL-7 and  $\beta$ -defensin and also induces nitric oxide production (Herscovitch et al., 2014; Fayyazbaksh et al., 2017; Sattary et al., 2019).

Vitamin E is an anti-oxidant that express anti-inflammatory property. They are usually added into the polymer to render it biocompatible. Initially, it was assumed that vitamin E possess anti-bacterial activity. Campoccia et al. (2015), observed that the addition of vitamin E or vitamin E acetate reduced the adhesion of *S. aureus* and *S. epidermidis*. Similarly, when vitamin E acetate and vitamin E phosphate, vitamin E acetate have better anti-adhesive property towards *S. epidermis* but vitamin E phosphate has better anti-adhesive property towards *S. epidermis* than *S. aureus*. Vitamin E phosphate has excellent prevention of biofilm towards *S. epidermis* than *S. aureus*, than vitamin E acetate. Vitamin E acetate and vitamin E phosphate does not have anti-adhesive property and does not prevent the biofilm formation of *P. aeruginosa* (Bidossi et al., 2017). When vitamin E phosphate is coated on titanium implant, they stimulated bone deposition even in presence of high bacterial load of  $3 \times 10^4$  CFU of *S. aureus* in the rat model (Lovati et al., 2018).

Vitamin B2 or otherwise called riboflavin is a water-soluble vitamin that usually gets absorbed in the small intestine into blood. Riboflavin supplemented diet can prevent pathogenic infection. Experiments suggested that riboflavin has shown a higher zone of inhibition towards both the Gram strain of bacteria along with *C. albicans* (Ahgilan et al., 2016). Riboflavin-low density polyethylene composite at 5% percent loading showed 99.44% reduction of *E. coli* and 94.31% reduction of *S. aureus* (Orusuwan et al., 2019).

Vitamin C is a water-soluble anti-oxidant, that aides in collagen synthesis necessary for bone formation. It also

inhibits osteoclasts and various types of cancers including lung, pancreas, oral, cervical, and esophageal. To know the effect of vitamin C on effective bone formation, ovariectomized rats were subjected to diets with Vitamin C. It was observed that vitamin C has increased the calcium content in bones, bone mineral density and bone volume fraction. Osteoblast related protein expression like bone morphogenic protein-2 (BMP-2), osteocalcin, collagen-I has increased and reduced osteoclast related protein expression (Choi et al., 2019). Vitamin C also exhibited anti-bacterial property against *E. coli* and *S. aureus* (Voss et al., 2018). Some studies show that vitamin C at a concentration of 0.31 mg/ml inhibits *P. aeruginosa* and at 0.15 mg/ml inhibits *S. aureus* growth. Even at near neutral pH, vitamin C has potent anti-microbial activity against streptococci (Mousavi et al., 2019).

Vitamin K is lipophilic and has osteoinductive properties, whose dietary intake increases bone mineral density by producing more osteocalcin and other osteoblast specific genes and reduce osteoclastogenesis. Vitamin K5 has anti-microbial activity. At concentrations of 80, 120, 140, and 220 ppm they are bactericidal towards *S. aureus*, *B. subtilis*, *P. vulgaris*, *P. fluorescens*, and *E. coli* (Merrifield and Yang, 1965). The reason is lipophilic substances permeabilize the cell membrane of bacteria, damaging the membrane integrity, causing cell lysis and death (Andrade et al., 2017).

Vitamins along with antibiotics showed synergic effect against microbes. The presence of vitmins lowered the MIC of antibiotics. One such example is that vitamin B2 with oxytetracycline or linezolid was effective against MRSA (Shahzad et al., 2018). Similarly, vitamin B2 with ciprofloxacin and azithromycin was effective against MRSA by enhancing ROS production but also ensures host cells protection from inflammation damage (Dey and Bishayi, 2016). Even though vitamins had good anti-microbial property, their stability was the drawback. Factors like temperature, moisture, oxygen, light, pH, oxidizing and reducing agent, presence of metallic ions and other compounds, presence of other vitamins and the combinations of above affected their stability or activity (Ottaway, 2010). These factors can be lowered by using an appropriate encapsulation strategy using a polymer, porous nanoparticles or by intercalating between layered nanoparticle.

## Scaffolds With Phytochemicals

Natural compounds obtained from plant based extracts have been used to treat ailments for ages. Some of them possess osteogenic properties and has been documented in Indian and Chinese traditional medicine. For nearly 2 decades, the compounds responsible for osteogenic and anti-microbial activity has been individually identified and the research on this front is incomplete. Some of the phytochemicals or plant based extract that has both osteogenic and anti-microbial activities are discussed in this section.

Shrubs like *Cissus quadrangularis* possess vitamin C, vitamin A, carotene, and anabolic steroidal substances along with ions like calcium, phosphorus, zinc, iron, sodium, cadmium, copper and magnesium essential for their use in bone tissue engineering. They also possess anti-osteoporosis, anti-bacterial, bone fracture

healing activity along with free radical scavenging and anti-oxidant potential. The fresh and dried stem extracts of *C. quadrangularis* showed anti-oxidant activity and it is also observed to have high anti-bacterial activity against Gram-positive bacteria like *B. subtilis*, *B. cereus*, *S. aureus* than Gram-negative bacteria like *P. aeruginosa* and *E. coli* but they exhibited significantly higher resistance towards plant extracts (Chidambara Murthy et al., 2003). When patients with maxillofacial fracture were supplemented with 500 mg of *C. quadrangularis* thrice a day for 6 weeks, showed less tissue reaction, early callus formation and complete new bone formation in 7 weeks (Brahmkshatriya et al., 2015). *C. quadrangularis* diet inhibits bone loss in ovariectomized mice (Banu et al., 2012).

Medicarpin, cajanin, formonentin, isoformonentin, cladrin present in ethanol extract from the bark of *Butea monosperma* found to possess osteogenic activity (Maurya et al., 2009). Medicarpin treatment generated new bone in ovariectomized and osteopenic mice models by activating Wnt/canonical and notch signaling pathway (Dixit et al., 2015). They have osteoblastogenic, anti-osteoclastogenic activity, and increases bone volume fraction (Tyagi et al., 2010). Also, prevents bone loss in ovariectomized and collagen-induced arthritis models (Mansoori et al., 2020). Medicarpin also possesses anti-fungal activity towards *Cladosporium cladosporioides* (Bandara et al., 1989). They usually get distributed in the liver, bone marrow, small intestine, lungs, and kidney and gets excreted from the body via urine (Taneja et al., 2020).

*Dalbergia sissoo* also known as Indian Rosewood, whose leaves extract was rich in phytoestrogens, which exhibits bone forming property. Phytoestrogens are naturally occurring, chemically similar to 17  $\beta$ -estradiol, which are metabolized and can be eliminated from the body. Phytoestrogens possess osteoprotective and fracture healing properties (Khedgikar et al., 2017). It restores bone mineral density, bone volume fraction, and mineralization by enhancing osteoblast and reducing osteoclast activity in ovariectomized rats (Karvande et al., 2017). Ethanol extract from bark showed higher anti-bacterial activity towards *S. aureus*, *M. luteus* and *E. aerogenes* than leaves. Both bark and leaf extract showed little anti-fungal activity against *A. niger*, *A. alternate*, and *S. commune* (Majeed et al., 2019). *Dalbergia sissoo* also possesses polyphenolic compounds that belong to a class of neoflavonoids, exhibited anti-oxidant and anti-inflammatory properties. Dalbergin is a neoflavonoid enhanced ALP activity, mineralization, enhanced bone volume fraction, osteoblast, and reduced osteoclast expression in ovariectomized rats (Choudhary et al., 2016). *Dalbergia sissoo* based magnesium oxide particle showed good anti-bacterial activity against *E. coli* and *R. solanacearum* (Khan et al., 2020).

Ethanol extracts from the flower of *Pterospermum acerifolium* contains 17 compounds. Among them, pterospermin A (4'-(2-methoxy-4-(1,2,3-trihydroxypropyl))phenoxy luteolin), pterospermin C (3,5-dihydroxyfuran-2(5H)-one) and trans-triliroside showed higher ALP activity, mineralization and osteoblastic gene expression in the concentration range of 10–100  $\mu$ M (Dixit et al., 2011).

Similarly, seed coats of *Pterospermum acerifolium* contains two phytoceramides (A and B), two acylated phytosterol (A and B), and five other compounds. Among them, the two phytoceramides at 1 nM concentration, increased ALP activity, mineralization, and osteoblastic gene expression. When neonatal pups of 1–2 days old were treated with phytoceramides A and B at 10 mg/kg showed higher expressions of osteoblast (Dixit et al., 2012). Extracts from the bark of *Pterospermum acerifolium* showed promising antibacterial activity against *B. licheniformis*, *S. aureus*, *P. florescence* and *S. typhimurium* (Panda and Dutta, 2011).

Extract from fruits of *Cupressus sempervirens* contains sugiol, trans-communic acid, 15-acetoxy imbricatolic acid and imbricatolic acid had effects on calvarial derived osteoblast cells. Among them, sugiol has good mineralization potential and expressed high osteogenic markers like BMP-2. On ovariectomized rat, dietary intake of 1 mg/kg of rat exhibited osteoprotective property and also improved biomechanical properties of the bone (Khan et al., 2014). The methanol extract of *Cupressus sempervirens* showed anti-bacterial activity against *E. faecalis*, *S. aureus*, *K. pneumonia*, and *P. aeruginosa* (Selim et al., 2014).

Ethanol extract of *Peperomia pellucida* is found to have fracture healing property. When rats with drill-hole femur injury surgery were orally administered with 200 mg/kg showed higher calcein content and bone volume fraction. *In vitro* study with BMSC also confirms high mineralization and osteoblastic gene expression (Ngueguim et al., 2013). Mineral content in aqueous extract contains potassium, phosphorous, magnesium, calcium, and sodium. Administration of aqueous extract increased calcium level and ALP in serum and bone, concluding that it has dose-dependent fracture healing property (Florence et al., 2017). Essential oil and extract from leaves, stem, flowers, and even the whole plant has anti-bacterial and anti-fungal properties (Alves et al., 2019).

*Ulmus wallichiana* also known as Himalayan elm is used to heal bone fractures in Indian traditional medicine. The bark of *Ulmus wallichiana* contains a flavanol called (2S,3S)-Aromadendrin-6-C- $\beta$ -D-glucopyranoside that stimulates osteoblast expression, maturation and also during estrogen deficiency. It also inhibits bone marrow cells towards osteoclast differentiation giving them an osteoprotective property. It also inhibits adipogenic differentiation and induces apoptosis and does not evoke undesirable effects in the uterus (Swarnkar et al., 2011). Similarly, compounds like quercetin-6-C- $\beta$ -D-glucopyranoside, ulmoside A, and B were also found to have an osteogenic property (Rawat et al., 2009). Also, naringenin-6-C-glucoside increased ALP activity and osteogenic gene expression and maintains bone volume fraction in ovariectomized mice (Swarnkar et al., 2012). Ethyl acetate extract from the bark of *Ulmus wallichiana* has a better anti-oxidant property and showed greater anti-bacterial activity against *E. coli*, *B. subtilis*, *S. aureus*, *P. aeruginosa* and anti-fungal activity against *A. fumigates* and *A. flavus* than chloroform extract (Bora et al., 2018).

*Tinospora cordifolia* in ayurvedic medicine is considered as “nectar of immortality” because of various property it possesses.

Ethanol extract of *Tinospora cordifolia* increased ALP activity, mineralization in both MG3 and osteoblasts, and also acts as an osteoprotective agent (Abiramasundari et al., 2012). They possess anti-oxidant activity (Saha and Ghosh, 2012) and also different extracts of this species possess anti-microbial activity against *S. epidermidis*, *E. faecalis*, *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *E. coli*, *P. mirabilis*, *S. marcescens*, *C. freundii*, *C. koseri* and *E. cloacae* (Narayanan et al., 2011).

*Achyranthes bidentata* is used in one of the herb formula in Chinese medicine for bone and mineral metabolism. Butanolic extract from the root (He et al., 2010), alkaline crude polysaccharide ABPRB (Zhang et al., 2018a; Zhang et al., 2018b), AB70 water soluble polysaccharide (Zhang et al., 2019) in ovariectomized rat, fructan ABW50-1 (Yan et al., 2019) and polysaccharide ABPB-2 (Zhang et al., 2018a; Zhang et al., 2018b) in zebrafish model of glucocorticoid induced osteoporosis, induced osteoprotective effect. Oral dosage of 500 mg/kg/day for 16 week in ovariectomized- osteoporosis induced rats maintained bone volume fraction and bone mineral density (Zhang et al., 2012). Fructan ABW70-1 increased proliferation, ALP activity, mineralization and osteogenic gene expression in MC3T3-E1 (Zhang et al., 2019).

Scutellarian 7-O- $\beta$ -D-glucopyranoside present in butanol fraction of *Kigelia pinnata* between the concentration range of 10 nM and 1  $\mu$ M showed higher ALP activity and mineralization in calvarial osteoblast (Ramakrishna et al., 2017). The methanol extract of leaves and bark extract of *Kigelia pinnata* exhibited good anti-bacterial activity against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* and anti-fungal activity against *C. albicans* (Agyare et al., 2013).

*Withania somnifera* also known as Ashwagandha in Ayurveda has multi-therapeutic purposes. The powder of root contains phytosterols, polyphenols, flavonoids, and vitamin C that have anti-arthritis properties (Rasool and Varalakshmi, 2007). Ethanol root extract possesses anti-osteoporotic activity (Nagareddy and Lakshmana, 2006). Withaferin A present in leaves of *Withania somnifera* is known to reduce osteoclastogenic expression and promotes bone regeneration in ovariectomized mice (Khedgikar et al., 2013; Khedgikar et al., 2015). Water and chloroform extract from the unripened and ripened fruit of *Withania somnifera* possess anti-bacterial activity against *P. aeruginosa* (Singariya et al., 2012) and methanolic leaf extract inhibits MRSA (Bisht and Rawat, 2014).

Curcumin, a phenolic compound obtained from *Curcum longa* is known for its bone healing properties according to Indian traditional medicine. They also exhibit anti-oxidant, anti-inflammatory (Zambrano et al., 2018), anti-tumorigenic and anti-microbial properties. The synergistic effect of curcumin along with antibiotics like oxacillin, ampicillin, ciprofloxacin, norfloxacin and erythromycin reduced the MIC of antibiotics against MRSA (Zhou et al., 2017).

Epigallocatechin-3-gallate, a polyphenolic compounds from dried tea leaves has anti-tumor, anti-oxidation and anti-microbial properties. This polyphenolic compound have been studied *in vitro* towards dental adhesive, barrier membrane, bone substitute material, anti-caries applications. They inhibit bacterial colonization, improves cell viability, compatibility, osteogenic activity, and reduces ROS production (Liao et al., 2020).

The listed phytochemicals in this sections are not the only phytochemicals that has both osteogenic (Suvarna et al., 2018) and anti-microbial property. The research in the usage of phytochemicals are still *in vitro*. The *in vivo* effect of phytochemicals and the mechanism that makes them osteogenic, the maximum dosage and their release kinetics is still needed to be evaluated. Along with this, the overall cost of phytochemicals, the shelf life of phytochemicals based scaffold should be further evaluated.

## Polymer Scaffolds With Anti-Microbial Peptide

To tackle narrow spectrum of antimicrobial agent and microbial resistance, new class of molecules called anti-microbial peptide (AMP), derived from immune responses of multicellular organisms is gaining attention. Many recent studies have been focused on incorporating AMPs into scaffold for orthopedic application to make them anti-microbial. AMPs are cationic amphiphilic molecule with 10–50 amino acid residues on their structure. AMPs disrupt microbial membrane by inserting their hydrophobic residue through the lipid bilayer and some AMPs inhibit nucleic acid, protein or cell wall synthesis. The limitations of AMPs are their *in vivo* stability, susceptible to inactivation and undesired cytotoxicity. These limitations can be overcome by using nanoparticles as the drug delivery system for AMPs (Makowski et al., 2019), by grafting or immobilizing AMP on the surface or by attaching non-natural polyglycine (Hasan et al., 2020a; Hasan et al., 2020b; Castelletto et al., 2020) to offer protease resistance and enhanced lipid membrane permeability. Many studies regarding surface grafting on titanium surface is reported in the literature. When AMP of polyphemusin I was grafted with diphosphoserine, they inhibited dental plaque biofilm caused by *S. mutans* because the modifications caused tooth binding ability without affecting the cytocompatibility (Zhang et al., 2019). Similarly, when cationic peptide LL37 binded with HA to make PCL/HA composite, LL37's cytotoxicity was circumvented along with retaining bactericidal activity against *S. mutans* (Fateme et al., 2019). Also, LL37 was found to promote odonto/osteogenic differentiation of apical papilla stem cells (Cheng et al., 2020). When AMP of ponericin G1 and BMP-2 is encapsulated by polydopamine and coated on PLGA, along with osteogenic differentiation and mineralization of MCT3-E1, the scaffold exhibited anti-bacterial activity against *E. coli* and *S. aureus* (Chen et al., 2019).

## Polymer Scaffolds and Its Chemical Modifications

To design an anti-microbial resorbable scaffold, either the polymer base should possess anti-microbial property or the scaffold should have anti-microbial nanofiller or extracts within or chemical grafting antibiotics on its surface. Surface grafting of antibiotics, anti-microbial polymer or addition of phytochemical extract on the scaffold has limited shelf life. Therefore there is a need for polymers that has either innate anti-microbial property or their chemical modification renders the polymer its permanent anti-

microbial property and also to reduce the need for anti-microbial nanofiller or extracts or antibiotics.

Chitosan is a deacetylated form of chitin usually derived from the exoskeleton of crustaceans has innate anti-microbial property. They get dissolved only at slightly acidic condition and their surface charge becomes positive in acidic medium but not at neutral pH. Amine group, secondary hydroxyl and primary hydroxyl group in C-2, C-3 and C-6 position are nucleophilic functional groups in chitosan. Therefore, various modifications can be done to incorporate permanent positive charge at neutral pH and enhancing solubility in the aqueous medium. Addition of alkyl, aromatic, carboxyalkyl, quaternary ammonium groups, amino acids, and peptides into chitosan are the various forms to increase its anti-microbial potential. Four models are proposed for their anti-bacterial mechanisms, 1) electrostatic interaction between positively charged chitosan derivative with negatively charged peptidoglycan causing them to hydrolyze, 2) change in permeability of outer membrane because of ionic interaction leading to restriction in nutrient transport and osmotic pressure buildup, 3) perforating cell wall and interacting with DNA by preventing its transcription and disturbing protein and mRNA synthesis and 4) higher chelating ability with metal ions and hindering with microbes overall growth (Sahariah and Masson, 2017).

Cellulose being a highly renewable biopolymer does not possess inherent anti-microbial property. Imparting positive charge on the backbone of cellulose can be done through quaternary ammonium moiety, aminoalkyl, or aminosioane, quaternized halamine incorporation. Quaternary ammonium moiety interacts with cell membrane through electrostatic interaction. They bind to cell membrane, compromise their permeability leading to bacterial apoptosis. Aminoalkyl functionalization on cellulose not only imparts polycationic nature but also lipophilic. Its lipophilicity increased with increased chain length which helped the functional groups to penetrate into cell membrane, therefore, further enhancing its anti-bacterial effect. Quaternized halamine incorporation into cellulose releases halogens, thereby, inactivating bacterial cells of their growth (Tavakolian et al., 2020).

Biopolymers like silk has attracted researchers in orthopedics for their mechanical property and good processibility. They are biocompatible and their slow degradation helpful in repairing critical size defects. Silk provides a better surface for cell adhesion and mineralization (Bhattacharjee et al., 2017). Anti-bacterial property of silk has conflicting results. Cocoon components before and after processing do not resist *E. coli* growth. The presence of processing chemicals may inhibit or kill bacteria (Kaur et al., 2014). Silk spun by genetically modified silkworm has inherent antibacterial activity against *E. coli* (Saviane et al., 2018).

PCL is one of the extensively studied polymer in tissue engineering. Even though their performance is excellent *in vivo*, they lack anti-microbial property. This lacuna is usually overcome by adding metal, metal oxides or carbon based filler. A recent study on grafting poly (Z-Lysine) with PCL showed anti-bacterial activity. The positively charged lysine get attracted to the negatively charged cell membrane. PCL being hydrophobic gets inserted into the cell membrane causing them to rupture and leak

the bacteria's intracellular contents. The balance between cationic charge and hydrophilic chain is the key for effective bactericide (Zhou et al., 2019).

Citrate based polymers do possess anti-microbial activity. Citric acid in the polymer lowers pH in the cell, chelates with metal ion and causes damage to proteins, DNA and cell membranes. Poly(octamethylene citrate) and poly(octamethylene maleate anhydride citate) has good anti-bacterial activity against *E. coli* and *S. aureus* (Su et al., 2014). Polycitrate and silica polycitrate also showed anti-bacterial activity against *S. aureus* (Du et al., 2015). When poly(octanediol-citrate) crosslinked with poly- $\epsilon$ -lysine, they showed excellent anti-bacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis* (Li et al., 2018).

Similarly, chemical modifications are made to the naturally available gums that have weak anti-microbial property. Polyanionic polysaccharides like chichi gum from *Sterculia striata* upon acetylation have shown to have higher inhibitory effect against *C. albicans*, *E. coli*, *P. aeruginosa*, *S. typhimurium* and *K. pneumonia* (Braz et al., 2020). Anionic heteropolysaccharide like cashew gum from *Anacardium occidentale*, with increasing quaternization degree showed higher anti-staphylococcal activity (Quelemes et al., 2017). Non-ionic *Guar gum* when crosslinked with polyacrylic acid strongly inhibits *E. coli* and *S. aureus* (Kaith et al., 2015).

## TESTING SCAFFOLDS FOR ANTI-MICROBIAL PROPERTY

Anti-bacterial activity of scaffolds can be analyzed *in vitro* using disc diffusion assay and dynamic contact assay. In disc diffusion assay, the bacterial suspension is evenly spread across the agar plate, followed by placing a sterilized scaffold in the middle and then incubated at 37°C for a particular duration, and then the diameter of inhibition zone is noted. In dynamic contact assay, the scaffold is dipped and incubated in the bacterial suspension. Then the serially diluted bacterial solution was spread on a nutrient agar plate and cultured at 37°C and CFUs can be counted (Bai et al., 2020). Similarly, *in vitro* adhesion studies of bacteria or bacteria and host cells (osteoblasts and/or macrophages) co-culture incubated at 37°C for a specific period, to analyze the bacteria and cell's attachment and proliferative ability on surface through live/dead staining (Mehrjou et al., 2019). To analyse bacteria colonization and biofilm formation on various substrates and antimicrobials efficacy, microtiter plate based model (Nett et al., 2011) can be used to mimic *in vivo* activity. Microtiter plate based model is one of the cheap but labour intensive study that can be used to analyse biofilm mass and their metabolic activity under the influence of antimicrobial agents (Vandecandelaere et al., 2016). To simulate *in vivo* environment, 3D *in vitro* models are created to effectively reproduce the bacteria and host interaction in osteomyelitis (Hofstee et al., 2020).

For chronic osteomyelitis animal model, the Nordon method was employed. Hole is drilled into the medullary cavity of rabbit's right tibia and 0.1 ml of  $3 \times 10^7$  CFU/ml of *S. aureus* suspension in nutrient media is injected and sealed using bone wax for 4 weeks. Then rabbits were treated with debridement and the scaffold is inserted. After

implantation at specific time points, general observations, X-ray imaging, gross pathology, Micro CT evaluation, and histological evaluation can be conducted (Wang et al., 2017).

## CONCLUSION AND FUTURE SCOPE

This review discussed various nanoparticles be it ceramic, metal or carbon based along with polymers and its chemical modification that were used in scaffolds to improve their anti-microbial and osteogenic activity. These nanoparticles are extensively studied over years both *in vitro* and *in vivo*. The extent of substitution of metal ion(s) into the ceramic changed the crystal structure and its solubility. Also, in the case of scaffold with metal and metal oxides and carbon based nanofiller, the release kinetics was determined by the degradation of the polymer, size and concentration of nanoparticle that had a potential impact in the scaffolds anti-microbial and osteogenic property. These nanoparticles, have wide range of applications from bio-imaging, tissue regeneration, drug delivery, and so on. Due to their small size, large surface to volume ratio, surface charge, and reactivity, they produce ROS, act as nanoknives, and their surface charge either repel away microbes or destabilize their cell membrane. But they get distributed to all the organs, present *in vivo* for long term, and mainly produce massive ROS which becomes cytotoxic till they are degraded by macrophages *in vivo* (Yang and Zhang, 2019).

Biomolecules like vitamins, even though they possess dual properties like anti-microbial and osteogenic activity *in vitro*, their *in vivo* release kinetics and efficacy have to be further explored. Similarly, AMPs have good antimicrobial property but their cytotoxicity can be overcome by modulating their release kinetics by using suitable scaffold design. Their drawbacks are their storage conditions, stability, and shelf life are a major concern.

Phytochemical extract is a cocktail of various vitamins, metal ions, and phytochemicals. Their performance *in vivo* shows an excellent osteogenic property. But very few phytochemical extracts were translated into scaffolds. Therefore, there is a need to elucidate the osteogenic potential of different components in the extract. Many traditional Indian plants are used in households of Indian family for their positive effect in bones. But extensive scientific studies do lack in that front. There is a need to gather information about various plants and their phytochemicals from different native traditions and their potential use in bone tissue engineering. Drawbacks are difficulty in extraction, separation, purification, and their large scale production.

Polymers are the base of a degradable scaffold. Except for chitosan, many polymers requires either chemical modification or addition of bioactive fillers to impart anti-microbial property. Currently, it is encouraging to observe the research being focused on developing and modifying already existing polymers to possess anti-microbial properties. But ease of synthesis and reproducibility is one of the concerns in this front. Inspiration from naturally occurring monomers like citric acid, maleic acid and tartaric acid are used in polymer synthesis with anti-microbial property.

While manufacturing and implanting scaffold *in vivo*, it's important to maintain chemical homogeneity of the scaffold and also to map the spatial-temporal interaction between scaffold and various cellular activities by it, on it and around it. Various state of art characterization tools can be employed to study the biological ultrastructure (using volume electron microscopy and super resolution optical microscopy) and biochemical composition of extra cellular matrix (using Raman spectroscopic mapping, fluorescence imaging and mass spectroscopic imaging). Similarly one can also employ micro-computed tomography to track the bone formation in an osteomyelitis model. Also, machine language tools can be incorporated while studying the images of different phases of scaffold interaction with host and bacteria cells and the information between different individuals and be processed to give a predictive model that can be employed to troubleshoot a similar orthopaedic complications in future (Armstrong and Stevens, 2019; Kim et al., 2019).

All the advantages that were discussed above are suitable to minimize microbial adhesion and kill them on contact on the scaffold. But the dead microbes shield the incoming microbes from scaffold's anti-microbial property and provide them a suitable site for biofilm growth (Goodwin et al., 2016). Therefore, the nanofiller incorporated inside scaffold upon degradation, should be able to penetrate inside biofilm and destabilize them [Example ZnO (Mahamuni-Badiger et al., 2020) and Carbon dot (Ran et al., 2019)]. Also, the nanoparticle should be provided with an additional functional group by attaching biomolecules (Turcheniuk et al., 2015) or antibiotics on their surface to enhance their properties against biofilm elimination and reduce microbial mutation against nanoparticle (Shkodenko et al., 2020).

In spite of the presence of various options to eliminate microbes and support osteogenesis *in vitro*, the *in vivo* efficacy for a longer term needs to be evaluated. To reduce the antibiotic toxicity and effective osteomyelitis treatment, a cocktail of iron oxide, CNT and gentamicin was used in bacterial capturing and effective microwave assisted bacterial killing of *E. coli* and MRSA (Qiao et al., 2020). Similarly, studies that involves a cocktail of nanofillers with biomolecules on scaffold have to be designed to not only treat osteomyelitis but also support osteogenesis and their long term *in vivo* efficacy can be viewed as an opportunity that can open newer avenues for an all in one patient specific scaffold that is lacking in the market for the bone graft.

## AUTHOR CONTRIBUTIONS

AP Literature review and manuscript writing. JB Literature review, manuscript writing, and evaluation.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmats.2020.594686/full#supplementary-material>.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## GLOSSARY

↑ Increase

↓ decrease

**A** Adhesion

**ADSC** Adipose derived stem cell

**ALP** Alkaline phosphatase

**AMP** Anti-microbial peptide

**Ang** Angiogenesis

**BMP-2** Bone morphogenic protein-2

**BMSC** Bone marrow derived MSC

**BV** Bone volume

**CD** Carbon dot

**CFU** Colony forming units

**CNT** Carbon nanotube

**Col-I** Collagen I

**HA** Hydroxyapatite

**L132** Human cervix carcinoma cells

**M** Mineralization

**MC3T3-E1** Osteoblast precursor cell line from mouse

**MG63, SaOS-2, U-2 OS** Different osteosarcoma derived cells

**MIC** Minimum inhibition concentration

**MRSA** Methicillin-resistant *S. aureus*

**MSC** Mesenchymal stem cell

**MW** Multiwall

**ND** Nanodiamond

**OB** Osteoblast

**OC** Osteoclast

**OD** Osteogenic differentiation

**P** Proliferation

**PCL** Poly( $\epsilon$ -caprolactone)

**PEEK** Polyetheretherketone

**PEG** Polyethylene glycol

**PGA** Poly glycolic acid

**PLCL** Poly (lactide -co- caprolactone)

**PLLA** Poly(l-lactic acid)

**PLGA** Poly (lactic -co- glycolic acid)

**PMMA** Polymethyl methacrylate

**PU** Polyurethane

**PVA** Polyvinyl alcohol

**PVDF** Polyvinylidene fluoride

**PVP** Polyvinylpyrrolidone

**S** Solubility

**SW** Single wall