Review

Metallic Nanoscaffolds as Osteogenic Promoters: Advances, Challenges and Scope

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Abstract: Bone injuries and fractures are often associated with post-surgical failures, extended healing times, infection, a lack of return to a normal active lifestyle, and corrosion associated allergies. In this regard, this review presents a comprehensive report on advances in nanotechnology driven solutions for bone tissue engineering. The fabrication of metals such as copper, gold, platinum, palladium, silver, strontium, titanium, zinc oxide, and magnetic nanoparticles with tunable physico-chemical and opto-electronic properties for osteogenic scaffolds is discussed here in detail. Furthermore, the rational selection of a polymeric base such as chitosan, collagen, poly (L-lactide), hydroxyl-propyl-methyl cellulose, poly-lactic-co-glycolic acid, polyglucose-sorbitol-carboxymethyl ether, polycaprolactone, natural rubber latex, and silk fibroin for scaffold preparation is also discussed. These advanced materials and fabrication strategies not only provide for appropriate mechanical strength but also render integrity, making them appealing for orthopedic applications. Further, such scaffolds can be functionalized with ligands or biomolecules such as hydroxyapatite, polypyrrole (PPy), magnesium, zinc dopants, and growth factors to stimulate osteogenic differentiation, mineralization, and neovascularization to aid in rapid healing. Future directions to co-incorporate bioceramics, biogenic nanoparticles, and fourth generation biomaterials to enhance biocompatibility, mechanical properties, and rapid recovery are also included in this review. Hence, the further development of such biomimetic metal-based nano-scaffolds at a lower cost with reduced risks and greater efficacy at regrowing bone can revolutionize the future of orthopedics.

Keywords: metal nanoparticles; implants; biopolymers; functionalized ligands; osteogenic differentiation; bone tissue engineering

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1. Introduction

Recent advances in the field of bioengineering have employed nanotechnology to revolutionize conventional therapies which are often ineffective and fail to provide long term relief to patients [1]. The natural hierarchical structures of human tissues at the nanoscale emphasize the need to explore the “small” to fix the bigger problems. Hence, nanomaterials (ranging from 1 to 100 nm in size) are advantageous as they can mimic the tissue nano-environment and significantly modulate biological responses [2]. Nanostructured vehicles have been reported as efficient drug delivery systems that can traverse biological barriers, such as the small intestine, nasal and oral mucosa pathways, and the skin [3]. Additionally, the larger surface area to volume ratios of nanoparticles can be multifunctionalized with drugs, growth factors, targeting ligands, contrast agents, and other bioactive agents to make multifunctional materials [4,5]. Such nanocomposites which can accomplish both therapy and diagnosis are termed theranostic agents [6–8]. Such nanoparticles coupled with conjugating moieties, such as peptides, aptamers, and small molecules, help to deliver drugs specifically to the target tissue, thereby preventing adverse effects of non-specific drug accumulation and toxicity [9,10]. More recently, nanoparticles have been used for fabricating multifunctional scaffolds for tissue engineering applications [11–16].

Extensive research on the fabrication of advanced biomaterials with attractive surface properties, mechanical strength and durability has resulted in nanotechnology-based strategies to ameliorate bone related diseases [17–19]. Conventional clinical therapies often fail to cure skeletal-related disorders, such as osteoarthritis (OA), osteoporosis (OP), non-union bone defects, bone cancer, and myeloma-related bone diseases [20–23].

In the USA alone, approximately 52.5 million adults suffer from clinical OA while almost 10.2 million adults aged 50 years and older suffer from OP. 43.4 million adults have a high risk of developing OP due to low bone mass [21]. Certainly as well, even in those cases where clinical unions occur, after implantation, patients do not return to an active lifestyle [1,22]. Novel materials which can promote osteoblast (or bone forming cells) differentiation, proliferation, adhesion, and integration have received wide attention which may solve these persistent problems with conventional orthopedic implants [24,25].

Biomaterials employing nanoengineering and nanofabrication technologies present promising biomimetic approaches as they resemble natural biological tissues with respect to structural, mechanical, and sometimes chemical properties. The adherence and growth of cells with desired functionalities can be controlled by the chemical nature, spatial geometry, porosity, and complexity of such 3D nano-scaffolds. Nanostructures with exotic shapes such as fibers, spheres, sheets, hollow tubes, and nets can be used for fabricating unique scaffolds for orthopedic tissue engineering including bone, cartilage, tendons, and ligaments [26]. Hard tissue engineering related to bone ideally requires nanomaterials with great mechanical strength, such as ceramic-based nanomaterials including hydroxyapatite, calcium phosphates, nanosilicates, and bioactive glasses. Nanoscale hydroxyapatite (HAp) has been extensively used as orthopedic implants for bone regeneration [27]. Similarly, carbon-based nanomaterials, such as carbon nanotubes (CNTs), graphene (G), and nanodiamonds (NDs), have also been incorporated in scaffolds due to their superior ability to induce osteogenic differentiation in stem cells [28,29].

However, metallic nanoparticles are preferred for bone implants due to their well-established presence in the field, durability, mechanical strength, and biocompatibility [30]. This review provides a comprehensive account of advances in the field of developing nanoscaffolds using metallic nanoparticles for bone tissue regeneration. Several nanoscale metals such as copper, gold, iron oxide, platinum, palladium, silver, strontium, titanium, zinc, and their oxides are discussed with respect to their promising role in bone tissue regeneration as listed in Table A1. Further, strategies for incorporating these nanoparticles within polymers such as poly (lactic-co-glycolic acid) (PLGA), poly (L-lactic acid) (PLLA), poly (caprolactone) (PCL), collagen, hyaluronic acid, silk, alginate, and fibrin to enhance mechanical properties, bioactive characteristics, and biocompatibility are presented. Critical comments on the future of such developments and additional modification ideas for nanoscaffolds to serve as biomimetic materials for drug delivery with better resorption and enhanced bone tissue healing properties are also included.

2. Systematic Review of Literature

Literature Search

A structured review of research articles was conducted in accordance with the methodology described by van den Bosch et al. (2017) [31]. An electronic literature search was conducted and finalized in June 2021, using the databases Scopus, PubMed, and Web of Science. The search was limited to peer-reviewed articles written in English. The results were filtered by study type to include both research articles and reviews. Additionally, both in vitro studies and in vivo studies were taken into account while assessing bone tissue regeneration efficiency.

No other filters were applied. Search terms were related to: (i) metal nanoparticles; (ii) scaffolds; and (iii) bone tissue regeneration and were combined and searched for in keywords, topic, title and abstract, or MeSH-terms (PubMed). In order to widen the search beyond the protocol, snowball sampling and scanning of the bibliographies of identified articles were carried out. Research on bone tissue regeneration using metallic

nanoparticle-based scaffolds was the basis of the selection of search terms that relied on existing metabolic pathways [32].

Further, the titles and abstracts of articles, identified by the searches, were reviewed by the authors independently. Entire articles were selected for this review only if at least the retrieved abstract fulfilled the inclusion criteria which was based on the AMSTAR tool to ensure that only high-quality articles were included [33].

We applied the following inclusion criteria:

- Systematic reviews and research articles
- Including a quality evaluation of supportive experiments
- Published in a peer-reviewed, scientific journal
- Written in English
- Establishing the underlying mechanism of bioactivity

Articles not identifying the search terms used, not providing synthesis methods and characterization data of nanometals, and/or not describing proper control experiments were excluded. As the aim of this review was to highlight the potential of metal nanoparticles-based scaffolds mainly for bone tissue regeneration, we did not consider reviews on other ailments. We also excluded any reviews on polymeric scaffolds that did not have metallic nanoparticles. Equally, we excluded research articles on carbon based and ceramic based nanoparticles used for tissue engineering.

3. Data Extraction

Each and every article was scrutinized by the authors for strict fulfilment of inclusion criteria with the extraction sheet including data on the type of metal nanoparticle, size in nanometer (nm), type of matrix used for scaffold fabrication, and the type of in vitro and/or in vivo studies used in order to support the claim for bone tissue regeneration. A summary of the results from the extraction sheet is provided in Table A1. As our review considers one type of “intervention” (scaffolds), though of varying composition (based on diverse metallic nanostructures), and its effect on osteogenic induction, mineralization and tissue differentiation, we considered extracting the results from diverse types of metallic nanoparticles to highlight the heterogeneity in the type of nanoscaffold as a function of metals and matrix used [34].

4. Results

The initial search resulted in 286 retrievals. A first scan of titles and abstracts was carried out limiting the amount of potentially relevant papers to a total of 185. The remaining potentially eligible full papers were reviewed and evaluated as fulfilling inclusion criteria or not, resulting in a final inclusion of 115 articles as illustrated in the flow chart in Scheme 1. The research articles involving polymeric nano/micro structures, mesoporous non-metallic nanomaterials and bioceramics were not analyzed and were excluded from the review. Results were extracted for each respective article separately based on the type of metallic nanoparticle, their morphological features, nature of the polymeric matrix for impregnating the nanometals, and the in vitro and/or in vivo osteogenic activity.

Scheme 1. Process for article selection based on the inclusion criteria.

5. Copper

Among many vital metals, copper is noteworthy due to its significant physiological role in enzymatic reactions and electron transfer associated energy generation. Copper nanoparticles (CuNPs) are biocompatible and possess enhanced antioxidant and antidiabetic activity advantageous for wound healing [35–37]. It can induce the migration and proliferation of endothelial cells and can also stimulate angiogenesis. Moreover, copper treated wounds exhibited enhanced tissue regeneration, thereby facilitating the healing process [38]. Hence, copper is considered as a promising metal to be incorporated in nanoscaffolds for bone tissue regeneration applications.

D'Mello et al. (2014) incorporated copper into chitosan scaffolds to enhance bone tissue regeneration [39]. Initially, chitosan sponges were prepared by dissolving chitosan in a 0.3 M acetate buffer at a concentration of 2% *w/v* which was then lyophilized. Furthermore, copper-chitosan scaffolds were fabricated by reacting the chitosan solution with a 0.625 mM copper solution for 4 h. Pores $104 \pm 5 \mu\text{m}$ in diameter in the chitosan scaffolds formed interconnecting networks, while the incorporation of copper in the scaffolds did not alter such micro-structural aspects. Uniform mineralized bone tissue formation was evident 4 weeks after implantation of copper-chitosan scaffolds into critical-sized calvarial defects (5 mm diameter) in Fisher 344 male rats. This enhanced osteogenesis might be attributed to the macroporous nature of the scaffolds that facilitated the infiltration of cells to migrate to the site of injury. Moreover, the walls of the porous matrix provided support for the attachment and anchoring of cells. Larger pore sizes allowed for adequate space for cell growth, proliferation, differentiation, and angiogenesis, resulting in better bone tissue development.

Zhang et al. (2016) reported enhanced osteoinductive properties of graphene oxide-copper nanocomposite (GO-Cu) coated porous calcium phosphate (CaP) scaffolds. GO

was prepared by the oxidation of graphite [40]. Then, an aqueous CuSO_4 solution reacted with GO and ascorbic acid at 60°C followed by centrifugation to recover the nanocomposites comprised of crystallized $\text{CuO}/\text{Cu}_2\text{O}$ nanoparticles $\approx 30\text{ nm}$ in diameter. The nanoparticles were uniformly distributed onto the surface of the porous scaffolds and exhibited a sustained release of Cu ions as shown in Figure 1. The calcium phosphate cement (CPC) scaffolds with 70% porosity, 5 mm diameter, 3 mm height, and $150\text{--}400\text{ }\mu\text{m}$ pore diameters were immersed in 1.50 mg mL^{-1} GO or a GO-Cu solution at 37°C for 10 min and were vacuum dried to obtain nanocoated scaffolds. The GO-Cu coating not only enhanced the adhesion and osteogenic differentiation of rat bone marrow stem cells (BMSCs), but it also activated the Erk1/2 signaling pathway and upregulated the expression of hypoxia-inducible factor-1 alpha ($\text{Hif-1}\alpha$). It is significant to note that angiogenesis and osteogenesis are tightly coupled during bone development and regeneration where the $\text{Hif-1}\alpha$ pathway plays a critical role. Wan et al. 2010 reported that the overexpression of the $\text{Hif-1}\alpha$ subunit results in its accumulation in the cytoplasm, which then translocates to the nucleus and dimerizes with the $\text{Hif-1}\beta$ subunit [41]. This dimer then binds to a highly conserved hypoxia-response element (HRE) within promoters of hypoxia-responsive genes. Functional HREs encode proteins involved in angiogenesis (VEGF, endothelin-1), maturation of red blood cells (erythropoietin and transferrin), energy metabolism (glucose transporter 1 and 3), and cell proliferation and viability (insulin-like growth factor 2, p21). This leads to an increase in the secretion of VEGF and BMP-2 proteins. Upon transplantation into critical-sized calvarial defects in rats, the GO-Cu-coated CPC scaffolds notably promoted both angiogenesis and osteogenesis. Phagocytosis of the GO-Cu nanocomposites by multinucleated giant cells indicated significant vascularized bone regeneration.

Figure 1. Schematic of the experimental protocol followed for the fabrication of GO-Cu-coated CPC scaffolds. (a) The GO pattern; (b) Premature Cu nanoparticles on GO films; (c) Mature nanoparticles on GO films; and (d) GO-Cu coated porous CPC scaffolds. Reproduced from [40], with permission of WILEY-VCH 2016.

Nanocomposites with a dual role of bone tissue regeneration as well as antimicrobial activity are more preferred biomaterials for orthopedics as they can promote bone healing as well as resist biofilm associated implant failure. Bari et al. (2017) incorporated copper within mesoporous bioactive glasses (MBGs) to facilitate both osteogenesis and angiogenesis apart from inhibiting bacterial growth [42]. The one-pot ultrasound-assisted sol-gel process was employed to modify the framework of a binary $\text{SiO}_2\text{-CaO}$ mesoporous glass with a copper ion concentration of 2 and 5% mol denoted as $\text{Cu_MBG 2\%}$ and $\text{Cu_MBG 5\%}$, respectively. In this method, calcium nitrate tetrahydrate and copper chloride were added to a methanolic cetyltrimethylammonium bromide (CTAB) solution which was further reacted with tetraethylorthosilicate (TEOS) under ultrasonication for 20 min followed by stirring at room temperature for 24 h. The resulting precipitate was recovered, dried and

eventually calcined at 600 °C for 5 h to obtain the final nanocomposite (Cu_MBG). 2% Cu_MBG uniformly spherical 170 to 200 nm diameter particles exhibited a worm like system of mesopores throughout the inner structure as seen in Figure 2. The Cu ions were evenly distributed within the nanocomposite. The 5% Cu_MBG were also spherical but slightly bigger in size ranging from 180 to 230 nm with a core-shell morphology. The shell was composed of a regular mesoporous structure while the internal core showed larger and irregular cavities with more Ca and Cu content. The Cu_MBG nanoparticles exhibited a promising antibacterial effect against *E. coli*, *S. aureus*, and *S. epidermidis*. Further, the nanoparticles significantly inhibited and dispersed the *S. epidermidis* biofilm. It was speculated that this advanced material of a mesoporous nature can effectively treat bone defects.

Figure 2. Scanning electron microscopic image of Cu_MBG 2% (a), EDS spectrum (b), TEM image (c) and EDS mapping

analysis of 2% Cu_MBG (d), Si (red), O (blue), Cu (green) and Ca (yellow) were uniformly distributed in the nanoparticles; SEM image of 5% Cu_MBG (e), related EDS spectrum (inset), and TEM image (f). Reproduced from [42], with permission of Elsevier 2017. Abbreviations: Cu_MBG, copper within mesoporous bioactive glasses.

6. Gold

Gold nanoparticles (AuNPs) are biocompatible and have shown tremendous therapeutic potential in the area of cancer, diabetes, other oxidative stress diseases, and tissue engineering [43]. AuNPs are being used for sensing, bioimaging, targeted drug delivery as well as photothermal and photodynamic therapies. Several top-down and bottom-up synthetic approaches have been employed to obtain tunable sizes and shapes. Since the bioactivity of AuNPs are largely size and shape dependent, processes are being optimized to obtain desirable morphological features. Green synthetic approaches employing bacteria, fungus, algae, plant extracts, and their metabolites aim to increase biocompatibility of the therapeutically promising AuNPs [44,45]. Moreover, their large surface area is ideal for multifunctionalization with drugs, targeting ligands and contrast agents.

AuNPs have also been used for developing scaffolds with promising biocompatibility and osteogenic potential. For example, Liang et al. (2019) fabricated a novel gold nanoparticle (AuNP)-loaded mesoporous silica nanoparticle (Au-MSNs) for accelerated bone regeneration [46]. MSNs were synthesized by reacting hexadecyl trimethylammonium bromide (CTAB) with 4 M NaOH and tetraethyl orthosilicate (TEOS) and then were recovered by centrifugation followed by calcination at 550 °C for 5 h. Next, the MSN with amidogen was obtained by dissolving the MSNs in methylbenzene followed by refluxing at 80 °C in the presence of N-(amino-ethyl)-amino-propyl trimethoxy silane (APTES). AuNPs were separately formed by the reaction of HAuCl_4 with a trisodium citrate solution at 100 °C for 20 min followed by the addition of polyvinylpyrrolidone (PVP). The resulting AuNPs were recovered, washed and reacted with MSNs for 12 h to obtain an Au-MSNs composite. Further, the chitosan scaffolds were immersed for 24 h in the Au-MSN solution to obtain Au-MSN coated chitosan scaffolds. Both MSNs and Au-MSNs were 80 to 110 nm in size with well aligned mesoporous structures.

However, in the case of Au-MSNs, several nanoparticles with a diameter of 15 nm were uniformly distributed across the surface of the MSNs. The Au-MSNs generated a favorable immune microenvironment by stimulating an anti-inflammatory response and promoting the secretion of osteogenic cytokines (IL-1ra, IL-10, and Arg) by Murine-derived RAW 264.7 cells. This resulted in more osteogenic differentiation in preosteoblastic MC3T3 cells as indicated by higher levels of osteogenic markers such as alkaline phosphatase (ALP) and calcium deposition. Similarly, the Au-MSNs also enhanced the formation of new bone in a critical-sized cranial defect site in rats. Hence, these novel gold supplemented nanoscaffolds promoted osteogenesis remarkably better than conventional approaches, resulting in better bone tissue repair and regeneration.

Tsai et al. (2013) synthesized AuNPs using sodium citrate with an average size of 10.76 ± 1.4 nm [47]. Upon treating MG63 osteoblast-like cells (human osteogenic sarcoma cells) with 1 ppm and 10 ppm of AuNPs, it was observed that most of the AuNPs aggregated and were localized in the late endosomes in cells which might be attributed to vesicle fusion as shown in Figure 3. Importantly, the AuNPs didn't induce apoptosis or necrosis. The appearance of specific nodule-like phenotypes along with the expression of bone associated genes in the MG63 cells, even after treatment with AuNPs, indicated regular osteogenetic differentiation without any toxicity or metabolic alteration. Hence, AuNPs based nanoscaffolds may provide biocompatible materials for improved orthopedic regenerative medicine applications.

Figure 3. Laser scanning confocal microscope (LSCM) fluorescence images of MG63 cells after uptake of AuNPs. LSCM fluorescence images of (a) MG63 cells treated with AuNPs at a concentration of 1 ppm, (b) 10 ppm, and (c) normally cultured cells. Cytoskeletal F-actin (red) were stained with Texas Red-X phalloidin. Cell nuclei (blue) were stained with Hoechst 33258. The green spots in the circled areas are AuNPs. Reproduced from [47] under Creative Commons Attribution License.

The underlying mechanism for AuNPs osteogenic promotion was reported by Yi et al. 2010 [48]. The synthesized AuNPs were spherical in shape with an average diameter of 20 nm that showed a concentration dependent promoted osteogenic differentiation of MSCs. The AuNPs entered the cells by receptor mediated endocytosis after adsorption of serum protein onto the external surface of the nanoparticles. Figure 4 shows that the AuNPs were grouped into intracellular compartments in the cells, particularly inside the perinuclear compartments and vesicular structures close to the cell nucleus. The AuNPs modulated cellular signaling pathways by interacting with the cytosolic proteins. AuNPs treated cells showed a mechanical stress mediated marked enhancement in ALP activity, mineralized nodule formation, up-regulation of p38 protein, integrin family genes (integrin $\alpha 2$, integrin $\alpha 2b$, integrin $\alpha 3$, integrin αM , and integrin αV), matrix metalloproteases (such as Mmp2, Mmp8, and Mmp9), cell-cell adhesion related genes (Cd36, Cdh11, and Vcam1), and the lineage commitment gene Runx2 along with other osteogenic markers (BMP1, BMP2, BMP4, OCN and Col1a2). Thus, it is now well established that osteogenic differentiation due to AuNP exposure can be attributed to mechanical stimuli-initiated signal transduction via the p38 MAPK pathway.

Zhang et al. (2017) established that the AuNPs which promoted the osteogenic differentiation of MSCs and osteoblasts were size dependent [49]. Interestingly, it was noticed that the treatment of human periodontal ligament progenitor cells (PDLFs) with smaller dimensions than the AuNPs (5 nm), significantly reduced alkaline phosphatase activity, mineralized nodule formation and expression of osteogenic genes. On the contrary, treatment with AuNPs of a comparatively larger size (13 and 45 nm) resulted in the increased expression of osteogenic markers. Osteogenic differentiation was significantly promoted by 45 nm compared to 13 nm AuNPs. Further, the up-regulation of autophagy was facilitated by 13 and 45 nm AuNPs while 5 nm AuNPs blocked the same events clearly indicating that the AuNPs modulated the osteogenic differentiation of PDLFs in a size-dependent manner,

with autophagy playing a major role. Hence, the morphological features of AuNPs should be taken into consideration when designing scaffolds for periodontal bone regeneration.

Figure 4. Transmission electron microscope (TEM) images of the internalization of AuNPs. (a–d): Cells were treated with OS as a control; (e–h): cells were treated with OS and 1.0 nM AuNPs. Arrows indicate AuNPs. The images are representative of three independent experiments. Reproduced from [48], with permission of American Chemical Society 2010. Abbreviation: OS—osteogenic induction supplement.

AuNPs and alloys of gold can be attractive nanomaterials for promoting bone tissue regeneration. For example, Zhang et al. (2019) fabricated porous gold-palladium (pAuPd) alloy nanoparticles to facilitate bone regeneration due to hyperthermia (via a mild localized heating effect) up to 40–43 °C under NIR laser irradiation as shown in Figure 5 [50]. An effective wet-chemistry strategy was used for the synthesis of pAuPds that involved ascorbic acid, phloroglucinol, citric acid, sodium citrate, or glucose as reducing agents. Nearly spherical nanostructures with a solid core and densely branched shells were obtained when the molar ratio between Pd and Au was equivalent to 2:2. The pAuPds were considered safe as they didn't display any cytotoxicity up to concentrations as high as 200 $\mu\text{g mL}^{-1}$. Their application in photothermal therapy (PTT) was confirmed as the temperature of preosteoblast-MC3T3-E1 cells treated with pAuPds increased upon exposure to near-infrared (NIR) irradiation. Further, pAuPds were used for checking their healing effect on critical-sized cranial defects (8 mm in diameter) in rats. It is significant to note that after being engulfed by cells, pAuPds showed mild hyperthermia under NIR treatment that greatly accelerated cell proliferation and bone regeneration sealing 97% of the cranial defect by newly formed bone after six weeks of PTT. It was speculated that mild local heat could activate the Wnt signaling pathway for triggering enhanced in situ bone regeneration.

More recently, Zhang et al. (2021) reported superior osteogenic differentiation by PEGylatedAuNPs [51]. In this study, AuNPs of various sizes were synthesized by two different methods. Gold ions were reduced to AuNPs 4 nm in diameter in the presence of NaBH_4 and trisodium citrate. On the other hand, AuNPs 18 nm and 45 nm in size were fabricated using a citrate reduction method. The as-synthesized AuNPs were further PEGylated using poly(ethylene glycol) thiol via the high affinity site of the $-\text{SH}$ groups for AuNPs. The resulting nanoparticles of all three different sizes exhibited excellent biocompatibility and osteogenic differentiation properties on MC3T3-E1 cells which were found to be a function of their size. PEGylated AuNPs 45 nm in size enhanced alkaline phosphatase (ALP) activity, mineralized nodule formation and the expression of osteogenic marker genes as well as up-regulated β -catenin and p-GSK-3 β genes, compared with

the 18 nm group. On the other hand, nanostructures 4 nm in size significantly reduced osteogenic effects. The molecular events indicated that the activation of the Wnt/ β -catenin signaling pathway might be the underlying mechanism for the induction of osteogenic differentiation by larger PEGylated AuNPs, while smaller nanostructures inhibited such action. Further, AuNPs conjugated with PEG-hydrogel resulted in the rapid regeneration of bone in rabbits with 4-mm femur defects.

Figure 5. Schematic illustration of pAuPd for the PTT of cranial defects. Reproduced from [50], with permission of American Chemical Society 2019. Abbreviation: PTT, photothermal therapy.

7. Magnetic Nanoparticles

Although various metals have been used for developing scaffolds for supporting bone tissue regeneration, magnetic materials are considered much more attractive due to their mobility in response to a magnetic field [5,52]. Magnetic iron oxide nanoparticles (IONPs) can be multifunctionalized with growth factors, drugs, antibodies, or other biological agents for efficient targeting, hyperthermia, triggered release and imaging [53].

In one of the studies, Bock et al. (2010) fabricated a magnetic biomimetic scaffold using ferrofluids containing aqueous dispersions of magnetite nanoparticles (200 nm in size) [54]. The scaffolds further contained hydroxyapatite (HA) and collagen (COLL) crosslinked with 1,4-butanediol diglycidyl ether (BDDGE), as homogeneous, micro- and macroporous pellets (diameter 12.2 ± 0.8 mm and height 4.7 ± 0.8 mm). Similarly, scaffolds referred to as COLL contained 100% collagen that were homogeneously formed into porous pellets (diameter 12.3 ± 1 mm, height 2.8 ± 0.9 mm). These magnetic scaffolds supported the adhesion and proliferation of human bone marrow stem cells in vitro.

Cai et al. (2015) also reported enhanced osteogenic differentiation under a moderate-intensity (1 mT–1 T) static magnetic field (SMF) when treated with electrospun nanofibrous structures made out of poly(L-lactide) (PLLA) and ferromagnetic Fe_3O_4 nanoparticles (NPs) [55]. The PLLA/ Fe_3O_4 composite nanofibers 600 to 1000 nm in diameter could significantly increase cell adhesion, and promote the proliferation and osteogenic differentiation of MC3T3-E1 cells. Increased ALP activity and calcium deposition in

treated cells further confirmed the positive role of PLLA/Fe₃O₄ composite nanofibers for osteogenic differentiation.

Advancements in the area of nanotechnology and nanomedicine have led to the development of nanoinks ideal for 3D printing of tissues and organs. Rational tuning of the porosity and surface properties of 3D printed materials help to mimic the tissue microenvironment [56,57]. Although, iron based magnetic nanomaterials are ideal for bone healing, the uncontrollable architecture and stiffness of the nanostructures achieved during conventional synthesis have been major limitations. This problem was addressed by Yang et al. (2018) where a 3D printing technique was employed to control the macropore structure of scaffolds more precisely [58]. Figure 6 shows the morphological features of the HA coating on the Fe scaffolds under various experimental conditions. Interestingly, the pH value of the initial solution played a major role in determining the shape of the HA coating. A nanosheet structured HA was obtained at pH 6 (Figure 6b) while a nanorod structured HA was obtained at pH 9 (Figure 6c) and pH 13 (Figure 6d). These innovative scaffolds with $66.2 \pm 4.9\%$ porosity significantly improved the viability, alkaline phosphatase (ALP) activity and osteogenic differentiation of rabbit bone marrow mesenchymal stem cells (rBMSCs) on the scaffolds.

Figure 6. Scanning electron microscope (SEM) images of HA coated scaffolds prepared in an initial solution with different pH values at 120 °C for 12 h: (a) A Fe scaffold without HA coating and (b–d) HA coating generated at pH 6, 9, and 13, respectively. Reproduced from [58], with permission of American Chemical Society 2018. Abbreviation: HA, Hydroxyapatite.

More recently, Zhao et al. (2021) fabricated superparamagnetic iron oxide nanoparticles (SPIONs) encapsulated into poly-lactic-co-glycolic acid (PLGA) microspheres to

regulate the osteogenic differentiation of bone mesenchymal stem cells (BMSCs) [59]. In this study, three different types of PLGA microspheres (PFe-I, PFe-II, and PFe-III) were synthesized with the amount of loaded SPIONs equivalent to 1.83%, 1.38%, and 1.16%, respectively. The microspheres were in a range from 160 μm to 200 μm with notable porous and rough surfaces. The PFe-II microspheres served as ideal surfaces as indicated by the enhanced adhesion of BMSCs after two days of co-culture. Remarkable osteoblast differentiation of BMSCs as indicated by a high expression of ALP, COL-I, OPN, and OCN was observed after 2 weeks of co-culture. Under an external magnetic field, notable bone mineral density (BMD, $263.97 \pm 25.99 \text{ mg/cm}^3$), trabecular thickness (TB. TH, $0.58 \pm 0.08 \text{ mm}$), and bone tissue volume/total tissue volume (BV/TV, $78.28 \pm 5.01\%$) were found six weeks after surgical implantation of PFe-II microspheres into femoral rat bone defects.

Wang et al. (2016) showed osteogenic differentiation of hBMSCs can be promoted by IONPs in response to the MAPK pathway [60]. Initially, the IONPs were synthesized by employing a classic chemical co-precipitation method using ferrous chloride hexahydrate and ferrous chloride tetra-hydrate. Further, the nanoparticles were modified to obtain an IONP core and polyglucose-sorbitol-carboxymethyl ether (PSC) shell. The core-shell type magnetic nanoparticle exhibited an average size of 7 nm. Cellular uptake of IONPs by hBMSCs was time- and dose-dependent where 100 mg/mL of the particles were most suitable for promoting hBMSCs osteogenic differentiation. A notable (4–12 fold) up-regulation of osteogenic markers, such as ALPL (ALP liver), RUNX2 (runt-related transcription factor 2, also known as CBFA1), BMP2 (bone morphogenetic protein 2), OMD (osteomodulin), FOXO1 (forkhead box O1) and ATF4 (activating transcription factor 4), was observed. On the other hand, the MSC markers, such as MCAM (melanoma cell adhesion molecule), ENG (endoglin), CLCF1 (cardiotrophin-like cytokine factor 1), TPM1 (tropomyosin 1) and CD44, were down-regulated 2.5–5 fold. Likewise, mRNA expression of other significant genes like FGFR1, KRAS, MAP3K8 (Tpl2/Cot), RPS6KA1 (p90RSK1) and RPS6KA3 (p90RSK3) were all up-regulated, whereas MAP2K2 (MEK2) was down-regulated suggesting the role of the classical MAPK pathway.

A static magnetic field (SMF) stimulated osteoblastic differentiation and bone formation much better when magnetic nanoparticles were incorporated into the scaffolds [61]. Magnetism has recently been implicated to play a significant role in the regulation of cell responses. A magnetic nanocomposite scaffold comprised of polycaprolactone and magnetic nanoparticles significantly promoted primary mouse calvarium osteoblastic differentiation as noted by the simultaneous expression of bone-associated genes (Runx2 and Osterix) and alkaline phosphatase activity. Integrin signaling pathways were activated showing the up-regulation of focal adhesion kinase, paxillin, RhoA, mitogen-activated protein kinase, and nuclear factor-kappaB, along with the enhanced expression of BMP-2 and phosphorylation of Smad1/5/8. Furthermore, apart from osteogenic promotion, the SMF/magnetic scaffolds also stimulated the angiogenic responses of endothelial cells with a simultaneous expression of vascular endothelial growth factor (VEGF) and angiogenin-1 genes. Capillary tubes were also formed upon treatment. Upon implantation into mouse calvarium defects, the magnetic scaffolds remarkably promoted new bone formation after six weeks. Considering their advantages and enhanced biocompatibility, magnetic nanoparticles can definitely serve as a good candidate for developing nanometal based scaffolds for osteogenic induction and bone tissue regeneration.

8. Platinum and Palladium

Among various noble metal nanostructures, platinum nanoparticles (PtNPs) and palladium nanoparticles (PdNPs) are most noteworthy due to their unique therapeutic properties that can be used for numerous antimicrobial, bioengineering, and biosensing applications. These nanoparticles are known to ameliorate oxidative stress by scavenging reactive oxygen species (ROS) which is critical to prevent several diseases [62–64]. Hence, these medically significant nanoparticles can protect one from neurodegenerative disorders, such as Alzheimer's disease, and also protect cells from oxidation-induced inflammation

and induced bone loss by decreasing osteoclastogenesis [65]. However, less work has been done towards the incorporation of PtNPs and PdNPs for bone tissue regeneration.

In one study, calcium phosphate platinum nanoparticle (CPs-PtNPs) scaffolds were fabricated by heat sintering where a poly vinyl alcohol solution (2 wt%) was used to create a ceramic slurry and 2.5% glutaraldehyde was used as a cross-linker. The casted slurry was initially dried at 100 °C for 24 h followed by sintering at 400 °C. Superior porosity between 30–35% with pore sizes between 100 to 400 µm was found in the scaffolds designed with 20 wt% CPs. The scaffolds were biodegradable and 10% PtNPs significantly enhanced cell attachment and proliferation on the scaffold surface [65].

In another study, novel hybrid 3D scaffolds were constructed which were composed of nanohydroxyapatite crosslinked with chitosan in a matrix of poly (lactic acid) nanofibers (PLA) and doped with PtNPs, AuNPs, and TiO₂NPs together [66]. The PtNPs with sizes ranging from 10 to 20 nm with round, square and triangular shapes were homogeneously dispersed in the scaffold matrix due to their high affinity for the PLA fibers creating a “spiderweb-like” structure. The nanostructures enhanced the biomineralization process, cell adhesion, cell proliferation and osteogenic differentiation in MG-63 cells. Hence, these nanoparticles may help provide appropriate architectural requirements for numerous bone-tissue regeneration applications.

PdNPs along with polypyrrole (PPy; a conductive polymer) were non-covalently functionalized on two-dimensional reduced graphene oxide (rGO) in order to fabricate a Pd/PPy/rGO nanocomposite (NC) scaffold for bone tissue engineering [67]. 2–5 nm PdNPs were uniformly dispersed and strongly anchored on the PPy/rGO surface. It inhibited biofilm formation by bacteria, such as *E. coli*, *B. subtilis*, *P. aeruginosa*, and *K. pneumoniae* indicating its promise to resist biofilm associated implant failure. Further, the biocompatible scaffold promoted the proliferation of Saos-2 cells. The NC substrates supported the development of osteo (Saos-2) cells which was indicated by the attachment of more osteoblasts to the walls of Pd/PPy/rGO after seven days of incubation.

Calabrese et al. (2020) reported that multi-metal nanoparticle-functionalized hydroxyapatite scaffolds could improve bone regeneration [68]. The cylindrical magnesium doped hydroxyapatite (Mg-HA) scaffolds 8 mm in diameter and 5 mm in height were composed of a bilayer structure where the upper layer (3 mm in depth) was made of equine type I collagen (Coll) that was deposited on a second layer (2 mm depth) comprised of a mineralized blend of type I Coll (30%) and 70% of Mg-HA. The collagenous layer in the scaffold had interconnected pores and larger channels with diameters up to 300 µm and 600 µm, respectively. Further, these scaffolds were functionalized with gold nanorods (AuNRs), palladium nanoparticles (PdNPs) and maghemite nanoparticles (MAG NPs) which were 15 ± 5 nm, 3.4 ± 1 nm and 10 nm in diameter, respectively. MAG nanofunctionalization substantially improved adipose-derived mesenchymal stem cell (hADSCs) proliferation up to 70%. However, both PdNPs and AuNRs inhibited cell growth up to 89% and 94%, respectively, which was probably attributed to the cytotoxic effects for both PdNPs and AuNRs.

Similarly, Heidari et al. (2020) reported palladium supplemented hybrid ZnO-hydroxyapatite nanocomposite-based scaffolds where chitosan was used as a binder [69]. The HA/ZnO/Pd nanoparticles were irregular in shape with an average particle size of 70 nm. The PdNPs distributed on the surface of HA/ZnO were smaller than 10 nm. However, the supplementation of the nanoparticles significantly reduced the number of viable human dental pulp stem cells (hDPSCs) indicating their low biocompatibility. On the other hand, the scaffolds showed notable inhibition of *Pseudomonas aeruginosa* which is a potent nosocomial pathogen responsible for post-surgical complications.

9. Silver

Silver nanoparticles (AgNPs) have significant antimicrobial properties that make them more suitable to introduce into scaffolds to resist post-surgical pathogenic infections [70,71]. Various physical and chemical biological methods have been employed to synthesize AgNPs with well-defined sizes and shapes. Similarly, the synthesis of biocompatible

AgNPs was reported using bacteria, fungi, algae and medicinal plants [72–75]. Thus, the incorporation of nanostructured silver and their alloys into scaffolds at sub lethal dosages can enhance the shelf life of the material and possibly prevent post-operative pathogenic infections [76,77]. AgNPs are well known microbicidal agents and are used in implants to avoid biofouling due to bacterial biofilm formation. Hence, they can be used for surface modification and developing microbe resistant coatings. Cao et al. (2017) reported titanium supported AgNPs for the promotion of osteogenesis [78]. Initially, AgNPs were in situ fabricated and immobilized onto titanium by using a plasma immersion ion implantation process. Three nanoparticle coated supports denoted as Ti-Ag1, Ti-Ag2, and Ti-Ag3 were generated by silver plasma immersion ion implantation (Ag PIII) at 20 kV for 0.5, 1.0, and 1.5 h, respectively. The particle diameters for the Ti-Ag1 and Ti-Ag2 groups were in the range of 4–8 and 4–19 nm, respectively, whereas the Ti-Ag3 group could be divided into two subranges, i.e., 4–7 and 9–13 nm. Rat bone marrow stem cells (BMSCs) grown on Ag PIII treated groups expressed higher levels of filamentous F-actin indicating better cell adhesion. In the presence of Ti-Ag3, enhanced expression of osteogenic markers, like alkaline phosphatase (ALP) and osteocalcin (OCN), was evident after seven days. Further, expression of the integrin $\alpha 5$ was up-regulated when BMSCs were cultured on Ag PIII groups from day one to day seven indicating a population-dependent capability of the nanomaterial for activating the integrin $\alpha 5$ orchestrated MAPK/ERK signal cascade resulting in superior osteoblast differentiation. In order to determine the influence of AgNPs immobilized on titanium on osteointegration, indwelling cylindrical implants were used in the femurs of rats followed by sequential labelling with fluorescent dyes as illustrated in Figure 7. The Ti-Ag3 group exhibited promising osteointegration as the implants were completely covered with new bone as shown in Figure 7b. Calcein (as indicated by the green color) was injected at the sixth week which revealed significant thickening of the bone around the Ti-Ag3 implants. Also, bone remodeling was confirmed by a large overlapping region around the Ti-Ag3 group after the six-week period. Hence, these nanomaterials can be used for engineering durable implants with antibacterial and osteogenic potential.

Further, Castiglioni et al. (2017) reported the use of AgNPs for orthopedic applications where Saos-2 cells were found to be rather resistant to the cytotoxic effects of AgNPs alone, with a half maximal inhibitory concentration (IC_{50}) of approximately 25 $\mu\text{g/mL}$ [79]. Moreover, after a month-long treatment, the hMSCs adapted to the presence of the AgNPs, which was attributed to the upregulation of hsp70 resulting in the restoration of normal cell proliferation. Interestingly, Jiang et al. (2016) reported a concentration and time dependent release of Ag^+ ions from a porous nanohydroxyapatite/polyurethane (n-HA/PU) composite scaffold which was incorporated with silver phosphate (Ag_3PO_4) [80]. The release of ions continued even beyond three weeks and, thus, led to strong bactericidal effects against *Staphylococcus aureus* and *Escherichia coli*. The scaffolds facilitated the proliferation and adherence of MG63 cells, derived from human osteosarcoma and expressed characteristic features of osteoblasts, indicating its cytocompatibility and potential strong use for infection-associated bone defect repair.

Another powerful strategy to develop an antimicrobial bone tissue regenerating scaffold was reported by Jin et al. (2018) where electrospun silver ion-loaded calcium phosphate/chitosan antibacterial composite fibrous membranes were used [81]. Although an initial burst release was observed within the first day, augmentation of soaking time thereafter could control the amount of the Ag^+ released from nanofiber membranes. A strong inhibitory effect was observed against *Porphyromonas gingivalis*, which is the main pathogenic bacteria of peri-implantitis or periodontitis. Likewise, the membranes significantly inhibited the adhesion of *Streptococcus mutans*. Further, the membranes promoted the attachment of BMSCs which could greatly spread and grow cells on the membranes as shown in Figure 8. As the fibrous framework of the biodegradable polymer membrane, the micro-nanostructure can act as an ECM for improved cell attachment and adhesion.

Figure 7. Immobilized AgNPs accelerated the osteointegration properties of titanium: (a) schematic of the surgical procedure, (b) sequential fluorescent labelling of the implant-bone boundaries of Ti and the Ti–Ag3 group, (c) percentage of new bone stained with Alizarin Red S (ARS) and calcein in (b); ★ represents $p < 0.05$. Reproduced from [78], with permission American Chemical Society 2017.

Biocompatible natural rubber latex (NRL) can also be used for delivering AgNPs for bone regeneration. Marques et al. (2020) incorporated AgNPs 10–20 nm in size in the NRL membrane which exhibited enhanced bone tissue regeneration in rat calvaria bone defects [82]. Osteogenic activity was prominent mostly on the edges of the bone defect with the formation of short primary bone trabeculae near the wall of the defect. Also, the defects were progressively filled with connective tissue during the process of bone neoformation.

Natural antibacterial polymers (like silk fibroin combined with nanohydroxyapatite hydrogels) were supplemented with AgNPs and AuNPs in order to achieve superior bone regenerative properties [83]. Spherical monodispersed AgNPs and AuNPs with a size distribution ranging from 12.7 to 69.1 nm and from 9.3 to 54.7, respectively, were synthesized and combined using a silk fibroin solution derived from the cocoons of a *Bombyx mori* silkworm. Hydrogels with AgNPs exhibited very high antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*. Hydrogels with AgNPs at concentrations up to 0.5%, and the materials with AuNPs, supported the attachment and spreading of osteoblasts, with elongated morphologies and superior cell-to-cell contacts.

In another interesting study, Zhang et al. (2015) revealed the underlying mechanism behind the osteogenic effects of AgNPs [84]. Collagen scaffolds were developed with chemically synthesized AgNPs which exhibited early closure of a wound in a mouse femoral fracture model that was attributed to the formation of a fracture callus. It was hypothesized that the AgNPs may promote the formation of the callus and the subsequent joining of the fractured bone via multiple routes: (i) chemoattraction of MSCs and fibroblasts to migrate to the fracture site; (ii) induction of MSC proliferation; and (iii) induction of the osteogenic differentiation of MSCs via activation of TGF- β /BMP signaling in MSCs.

Figure 8. Scanning electron microscope (SEM) images of BMSCs on the surface of (a,b) CS, (c,d) A0, and (e,f) A1 and (g,h) A2 after (a,c,e,g) 4 days and (b,d,f,h) 7 days of incubation. Abbreviations: BMSCs, bone marrow stromal cells; CS, chitosan; SEM, scanning electron micrograph. Reproduced from [81], with permission of Jin et al. 2018. Abbreviations: CS, chitosan; A0, Ratio of CS to calcium phosphate is 4:1 and silver content is 0; A1, Ratio of CS to calcium phosphate is 4:1 and silver content is 0.075%; A2, Ratio of CS to calcium phosphate is 4:1 and silver content is 0.144%.

10. Strontium

Although various metal-based implants have been developed, they often fail to meet the desired porosity, interconnectivity, mechanical strength, bioactivity, and degradability. Hence, strontium (Sr), which is used for the treatment of osteoporosis due to its antiresorptive and anabolic effects, can be incorporated into scaffolds for better bone tissue engineering [85–87]. Sr can be incorporated into bone by two mechanisms: (a) surface exchange involving the incorporation of Sr into the crystal lattice of the bone mineral and (b) ionic substitution whereby Sr is taken up by ionic exchange with Ca in bone. Another benefit in using Sr is that it can significantly enhance collagen and non-collagen protein synthesis facilitating pre-osteoblast differentiation and inhibition of osteoclast differentiation and function [88]. Hemopoietic cells of the monocyte/macrophage lineage may differentiate into multinucleated osteoclasts cells (which are responsible for bone resorp-

tion) under control of the bone micro- and nano-environment. Vacuolar H^+ -ATPase in the ruffled border membrane of osteoclasts helps to acidify the bone resorbing area. Osteoclasts attach to the bone surface in a clear zone surrounding the ruffled border facilitating bone resorption. Hence, inhibition of osteoclast differentiation is critical to control osteogenesis and restrict bone destruction [89].

Zreiqat et al. (2010) fabricated novel calcium–silicon system-based scaffolds through the controlled substitution and incorporation of strontium and zinc resulting in Sr–Hardystonite ($Sr-Ca_2ZnSi_2O_7$, Sr-HT) [90]. The interconnected porous network (interconnectivity: 99%), large pore size (300–500 μm), and overall porosity of 78% were evident in the Sr-HT scaffolds. A remarkable induction of the attachment and differentiation of human bone derived cells (HOB) was achieved upon treatment with the Sr-HT ceramic scaffolds, which was attributed to the enhanced expression of alkaline phosphatase, Runx-2, osteopontin, osteocalcin and bone sialoprotein. Further, rapid new bone formation into the porous 3D scaffolds was reported when the Sr-HT scaffolds were implanted into tibial bone defects in rats.

In another study, Xin et al. (2009) used titanium osteoporotic bone implants as a Sr delivery platform [91]. Initially, titania nanotube arrays (TiO_2 -NT) were synthesized employing anodization of a chemically polished titanium plate, which were then subsequently subjected to a hydrothermal treatment in a $Sr(OH)_2$ solution. This eventually resulted in well-ordered $SrTiO_3$ nanotube arrays (Sr-NT) with a diameter of about 70 nm and length of about 1.3 μm . The $SrTiO_3$ nanotube arrays with superior biocompatibility could support cellular adhesion, proliferation and osteogenesis providing evidence that they can be ideal candidates as improved bone implants.

11. Titanium

Another important metal for triggering bone tissue regeneration and support is titanium. The large surface area and highly porous nature of nanostructured titanium and its alloys have made them ideal bone substitutes. Titanium based nanostructures are mostly hydrophobic and inert. Various strategies are being developed for the surface treatment of titanium-based nanocomposites to make them ideal surfaces for enhanced cell adherence and proliferation [92–94].

For example, Yavari et al. (2014) fabricated porous titanium alloy structures from a spherical pre-alloyed Ti6Al4V ELI powder using a selective laser melting technique [95]. The resulting nanostructures were further surface modified using two different techniques, namely alkali-acid-heat treated (AlAcH), acid-alkali (AcAl) and anodizing-heat treated (AnH). The porosity of the surface treated titanium alloys varied between 85 and 90%, while the mean pore and strut sizes were 575–600 nm and 160–175 nm, respectively. The diameter of the nanotubes was between 25 and 35 nm. Elongated human periosteum-derived cells (hPDC) were more homogeneously distributed and bridged the corners on AnH surfaces. The expression of Col1 and VEGF was enhanced on day seven and day one, respectively. AlAcH surfaces also showed improved expression of Runx2 (day 21), OCN (day 1), Col1 (day 21), and VEGF (day 21) as compared to AcAl surfaces. Further, AnH provided for high bone tissue regeneration and significantly better biomechanical stability in critically-sized femoral Wistar rat bone defects.

The surface topology of the titanium nanostructures can certainly play a vital role in modulating cellular responses towards osteogenesis. Zhao et al. (2012) demonstrated that micropitted/nanotubular structures of titanium can induce more prolific osteogenic differentiation [96]. Titania nanotubes (NTs) 25 and 80 nm in diameter were formed with an acid-etched Ti topography (AcidTi) and hierarchical hybrid micropitted/nanotubular topography. AcidTi was further anodized at 5 and 20 V to form hierarchical hybrid micropitted/nanotubular topographies denoted as Micro/5VNT and Micro/20VNT, respectively, to mimic the natural nanotextured bone extracellular matrix (ECM). Significantly, cell attachment and spreading, collagen secretion, ECM mineralization, and expression of osteogenesis-related genes were enhanced in the presence of the nanotubular surface.

However, most promising, the proliferation and osteogenic differentiation of MSCs could be induced by the Micro/20VNT sample. This finding strongly rationalized that cellular responses toward bone tissue regeneration area are related to the shape and surface feature on nanotextured materials, which needs to be carefully controlled.

Compared to bare titanium nanostructures, surface functionalized titanium dioxide nanocomposites can have a better osteoinductive effect. Zhou et al. (2013) grew strontium-doped hydroxyapatite ($\text{Ca}_9\text{Sr}_1(\text{PO}_4)_6(\text{OH})_2$, Sr₁-HA) nanorods with different lateral spacing (e.g., interrod spacing) values (67.3 ± 3.8 , 95.7 ± 4.2 , and 136.8 ± 8.7 nm) and nanogranulates on microarc-oxidized (MAO) microporous TiO_2 , respectively [97]. This resulted in a nanotopography with both a nano rod-shaped 3D pattern and a nanogranulated 2D pattern. The three coatings generated by the one-step MAO method were denoted as S67, S96, and S137, which exhibited interrod spacing values of 67.3 ± 3.8 , 95.7 ± 4.2 , and 136.8 ± 8.7 nm, respectively, as depicted in Figure 9. It is interesting to note that the interrod spacing values were inversely proportional to the electrolyte concentrations used. The microporous walls were nanorods with an average diameter of 70 nm. Interrod spacing in 3D patterned coatings below 96 nm significantly enhanced the proliferation and differentiation of hFOB1.19 cells (a human fetal osteoblast cell line). Likewise, the surface topology also regulated intracellular Ca^{2+} concentrations and, thereby, cellular metabolism for better osteointegration.

Figure 9. Scanning electron microscope (SEM) images of the surface morphologies of the microarc-oxidized (MAO) and hydrothermally treated coatings for: (a) S67, (b) S96, and (c) S137 (insets show the corresponding low-magnification images). (d) TEM bright-field image of the scratched nanorod from the S137 coating (inset showing the SAED pattern). Reproduced from [97], with permission of American Chemical Society 2013. Abbreviations: S67, nanorods with interrod spacing values of 67.3 ± 3.8 ; S96, interrod spacing values of 95.7 ± 4.2 ; and S137, interrod spacing values of 136.8 ± 8.7 nm.

In an interesting study, Palin et al. (2005) established the fact that natural nanoroughness of bone itself is an important parameter that can be considered while mimicking the nanofeatures of bone [98]. Increased osteoblast adhesion and proliferation was reported when poly-lactic-co-glycolic acid (PLGA) scaffolds incorporated with nanostructured titania were used. Initially, the nanophase titania (32 nm grain size) was converted into compacts 11 mm in diameter associated with a PLGA model cast polymer. Interestingly, the root-mean-square surface roughness of the nanophase titania and the PLGA mold of the nanophase titania were more closely related to the natural bone surface roughness than the conventional titania. The atomic force microscopic (AFM) root-mean-square surface roughness values for the $25\ \mu\text{m} \times 25\ \mu\text{m}$ scans of cortical bovine bone, the PLGA mold of nanophase titania, and the bare nanophase titania was 25 nm, 27 nm, and 22 nm, respectively, which were very close to each other. On the other hand, the root-mean-square surface roughness values of conventional titania of the identical dimensions was 11 nm indicating its striking difference to the native bone surface topology. Hence, it is important to note that rational modulation of the nanostructured surfaces of metal nanoparticles in the scaffolds can significantly alter osteoblast adhesion and proliferation and thereby promote rapid healing of the bone defects.

12. Zinc

Zinc is an essential element in our body which helps in the normal functioning of several metabolic enzymes and physiological reactions. ZnONPs have been reported to ameliorate oxidative stress by scavenging harmful free radicals [99]. Also, ZnONPs can be doped with other active elements for enhanced antidiabetic activity [100]. Functionalized ZnONPs and their alloys have been reported to have potential antioxidant, antidiabetic and antibacterial activity [101,102]. Hence, ZnONPs are promising nanomaterials that either individually or in functionalized form, can be incorporated within scaffolds to promote bone tissue regeneration along with the prevention of reactive oxygen species (ROS) associated damage and bacterial infection mediated failure of implants. Recently, Song et al. (2020) showed the effect of zinc silicate/nanohydroxyapatite/collagen (ZS/HA/Col) scaffolds on bone regeneration and angiogenesis [103]. The 3D printed scaffolds were fabricated by using a homogeneous mixture containing nano-HA/zinc silicate powders and collagen. An increase in zinc silicate concentration enhanced the roughness of the scaffold surface and overall strength. The sustained release of zinc ions and silicon was biocompatible with BMSCs and facilitated bone regeneration in rat critical-sized bone defects. A greater mass of newly formed bone with larger volumes of calcified tissue associated with several chondrocytes were evident when defects were treated with the composite scaffolds as seen in Figure 10. Interestingly, the cartilage gradually calcified to form bone tissue, accompanied by the degradation of the scaffolds during an 8 to 12-week period. Further, the high expression of osteogenesis-related genes Bmp-2 and Osterix were associated with the in vivo bone repair process. The composite scaffolds enhanced tartrate-resistant acid phosphatase (TRAP)-positive cells, nestin-positive bone marrow stromal cells (BMSCs), and CD31-positive neovessels, angiogenesis-related (Vegf- α and Cd31) genes and also activated the p38 signaling pathway in monocytes. Hence, the monocytes subsequently differentiated into TRAP+ cells and expressed higher levels of SDF-1, TGF- β 1, VEGF- α , and PDGF-BB, which recruited BMSCs and endothelial cells (ECs) to the defect areas. In view of this background, ZS/HA/Col based scaffolds can be considered as promising biomaterials suitable for bone tissue engineering applications.

Figure 10. (A) High-resolution microcomputed tomography (Micro-CT) imaging of rat critical-sized cranial defects implanted with ZS/HA/Col scaffolds containing different proportions of zinc silicate at 8 and 12 weeks post-surgery. No scaffolds were implanted in the blank group. Newly formed bone is indicated in yellow; the scaffolds are shown in gray [scale bar: 5 mm]. Histological analysis (Van Gieson staining) at 8 (B) and 12 weeks (C) after implantation [NB: neobone. S: scaffold. white arrows: cartilage. low-power images; scale bar: 2 mm (a,d). high-power images, scale bar: 200 μm (b,c,e)]. (D) Fluorescent double staining for alizarin red (red) and calcein (green) in the defect areas at 8 and 12 weeks after implantation [scale bar: 200 μm]. Reproduced from [103], with permission of American Chemical Society 2020. Abbreviations: 0 ZS, nano-HA/Col; 5 ZS, 5 wt% zinc silicate/nano-HA/Col; 10 ZS, 10 wt% zinc silicate/nano-HA/Col; 15 ZS, 15 wt% zinc silicate/nano-HA/Col.

13. Discussion

Nanomedicine is a field that has already revolutionized medicine, but it continues to garner interest from materials scientists, biochemical engineers, and biologists (to just name a few) suggesting the longevity of this field. The interdisciplinary nature of nanomedicine has enabled it to expand at a very rapid pace over the past few decades to where it is now finally being applied to develop improved regenerative medicine scaffolds. Still, it is in a latent phase where several nanoparticles are being used to deliver drugs and biomolecules to the site of injury for rapid healing. However, more recently, instead of bare nanoparticles, scaffolds are being developed where a compatible and durable polymeric matrix is impregnated with various nanoparticles that are functionalized with drugs, precursors, or biomolecules such as growth factors or immunomodulatory agents.

Although various nanomaterials have been developed for several bone tissue engineering applications, their features should be closely tuned so that they can mimic the tissue

micro- and nano-structure for the rapid attachment, proliferation, and differentiation of precursor MSCs facilitating osteogenic induction. Hereby, biomaterials should be modified into “biomimetic” nanostructures by a rational selection of the matrix, nanoparticles, or both. Type I collagen with more than 40 cellular binding sites can be used as a base material for the fabrication of scaffolds. Moreover, the scaffolds should be selectively functionalized with bone morphogenic proteins (BMPs) and proteins such as bone sialoprotein, osteocalcin, osteonectin, and matrix γ -carboxyglutamic acid (GLA) [104]. These biologically active minor components not only directly help in mineralization and integration of crystals in the collagen matrix, but also provide strong osteogenic signals to adjacent stimulating adult MSCs to differentiate into osteoblasts [105]. Further, functionalization with vascular endothelial growth factor (VEGF) can promote neovascularization in the wound site, and thereby accelerate healing. Designing scaffolds to incorporate blood vessels and nerves remain novel areas in orthopedic tissue engineering. Despite significant advancements in the field of bone tissue engineering, complete healing of traumatic bone defects remains a challenge (probably due to lack of attention to restore blood vessel and nerve networks within or next to bone implant materials). It should be noted that nerves enter bone alongside the arteries and remain closely associated with blood vessels inside bone, sharing common genetic pathways and stimuli. Thus, engineering innervated bone biomaterials for improved skeletal tissue regeneration would certainly avoid delayed or impaired recovery of bone fractures. Bone regulating neuropeptides such as calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), substance P (SP), and catecholamine can be functionalized on the nanotextured surface of the scaffolds to promote neurovascularized networks [106]. This innovative strategy can mimic native skeletal tissue in terms of stiffness, roughness and porosity more accurately, thus, improving the regeneration of bone tissue. Advanced technologies, such as 3D or 4D bioprinting (in which the fourth dimension is time control of implanted biomaterials), can be employed to create micro- and nano-patterned scaffold surfaces as the consequence of pits and grooves which critically control cell shape, proliferation rate, motility, differentiation, and also extra-cellular matrix production [107].

It is intriguing to ponder why in spite of their robust nature and high mechanical strength, metal nanoparticles can mimic the bone tissue microenvironment which in turn affects the adhesion and proliferation of osteoblasts, promoting the rapid healing of bone related defects. Mimicking the nanofeatures and surface roughness of the natural bone is critical when fabricating nanophase metals. This strategy would enhance osteoblast function on the scaffold surface ultimately increasing calcium deposits and bone density. Hereby, instead of using conventional metal or metal oxide nanoparticles, careful modification of the surface properties in terms of roughness and crystallinity of the nanomaterials can substantially improve orthopedic implant functionality [98].

Targeted drug delivery and triggered drug release are additional important parameters for bone tissue engineering for facilitating rapid wound healing. Surface modification of scaffolds is crucial for loading of drugs and bioactive agents such as growth factors. Several techniques such as plasma treatment, UV irradiation, chemical treatment, surface graft polymerization, and co-electrospinning of active agents and polymers can be employed either individually or in combination [108]. This might result in the generation and exposure of functional groups, such as primary amine and carboxyl groups on the scaffold surface, that can be consequently functionalized with the preferred drugs. Efficient targeted specific drug delivery can be achieved by programming stimuli responsive release systems. The conjugation of drugs on the scaffolds should be coupled with the selection of appropriate metal nanoparticles such that they can respond to a variety of stimuli such as pH, temperature, ultrasonic waves, magnetic fields, and light for triggering the release of the drugs even on-demand and only when needed [8,109]. This strategy will ensure sustained drug release, prevent unwanted circulation of drugs which dilute their active conventional at the site of need, inhibit premature drug degradation, and reduce non-specific side effects of drugs.

There are several limitations that make nanometals challenging for incorporation in scaffolds for bone tissue regeneration. The metals can leach out causing local toxicity and allergic reactions. Hereby, resorbable biomaterials are preferred as bone graft substitutes so that they can be replaced by mature bone without a transient loss of mechanical support. Since there is presently no material that can satisfy these conditions (for example, even MgO which is biodegradable suffers from toxic degradation by-products), non-resorbable metals such as stainless steel or titanium plates are frequently used to fix mechanically unstable bone defects. Hence, it is necessary to design scaffolds using resorbable nanostructures that can provide suitable mechanical strength, efficient bone replacement, and not release toxic degradation products [110–112].

Nanostructured ceramics (such as dicalcium phosphate, octacalcium phosphate, β -tricalcium phosphate, biphasic calcium phosphate, precipitated hydroxyapatite crystals, and β -calcium pyrophosphate) can ideally be incorporated into the scaffold matrix to make the biomaterial more resorbable due to cellular metabolism [113]. Likewise, the use of cellulose, hyaluronan, fibrin, collagen, and chitosan can make the scaffolds more resorbable as certain enzymes in the body can effectively degrade them [88].

Another limitation of metal nanoparticles includes their toxicity as various toxic chemicals are used during their synthesis and capping processes. Hence, more biocompatible nanoparticles should be incorporated into the scaffolds so that no toxicity is imposed on the organs in case the nanoparticles leach out of the scaffold surface. Biogenic nanoparticles synthesized using bacteria, fungi, algae, and medicinal plants can be used instead of chemically synthesized nanoparticles. Biogenic CuNPs, AuNPs, AgNPs, PtNPs, PdNPs, ZnONPs and their alloys can provide mechanical strength, resistance to microbial biofouling, and enhanced biocompatibility [114].

Fourth generation biomaterials that can exploit the role of bioelectricity in biological systems have a promising future in numerous biomedical applications if used for fabricating osteogenic scaffolds. These novel electrically-active materials could manipulate cellular bioelectric signals for tissue regeneration monitoring cellular responses and allow for communication with host tissues using bioelectric signals [115]. Electroactive biomaterials such as conductive polymers, piezoelectric materials, and carbon-based materials may serve as excellent components for scaffolds for more efficient electrical stimuli generation. Conductive polymers, such as poly(pyrrole) (PPy), may also ideally enhance bone formation due to electrical stimulation. Additionally, the piezoelectricity of bone-generated electrical fields, which are mainly attributed to aggregated charged ions and macromolecules in the bone interstitial fluid, can direct tissue-engineering scaffolds to enhance osteogenesis. Likewise, carbon-based materials, such as graphene, may also be used in scaffolds for electrical stimulation mediated tissue engineering. Of course, a dream for the use of electrically-active materials in bone tissue engineering scaffolds is to someday incorporate real time sensing of implant function and on-demand response to ensure implant success.

14. Conclusions

In this comprehensive review, we have described several metallic nanostructures that have been successfully used to fabricate scaffolds for improved bone tissue engineering. These composite nano-scaffolds can induce osteogenesis, promote mineralization, facilitate osseointegration, and also resist pathogenic biofilm mediated biofouling of implants. Further developments aim to tune the material, geometry, and architecture of these metal scaffolds so that they can mimic the tissue micro-environment, facilitate cell adhesion, proliferation, and differentiation, ensuring rapid healing of the fractures or bone defects. It is clear through this review that the wealth of knowledge created in the area of metallic nano scaffold-based tissue engineering will aid in the development of more sophisticated diagnostic and therapeutic strategies for bone injuries and defects in the decades to come.

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Appendix A

Table A1. Metal based nanoscaffolds for bone tissue engineering.

Nanoparticles	Size (nm)	Matrix	Functionalized Ligand	Cells	Reference
Copper nanoparticles	-	Chitosan	-	Rat calvarial defect	[39]
Graphene oxide-copper nanocomposites	30 nm	Porous calcium phosphate	-	Rat bone marrow stem cells (BMSCs)	[40]
Copper ion	-	Mesoporous bioactive glasses	-	-	[42]
Gold nanoparticles	80–110 nm	Mesoporous silica nanoparticles, chitosan	-	Preosteoblastic MC3T3 cells, critical-sized cranial defect site in rats	[46]
Gold nanoparticles	10.76 ± 1.4 nm	-	-	MG63 osteoblast-like cells (human osteogenic sarcoma)	[47]
Gold nanoparticles	20 nm	-	-	MSCs from Kunming mice	[48]
Gold nanoparticles	5–45 nm	-	-	Human periodontal ligament progenitor cells (PDLPs)	[49]
Ferrofluid	200	Collagen	Hydroxyapatite	MSC from human bone marrow (hBMSC)	[54]
Iron oxide nanofiber	600–1000	Poly(L-lactide)	-	MC3T3-E1	[55]
Fe powder	-	Hydroxypropylmethylcellulose	Hydroxyapatite	Rabbit bone marrow mesenchymal stem cells (rBMSCs)	[58]
Superparamagnetic iron oxide nanoparticles	160 µm to 200 µm	Poly-lactic-co-glycolic acid	-	Murine bone BMSCs, rat femoral bone	[59]
Iron oxide nanoparticles	7	Polyglucose-sorbitol-carboxymethylether	-	MSC from human bone marrow (hBMSC)	[60]
Magnetic nanoparticles	-	Polycaprolactone	-	Mouse calvarial osteoblasts	[61]
Platinum nanoparticles	-	Calcium phosphate, poly vinyl alcohol	-	Human osteoblast cells	[65]
Platinum nanoparticles	10–20 nm	Chitosan, poly (lactic acid)	Nanohydroxyapatite	Human osteosarcoma cell line MG-63 cells	[66]
Palladium nanoparticles	2–5 nm	Reduced graphene oxide	Polypyrrole (PPy)	Osteosarcoma osteogenic cell line-ATCC HTB-85 (Saos-2)	[67]
Palladium nanoparticles, gold nanorods, maghemite nanoparticles	15±5 nm, 3.4±1 nm and 10 nm	Type I collagen	Mg doped hydroxyapatite	Adipose-derived mesenchymal stem cells (hADSCs)	[68]
Palladium nanoparticles	10 nm	Chitosan	Zinc oxide nanoparticles impregnated hydroxyapatite	Human dental pulp stem cells (hDPSCs)	[69]

Table A1. Cont.

Nanoparticles	Size (nm)	Matrix	Functionalized Ligand	Cells	Reference
Silver nanoparticles	10–20 nm	Natural rubber latex	-	Mesenchymal stem cells of human deciduous dental pulp, bone defects in rat calvaria	[82]
Silver and gold nanoparticles	12.7 to 69.1 nm, 9.3 to 54.7 nm	Silk fibroin	Hydroxyapatite	Osteoblast-like cells (MG63 cell line)	[83]
Silver nanoparticles	5–15 nm	-	Collagen	Mouse mesenchymal stem cells (mMSC), mouse femoral fracture model	
Strontium and zinc		Ca–Si ceramic		Human bone derived cells (HOB), tibial bone defects in rats	[90]
Strontium	70 nm × 1.3 µm	Titania nanotube arrays	Hydroxyapatite	Saos-2 cells	[91]
Titanium	25 to 35	-	-	Human periosteum-derived cells (hPDC), critical-size femoral bone defects in Wistar rats	[95]
Titania nanotubes	25 and 80 nm (diameter), 5–10, 10–20 (width)			Mesenchymal stem cells derived from the bone marrow of Sprague Dawley rats	[96]
Titania	3–4 µm (diameter)	-	Strontium-doped hydroxyapatite	hFOB1.19 cells (human fetal osteoblast cell line)	[97]
Titania	32 nm	Poly-lactic-co-glycolic acid	-	Human osteoblast	[98]
Zinc silicate	-	Collagen	Nano hydroxyapatite	Rat BMSCs, monocytes, and aortic endothelial cells, rat critical-sized cranial defects	[103]

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