Application of Inorganic Nanocomposite Hydrogels in Bone Tissue Engineering

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SUMMARY

Bone defects caused by trauma and surgery are common clinical problems encountered by orthopedic surgeons. Thus, a hard-textured, natural-like biomaterial that enables encapsulated cells to obtain the much-needed biophysical stimulation and produce functional bone tissue is needed. Incorporating nanomaterials into cell-laden hydrogels is a straightforward tactic for producing tissue engineering structures that integrate perfectly with the body and for tailoring the material characteristics of hydrogels without hindering nutrient exchange with the surroundings. In this review, recent developments in inorganic nanocomposite hydrogels for bone tissue engineering that are of vital importance but have not yet been comprehensively reviewed are summarized.

INTRODUCTION

Bone defects caused by trauma and surgery are common clinical problems encountered by orthopedic surgeons. Severe bone defects can lead to delayed bone union and nonunion, which in turn contribute to a high rate of disability. Traditional bone defect repair methods, including autogenous and allogeneic bone transplantation, have deficiencies. Autogenous bone transplantation is the "gold standard" for bone repair, but limited materials, complications at donor sites, and the need for secondary surgery hinder its wide use. In addition, allogeneic bone transplantation may cause disease transmission and rejection reactions. Although in the past few years, rapid advancements in bone tissue engineering have solved the aforementioned problems to a certain extent, the main purpose of bone tissue engineering is to enhance bone metabolism, bone induction, osteogenesis, and bone integration to repair bone defects (Oryan et al., 2014). Due to the roles of stress-bearing capacity in bone tissue regeneration, the primary target of bone tissue engineering is to develop a hard-textured, natural-like biomaterial that enables encapsulated cells to obtain the much-needed biophysical stimulation and produce functional bone tissue (Stevens and George, 2005).

A scaffold of biomaterials is one of three elements in bone tissue engineering and exerts critical functions in cell proliferation and guiding new bone growth. A suitable three-dimensional (3D) scaffold can simulate the extracellular matrix (ECM) and support cell and tissue growth by providing a suitable microenvironment. Hydrogels, 3D networks comprising hydrophilic polymer chains embedded in a water environment, have become a study hotspot because their mechanical performance, chemical composition, and biological signals can be altered in a dynamic way to mimic specific ECMS (Chaudhuri et al., 2016). From the perspective of tissue engineering, hydrogels are capable of organizing cells into 3D structures, presenting stimuli to direct the formation of the required tissue and delivering bioactive molecules (Drury and Mooney, 2003). The softness and porous structure of hydrogels allow nutrients and oxygen to effectively diffuse into their structure. However, their lack of toughness, low mechanical stiffness, and limited recoverability restrict their biomedical applications (Mehrali et al., 2017). Moreover, the properties of hydrogels are generally modulated by changing the cross-linking type or density or the molecular weight of the polymer or by bringing in pendant functional groups, which can conversely restrict the transportation of nutrients throughout the matrix (Piantanida et al., 2019).

Nanomaterials are emerging as a new strategy for control and manipulation of the nano- and macroscale properties of hydrogels without hindering nutritive material exchange with the surroundings. Nanomaterials refer to synthesized or natural materials that are not more than 100 nm in size in at least one dimension, but for better medical application, the diameter should be in the range of 10–100 nm. Materials with a
diameter below 10 nm have reactive and toxic properties because of their larger specific surface area and activity. Materials with a diameter above 100 nm can result in embolism and can be phagocytized or separated with the help of the spleen. Moreover, it has been shown that nanotopography, rather than microtopography, is the primary influence on the structure and alignment of cells (Kim et al., 2014). Native tissues typically exist at the nanometer scale, and cells directly interact with and create a nanostructured ECM; thus nanomaterials have high biological activity (e.g., cell binding motifs) (Xavier et al., 2015), which is conducive to protein adsorption and cell adhesion and proliferation. Inorganic nanocomposite hydrogels can simulate the cell matrix microenvironment of human bone tissue. Bone tissues contain water and multiple proteins (collagen, fibronectin, laminin, and vitronectin) as soft hydrogels, whereas hydroxyapatite (HA) and Ca\(_{10}(PO_4)_{6}(OH)_2\) are inorganic components that compose hard bone tissues. Therefore, here, we focus on the application of inorganic nanocomposite hydrogels in bone tissue engineering. HA exists in the form of a linear nanocrystal with a length of 20–80 nm and a thickness of 2–5 nm, and proteins in the ECM are also at the nanometer scale. This bionic structure makes nanocomposite hydrogels more suitable for host cell colonization. The performance of obtained nanocomposite hydrogels can be the sum of the functions of the component nanomaterials and hydrogel or can be the result of new behavior, such as improved stiffness, stretchability, and cytocompatibility, potentially originating from synergistic interactions (Wang et al., 2019). For example, Liu et al. reported preparation of a hydrogel with a firm texture and good extensibility that could be elongated over 3,000% by the introduction of graphene oxide (GO) into the network (Liu et al., 2012). Nanoparticles (NPs) can not only function as chemical cross-linking agents but also be physically cross-linked with polymer chains, which allows dynamic system behavior and thus the formation of shear-thinning hydrogels. The resulting hydrogels exhibit temporarily reduced viscosity under shear stress, enabling injection through a plug flow system, whereas they return to their initial viscosity at low shear due to electrostatic interactions (Xavier et al., 2015). As a result of such properties, they can be applied in 3D bioprinting. Moreover, in addition to their structural characteristics, nanocomposite hydrogels can easily present gradients of biomolecules delivered by (porous) NPs. Piantanida et al. reported a nanocomposite hydrogel composed of a poly(amidoamine) network incorporated with mesoporous silica NPs capable of releasing chemokines, which have been observed to play an important role in tissue regeneration in vivo through their effect on mesenchymal stem cells (MSCs) (Piantanida et al., 2019).

To date, a range of inorganic nanomaterials have been synthesized for incorporation into hydrogels, including various clay-based platelets, black phosphorus (BP), ceramic NPs, magnetic nanoparticles (MNPs), graphene, and gold nanoparticles (GNPs). In this report, first, the properties of inorganic nanocomposite hydrogels for bone repair are described separately. Second, recent progress in the application of different inorganic nanocomposite hydrogels in bone repair is reviewed. The osteogenesis capacity, mechanical performance, and osteogenesis mechanism of these hydrogels, along with their advantages and current defects in osteogenesis induction, are described, and the application of these hydrogels in 3D bioprinting is highlighted. Then, we introduce nanocomposite hydrogel fabrication processes. Finally, we discuss the challenges and future development directions for inorganic nanocomposite hydrogels.

**PROPERTIES OF INORGANIC NANOCOMPOSITE HYDROGELS FOR BONE REPAIR**

**Basic Properties**

*Biocompatibility*

The best scaffold material for bone repair must not restrain the normal biological activities of cells, interfere with or inhibit molecular signaling pathways, or cause local or systemic toxicity during and after the implantation process (Williams, 2008). Furthermore, scaffold material should be capable of osteoinduction and facilitate bone remodeling and vascularization, which have positive significance for bone regeneration. Cells have good growth ability in a 3D microenvironment composed of NPs and hydrogel, which are capable of promoting protein adsorption, cell adherence, and growth due to the large specific surface area and the biomimetic structure of the nanocomposite components.

*Biological Absorbability*

The biological absorbability of scaffold materials is a very important feature for regeneration of bone tissues (Williams, 2008). An ideal scaffold material should completely degrade *in vivo* within a certain amount of time and should have variable absorptivity so that it can eventually prepare space for new bone generation. In addition, the time required for scaffold degradation should fulfill the adhesion demands. For instance, material used in spinal fusion should degrade after at least 9 months, whereas a material used...
in the skull or maxillofacial bones should degrade within 3–6 months. Nanoscaffold materials are porous, biodegradable, and provide mechanical support during the repair process (Khan et al., 2008).

Hierarchical Pore Structure

Biological tissues are hierarchical composite materials with tissue-specific chemical, mechanical, and topographical properties. Bone has ordered macropores and hierarchically ordered nano- and mesostructures composed of aligned collagen fibers and HA NPs. In the materials science and engineering fields, biomimetic materials have similar structural features, which are organized at very different length scales.

The interconnected porous structure of a material is a key factor in the design of bone repair scaffolds. The O’Brien research team proposed that when the diameter of the holes in a scaffold is 200–350 μm, osteoblast adhesion and proliferation are more favorable. Incorporating nanomaterials into a hydrogel network can improve the porosity, interconnectivity, and nano/micromorphology of the final 3D structure (Xavier et al., 2015). Pore size can be controlled by adjusting the diameter and content of NPs (Zhao et al., 2017). Designing integrated multilevel micro/nanostructures may meet more functional requirements and achieve better bone regeneration effects.

Mechanical Properties

Bone repair scaffolds should meet mechanical strength requirements and provide transfer properties. The mechanical strength of bone tissue ranges from cancellous to dense. Differences in the mechanical strength and geometric mechanics of bone tissue make designing ideal scaffolds difficult. Moreover, different types of nanomaterials can be incorporated into hydrogels, with multiple gradients and good mechanical properties, which may help in addressing this challenge. For example, Zhang et al. mixed aqueous solutions of ligands (bisphosphonates, BPs), polymer grafted with ligands, and metal ions and prepared a series of self-assembled metallic-ion nanocomposite hydrogels that were stabilized by the in situ formed ligand-metal ion (BPs-M) NPs. Specifically, the BPs grafted on hyaluronic acid (grafted-BPs) were embedded into the formed acrylated (Ac)-BPs-Mg NPs and strongly enhanced the interaction between NPs and hyaluronic acid-BPs macromers, hence increasing the mechanical properties of the hydrogels (Kunyu et al., 2019; Zhang et al., 2017a).

Osteoinductivity

NPs themselves have the ability to recruit stem cells and promote their differentiation into osteoblasts, thereby inducing bone regeneration. Motealleh et al. (2019) designed a gradient nanocomposite hydrogel and demonstrated that cells had higher affinity for periodic mesoporous organosilica nanomaterials (PMO)-incorporated AlgL hydrogels than for PMO-free AlgL hydrogels and showed enhanced cell growth at higher PMO concentrations. The PMO-based gradient hydrogels facilitated concentration-dependent cell migration toward the gradient hydrogel section that possessed a higher concentration of biopolymer-coated PMOs and subsequent osteogenic differentiation of human bone marrow mesenchyme stem cells (hBMSCs). The mechanism of osteoinduction depends on the NP type, which will be discussed in detail in the section Classification of Nanocomposite Hydrogels Based on Nanomaterials.

Properties Suitable for 3D Bioprinting

The promise of precisely controlling cell and biomaterial distributions to re-create the complexity of human tissue has made bioprinting a popular research area for tissue regeneration. Through computer-designed instructions, the 3D bioprinting technique can be used to fabricate biomaterials or cell-laden biomaterials layer by layer into well-defined 3D structures to generate tissue-like constructs.

To design advanced bioinks suitable for 3D bioprinting, certain vital parameters must be taken into consideration; these parameters are capable of dictating the interactions between individual materials based on their properties, including viscosity for uniform cell encapsulation, shear-thinning behavior to improve printability and avoid the use of highly viscous inks, gelation kinetics for structural fidelity, viscoelasticity to protect cells from shear stress, and biocompatibility to support cell viability and growth (Loo et al., 2015). Unfortunately, conventional hydrogels are weak and print poorly. Compromise between these ideals has led to bioinks with subpar printability and biocompatibility that are unable to be used to print structures taller than a few millimeters. Nanocomposite hydrogels can address two key weaknesses of conventional hydrogel networks: stress concentrations caused by a heterogeneous network structure and poor
mechanical energy dissipation capacity. NPs can be physically mixed with polymer chains, which allows dynamic behavior in the system and thus formation of shear-thinning hydrogels. Specially, the self-assembled injectable nanocomposite hydrogels designed by Zhang et al. mentioned in the section Mechanical Properties showed that some grafted BPs may loosely bind to the surface of NPs via dynamic and reversible coordination and contribute to the injectability and self-healing properties of the hydrogels. This dynamic dissociation and recombination enables HA-BP-Mg nanocomposite hydrogel to be effectively delivered and reshaped, thereby filling irregular-shaped bone defects through minimally invasive procedures (Zhang et al., 2017a, 2018).

Angiogenesis
Angiogenesis enhancement is a vital element in bone repair because of the high blood demand of bone tissues. In vivo, cells and tissues inside scaffolds must have a sufficient supply of oxygen and nutrients. In addition, the delivery of oxygen and nutrients can be hindered by incorrect or insufficient angiogenesis, which can induce cells to differentiate in uncontrollable directions or induce cell apoptosis (Malda et al., 2004). Some types of nanocomposite hydrogels are capable of inducing angiogenesis. For example, Filippi et al. reported that poly(ethylene glycol) (PEG)-based hydrogels incorporating iron oxide NPs and human adipose-derived stromal vascular cells prompted robust vascularization of tissue (Filippi et al., 2019).

The Ability to Produce and Release Oxygen
Oxygen (O₂) is the most important nutrient necessary for cell survival. Insufficient oxygen delivery can prevent cell migration and neovascularization, which then reduces cell growth and differentiation. In addition, in the case of insufficient oxygen in the tissue engineering structure, hypoxia often occurs, which reduces the efficiency of the engineering tissue. Oxygen-generating nanocomposite hydrogels, in which the release kinetics of oxygen can be controlled to occur over prolonged time periods, allow hypoxia to be avoided and cell viability to be maintained before the engineered tissue is vascularized (1–2 weeks) by the host system (Park and Park, 2018). Andisheh described a new injectable O₂-generating 3D biomaterial (BPO–AlgL) prepared by using an organic peroxide, namely, benzoyl peroxide (BPO), and Laponite incorporated into an alginate hydrogel (AlgL) (Motealleh and Kehr, 2020). BPO–AlgL was injectable and showed sustained release of O₂ over a period of 14 days. The results showed that BPO and its incorporation into a 3D network of AlgL have beneficial effects on cell viability under normoxic and hypoxic conditions. BPO-embedded AlgL was able to reduce hypoxia-induced cell death and enhance cell viability by providing sustained O₂ within the 3D AlgL scaffold.

Drug Loading and Release Performance
Due to the large and highly charged specific surface area generated by nanomaterials and a highly porous structure, nanocomposite hydrogels are capable of entrapping hydrophilic or hydrophobic drugs with high loading efficiency when compared with nanomaterial-free hydrogels and polymers. Various characteristics of the nanocomposite hydrogel network, such as drug encapsulation efficiency, drug release behavior, and drug stability, can be adjusted by modulating a variety of factors (including the size and pore size of nanomaterials, the stimuli-responsive characteristics of nanomaterials, and the cross-linking density of polymers that affect the porosity of the hydrogels) (Tutar et al., 2019). For example, taking advantage of the magnetic response characteristics of magnetic nanocomposite hydrogels, nanocomposite hydrogels can achieve high drug loading and prolong or control the release period. Nanodiamonds (NDs), a carbon-based nanomaterial, have been used as nanocarriers in GelMA hydrogel-based photocrosslinkable drug delivery systems and improved the mechanical properties of GelMA. The final GelMA–ND system was used for stem cell-guided bone regeneration via sustained release of the osteoinductive drug dexamethasone (Dex) into the surrounding GelMA from the NDs (Figure 1) (Pacelli et al., 2017). In addition, the design of injectable nanocomposite hydrogels allows delivery of drugs from specific sites to the tissues that most need the drugs.

Antimicrobial Effect
The problem of bacterial drug resistance is imminent, and therefore, before considering bacterial adhesion, biomaterials should be modified to promote tissue integration (Ribeiro et al., 2017). Some NPs, such as graphene oxide and GNP, have antibacterial activity. Thus, nanocomposite hydrogels can reduce bacterial infection during bone reconstruction surgery or hospital infection.
CLASSIFICATION OF NANOCOMPOSITE HYDROGELS

Mineral Nanocomposite Hydrogels

Clay minerals, also known as sheet silicate or phyllosilicate, are a family of inorganic layered nanomaterials traditionally defined as “minerals that impart plasticity to clay and that harden upon drying or firing” (Guggenheim and Martin, 1995). According to the chemical composition and morphology of the NPs, nanoclays can be divided into several categories, such as montmorillonite ([Al₃₋₂Mg₂₋₃] (Si₈O₂₂(OH)₄)Na₀₋₁), Laponite ([Mg₅₋₆Li₀₋₁] Si₈O₂₂(OH)₄)Na₀₋₁), hectorite ([Mg₅₋₆Li₀₋₁] Si₈O₂₂(OH)₄)Na₀₋₁), and halloysite (Si₂Al₂O₅(OH)₄·nH₂O).

Among these nanoclays, Laponite has dominated the field of bone tissue engineering. Laponite is composed of high-aspect-ratio nanoplatelets that are approximately 25 nm wide and 1–2 nm thick (Gaharwar et al., 2013) with a negatively charged surface (due to the presence of OH groups), allowing it to be easily dispersed in water at low concentrations (Jatav and Joshi, 2014; Mehrali et al., 2017). In addition, Laponite can induce hMSCs to differentiate into osteoblasts in culture medium without growth factors.

Figure 1. Modulation of Dexamethasone Release Using GelMA/ND Nanocomposite Hydrogels and Assessment of Osteogenic Differentiation of hASCs

(A) Schematic representing the different diffusion rate of dexamethasone with higher retention of the drug in the nanocomposite hydrogels compared with the scaffold without NDs.

(B) Comparative release profiles of dexamethasone-fluorescein isothiocyanate (Dex-FITC) from GelMA hydrogels and the nanocomposite scaffold over a week. Results are indicated as mean ± SD, (n = 4).

(C) In the first row, images are displaying alkaline phosphatase (ALP) staining of human adipose stem cells (hASCs) encapsulated in GelMA hydrogels after 14 days of culture in osteoconductive media. In the second and third rows, alizarin red staining of hASCs at 14 and 21 days of culture in osteoconductive media (scale bar, 100 μm).

(D) ALP quantification of hASCs after 14 days of culture in osteoconductive media showing a significant increase in ALP expression for the nanocomposite system compared with the control groups. Results are reported as mean ± SD (n = 3).

(E) Calcium content quantification after 14 and 21 days of hASC differentiation induced by osteoconductive media. Results are shown as mean ± SD (n = 3). Groups tested are GelMA hydrogels (Ctrl), GelMA/ND 0.2% w/v nanocomposite scaffold without Dex (ND), GelMA hydrogels containing the free drug (Dex), and the nanocomposite system containing the complex (ND/Dex) (**p < 0.01, ***p < 0.001). Open access. Reprinted with permission from Pacelli et al. (2017). Copyright (2020) Nature Research journals.

Mineral Nanocomposite Hydrogels

Nanoclay-Based Hydrogels

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is mainly controlled by the ion dissolution of intracellular Laponite and the subsequent increase in the concentration of ions and minerals such as magnesium, silicate, and lithium, and all degradation products are nontoxic (Gaharwar et al., 2013; Mihaila et al., 2014). Magnesium is related to the activation of osteogenesis-related pathways (HIF-1α and PGC-1α) and is also important for integrin adhesion to the surface of biological materials (Zreiqat et al., 2002). Silicate can increase calcification/mineralization of the bone matrix, promote collagen type 1 synthesis by increasing prolyl hydroxylase activity, and guide osteogenic processes in preosteoblast cells (Ha et al., 2014). Lithium can inhibit glycogen synthase kinase-3 (GSK3) and thus activate canonical Wnt-responsive osteogenic genes (Kim et al., 2013).

Various other models have been proposed to illustrate that the mechanism by which clay promotes osteogenesis is not related to clay dissolution (Figure 2). A common characteristic of enhanced osteogenesis related to clays is the stable and inchoate enhancement of CaP mineralization (Xavier et al., 2015). For instance, compared with the control, the mineralized matrix produced by cultivation of bone growth on a nanocomposite containing Laponite nearly doubled (Gaharwar et al., 2013). This might be because the heterogeneous and anisotropic charge structures of clay NPs and their aggregates, along with the affinity of silica for Ca²⁺, may provide favorable nucleation sites to promote Ca²⁺ and HPO₄²⁻ adsorption, thereby reducing the energy barrier for CaP deposition (Teng, 2013). Some studies have also shown evidence of the inherent “bioactivity” of certain clays, in which case this is understood as the particular capability to induce biomineralization in simulated body fluids (Wang et al., 2014). Ambre et al. (2011) tried to improve the effectiveness of biomineralization induced by clays by developing montmorillonite (MMT) clays modified with an amino acid. Similarly, due to its large and highly charged specific surface area, clay may also enhance osteogenesis by aiding in the intracellular absorption of biologically active molecules and stabilizing extracellular growth factor concentrations (Gibbs et al., 2014). For example, the ability of clay to take up and bind biologically active molecules has been used to initiate the formation of new vessels in a wound with the help of vascular endothelial growth factor localization and to stimulate osteogenesis at notably reduced...
bone morphogenetic protein (BMP) doses (Gibbs et al., 2016). Another example showed that stable Laponite@BMP-2 complexes can be formed by the strong static binding between Laponite nanoplatelets and BMP-2 to effectively preserve the intrinsic bioactivity of BMP-2 and prolong the release period (Kim et al., 2020; Zhang et al., 2020).

Clay nanocomposite hydrogels are innovative gels developed by Haraguchi (2007). These clays are capable of interacting with hydrogel networks through their electric charges. Compared with conventional hydrogels, clay nanocomposite hydrogels have superior properties, including mechanical properties, rheological properties, swelling capacity, biosorption, and bioadhesion. These properties allow generation of porous matrices with mechanical properties similar to those of a bone matrix without losing the inherent processability of the matrix. Hydrogel formation usually requires initiation of polymerization on the surface of a silicate; then a brush-like polymer grows from the flat surface and connects the surrounding nanosilicate particles to form a cross-linked network. The elongation at break of these hydrogels is at least 1,000% (Haraguchi and Takehisa, 2002). PNIPAAM/Laponite XLG nanocomposite hydrogels can be considered the most typical clay-based nanocomposite hydrogels. The tensile modulus, elongation rate, and tensile strength of PNIPAAM/Laponite XLG nanocomposite hydrogels are as high as 453 kPa, 1,000%, and 1.1 MPa, respectively, and the breaking energy is not less than 1,000 times greater than that of classical PNIPAAM hydrogels (Haraguchi and Li, 2006). The nanocomposite hydrogel synthesis method has an effect on the strength of the hydrogel. Highly ordered layered nanocomposites have high mechanical strength and can be made by using shear-flow-induced alignment of two-dimensional (2D) nanosheets at an immiscible oil interface (Zhao et al., 2020). This strategy, which can be readily extended to align a variety of 2D nanofillers, could lead to the development of high-performance composites.

The preosteoblast cell line MC3T3 and BMSCs have been cultured on nanocomposite hydrogels of Laponite and PEO, PGS, PEG, poly(ethylene glycol) diacrylate (PEGDA), L-pNIPAM-co-DMAC, PCL, PNAGA, GelMA, regenerated silk fibroin, chitosan, collagen, natural polysaccharides (hyaluronic acid and dextran), gelatin gum, and alginate, resulting in an improved osteogenic response and mechanical properties. Specifically, the addition of Laponite nanoclay led to a significant increase in the Young’s modulus of the resulting hydrogel—Laponite composite: a 1.9-fold enhancement for a PEGDA-Laponite composite, 3.3-fold enhancement for a gelatin-Laponite composite (Jin et al., 2017), 4-fold enhancement for a GelMA-Laponite composite (Xavier et al., 2015), and 7.4-fold enhancement for an alginate-Laponite composite. High incorporated concentrations of nanosilicates (usually no less than 2.5 w/v % of the polymer matrix) are indispensable for achieving an osteogenic effect (Xavier et al., 2015). Changing the silicate concentration within the clay nanocomposite system can modify cellular adhesion, spreading, and proliferation. Gaharwar et al. reported that enhancing the Laponite clay concentration in PEO from 40% to 70% resulted in a 10-fold enhancement in CaP mineralization and alkaline phosphatase (ALP) activity within 28 days, along with a remarkable increase in the expression of genes related to osteogenesis (Gaharwar et al., 2011).

Laponite can form reversible electrostatic bonds with charged polymers that are capable of forming and breaking dynamically, making Laponite a perfect candidate for use in designing shear-thinning injectable hydrogels. The reversibility of the electrostatic bonds allows the hydrogels to recover immediately after injection, thus restoring the prestrain modulus when shear is removed. When Laponite is mixed with hydrogel precursors, the resultant nanocomposite hydrogels can retain their self-supporting capacity, can be printed into 3D structures directly in air, and can retain their shapes before cross-linking (Ahlfeld et al., 2017). After that, the entire structure can be solidified in situ using proper cross-linking stimuli. Recently, methods have been developed that employ the dynamic mechanical properties that are conferred by clay NPs to optimize bioinks for 3D printing. By controlling the viscosity and shear-thinning characteristics (related to clay content) of the pregel solution, tough hydrogels with various complex structures have been formed. In addition, an extrusion-based direct-writing bioprinting strategy can be utilized to fabricate microstructured bone-like tissue constructs containing a perfusable vascular lumen. Byambaa et al. (2017) developed cell-laden cylinder elements made of GelMA hydrogel loaded with silicate nanoplatelets to induce osteogenesis and synthesized hydrogel formulations with chemically conjugated vascular endothelial growth factor to promote vascular spreading. The engineered construct was found to support cell survival and proliferation during in vitro maturation. Additionally, the entire construct demonstrated high structural stability during in vitro culture for 21 days. Moreover, the GelMA-Laponite hydrogels loaded with BMSCs showed no signs of cell apoptosis or inflammatory cytokine responses. During in vivo experiments using an immunocompetent rat model, the hydrogels showed minimal localized immune responses (Paul et al., 2016).
Laponite nanoclay can also be incorporated into DNA-based hydrogels to form a 3D network via electrostatic interactions. Sayantani et al. (2018) reported a DNA-based nanocomposite hydrogel whose noncovalent interactions involved interaction of the DNA backbone with 2D silicate nanodisks, and the hydrogel can be injected to regenerate bone and continuously release therapeutics (Figure 3). The silicate nanodisks enhanced the mechanical elasticity of the formulated hydrogels by creating additional network points through attractive electrostatic interaction with the DNA backbone. The elastic and yield stress of the thermostable hydrogel increased with increasing nSi concentration. These hydrogels can form self-supporting structures after being injected because of their rapid recovery upon removal of cyclic stress. Furthermore, the presence of nanosilicate can regulate the release of Dex. The biological activity of the released Dex was confirmed by the osteogenic differentiation of adipose-derived stem cells (ADSCs) in vitro and in vivo in a rat model of cranial bone defect osteogenesis.

Nanoclay-based hydrogels, a type of biological material, have remarkable potential for bone tissue engineering. To fully exploit the potency of Laponite in various skeletal regeneration strategies, future studies should focus on exploitation of stronger nanocomposites.

Ceramic-Based Nanocomposite Hydrogels

Ceramics are composed of ions (K, Ca, P, Mg, and Na) and have only minimal toxicity to human tissues in the presence of a physiological environment or ions (Al and Ti). As reported by Tanaka and Yamashita (2008), ceramics are commonly divided into two groups based on their chemical composition: calcium phosphate (CP) and others, including yttria (Y₂O₃)-stabilized tetragonal zirconia (ZrO₂) (YTZP), alumina (Al₂O₃), and some silicate and phosphate families of glasses and crystallized glasses (glass ceramics) (Tanaka and Yamashita, 2008). As HA largely constitutes the inorganic phase of bone (approximately 65% of the inorganic phase) and is regarded as one of the most biologically compatible substances among all orthophosphate molecules, it can act as a candidate material for bone grafting. The stoichiometric chemical formula of HA is Ca₁₀(PO₄)₆(OH)₂, and this material shares similarities with the mineral phase of bone.

Figure 3. Schematic Representation of the Design Strategy for the Development of Multifunctional Nanocomposite Hydrogels

DNA-nSi injectable hydrogels are formed via a two-step gelation method. The first step consists of an intermediate weak gel (pregel) formation by heating and subsequent cooling of double-stranded DNA. The denaturation of double-stranded DNA followed by rehybridization in a random fashion facilitates the development of interconnections between adjacent DNA strands (type A network points) via complementary base pairing. Introduction of nSi in the second step of the gelation process increases the number of network points (type B) via electrostatic interaction with the DNA backbone, resulting in a shear-thinning injectable hydrogel. Reprinted with permission from Sayantani et al. (2018). Copyright (2020) American Chemical Society.
Once nanoHA gradually degrades, it releases calcium (Ca$^{2+}$) and phosphate (PO$_4^{3-}$) ions, which are able to modulate cellular behavior in the microenvironment to regulate the bone mineralization process and bond to the surrounding tissues. Corresponding studies have reported increased expression of HA-mediated bone markers (such as osteopontin, osteocalcin, and ALP) due to the exceptional and native-like osteoinductive nature of HA (Sadat-Shojai et al., 2015).

However, the slow degradation rate of HA in bone remodeling limits its application as a bone filler. As a result, compared with HA, carbonate apatite can be applied as a more selective and better candidate for bone substitution purposes. Furthermore, a series of ceramic-based materials have been applied in bone engineering, including Mg-substituted carbonate HA, tricalcium phosphate (TCP), β-tricalcium phosphate (β-TCP), and biphasic calcium phosphate (BCP) ceramics. Based on their bioactive properties and mature preparation process, a range of ceramic implant materials have been introduced into the market. Ceramic is generally considered to be brittle, which indicates that pure materials may not match the mechanical requirements of complicated physiological environments; furthermore, their limited bioactive properties are unable to keep pace with clinical requirements.

Given such problems, ceramic-based nanocomposite hydrogels provide an alternative strategy for bone tissue engineering. This type of composite combines the osteoinductive properties of nanoceramics with the flexible and biocompatible properties of hydrogels to mimic the natural environment and components of bone tissues, resulting in excellent bone repair properties. A study performed by El-Ghannam et al. (1997) showed that the initial reaction of biologically active glass (a type of bioceramic) results in a local increase in pH, which can neutralize the acidity generated by the polymer biodegradation process. Natural hydrogels derived from the human body or animals, such as gelatin, collagen, alginate, and silk fiber, were composites with BCP to significantly improve the mechanical properties of scaffolds and cell adhesion and proliferation (Chen et al., 2018). In addition, compared with original BCP scaffolds or pure hydrogels, composite scaffolds show better mineralization ability and are more likely to form bone apatite during the bionic process (Thein-Han and Misra, 2009). Moreover, to access more tunable properties, synthetic polymers have also been chosen for construction of ceramic-based hydrogels. Yu et al. (2019) reported a PEGylated poly(glycerol sebacate)/β-TCP NP composite. As PEG itself exhibits osteoconductivity, when combined with β-TCP ceramic, the composite exhibits excellent inductivity. Moreover, PEG endows the hydrogel with surface attachment properties, which promote compatibility of implants with tissues.

Considering the multicomponent nature of natural tissue, a 3D environment similar to the bone matrix was designed by Sohier et al. (2010). Specifically, a biocompatible hydrogel (silated hydroxypropylmethyl cellulose) was used as an ECM, whereas BCP was used to replace the mineralized matrix. These homogeneous structures enabled cells to survive, proliferate, and interact. Furthermore, based on the heterogeneous structure of bone tissue, researchers have focused on gradient ceramic-based nanocomposite hydrogels to match the true microenvironment. For instance, a double-layer biohybrid gradient hydrogel scaffold with a gradient composition (TGF-β1 loaded in the top layers and β-TCP present in the bottom layers) was constructed to mimic the articular cartilage-subchondral bone architecture (Gao et al., 2018) (Figure 4). The resultant gradient scaffolds sustained remarkable fidelity and resolution of the emulated architecture, with highly interconnected porosity, ideal mechanical performance, and prominent biological compatibility. In vivo animal experiments have verified that biohybrid gradient hydrogel scaffolds can simultaneously promote regeneration of cartilage, subchondral bone, and load-bearing tissues.

Some authors (Eosoly et al., 2012; Zhang et al., 2014) have reported that the concentration of nanoHA incorporated into polymer-based composites can modulate the level of solubility; bone-like apatite layer formation; cell attachment, proliferation, and differentiation; and bone growth rate. Ferraz et al. reported that hydrogels with 30% nanoHA enhanced the proliferation of osteoblasts and the expression of osteogenic transcription factors, whereas hydrogels with higher concentrations (50% and 70%) decreased the osteogenic cell response (Barros et al., 2019). Roohaniesfahani et al. (2019) developed an injectable silk composite incorporated with a triphasic ceramic called MSM-10 (54 Mg$_2$SiO$_4$, 36 Si$_2$Sr$_5$, and 10 MgO [wt%]) at various weight percentages (0.1, 0.6, 1, and 2). Increased loading of MSM-10 was found to hinder the gelation kinetics of the silk hydrogel through a reduction in beta-sheet phase formation, which in turn affected the compressive strength, injection force, microstructure, and in vitro degradation rate of the hydrogels; in particular, the incorporation of MSM-10 (0.6 wt%) into a silk composite showed promising properties for bone regeneration. Kwon et al. (2018) prepared GelMA cryogels with varying concentrations of bioglass NPs. In vivo tests in a mouse cranial defect model, microCT, and histology indicated that the GelMA
cryogel with the highest concentration of bioglass NPs (2.5 w/w %) induced more bone formation than the other tested groups.

To summarize, with their marketable mechanical performance, great biological compatibility, and excellent osteoconductivity, ceramics are promising nanofillers for hydrogel-based orthopedic treatments. In addition to being biologically active, these materials also maintain physical integrity and can be organically integrated with the surrounding hard tissue. To extend the clinical application of ceramic-based hydrogel grafts, a large number of research achievements are needed to address the difficulties related to their sub-optimal degradability, mechanical strength, and elasticity.

**Conductive Nanocomposite Hydrogels for Bone Tissue Engineering**

**Magnetized Nanocomposite Hydrogels**

Magnetized nanocomposites are formed from a hydrogel and MNPs. Iron oxide NPs (IONPs) are the most commonly used MNPs. Their diameter ranges from 1 to 100 nm. When IONPs are in a certain displacement range and under special conditions, they are superparamagnetic, and thus, they are also called...
superparamagnetic iron oxide NPs (SPIONs). Currently, the SPIONs iron hematite (γ-Fe₂O₃), magnetite (Fe₃O₄), ferrite, and other iron-based oxides are the most commonly used.

An increasing number of studies have confirmed that IONPs have an effect on BMSC differentiation. On the one hand, magnetic scaffolds can promote bone regeneration, which can be explained by evidence showing that polymer/IONP scaffolds can promote bone regeneration in both the presence and absence of a static magnetic field (SMF). The mechanisms are as follows: cells in contact with magnetic scaffolds can experience nanoscale forces similar to mechanical forces, which stretch cell membranes and activate channels and receptors (Zhang et al., 2017b). In addition, IONPs exhibit catalase-like activity under cytoplasmic neutral conditions, decomposing H₂O to produce H₂O without cytotoxicity (Chen et al., 2012). This special nature of IONPs accelerates cell cycle progression in MSCs and promotes cell proliferation by activating the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Huang et al., 2009). Generally, IONPs decompose into Fe³⁺ and PSC molecules in the acidic environment of endosomes or lysosomes. These two components will not promote MSC differentiation into osteoblasts; therefore the promotion effect likely requires the participation of integral IONPs. On the other hand, magnetic fields might be capable of inducing membrane phospholipid rearrangement and triggering the material sensing system of cells, for example, by activating Ca²⁺ channels on the cell membrane and stimulating different signaling pathways, such as the BMP-2, integrin, and MAPK pathways (Zhu et al., 2017). Kim et al. explored the effects of electromagnetic fields (45 Hz, 1 mT intensity) and MNPs (50 μg/mL Fe₃O₄) on hBMSCs alone or in combination and found that using electromagnetic fields alone and MNPs alone can promote osteogenic differentiation and using MNPs combined with electromagnetic field exposure is more effective in increasing osteogenic differentiation (Min-Ok et al., 2015). In addition, the structure and orientation of ECM proteins can be influenced by an SMF. A specific protein complex (magnetoreceptor, MagR) with an intrinsic magnetic moment has been reported to sense the direction of the magnetic field and deflect with it. MagR is composed of two proteins: iron-sulfur cluster assembly protein (human homologous gene ISCA1) and cryptochrome (human homologous gene Cry1) (Qin et al., 2016). Studies have shown that the ISCA1 protein alone is not sufficient to induce the cell to respond to a magnetic field, and thus, the integrity of the protein complex is very important (Pang et al., 2017). Some magnetically responsive proteins are related to the actin cytoskeleton, focal adhesions, integrin binding, stress fibers, and cell responses to mechanical stimulation. When the MagR protein complex in the cell is aligned in the direction of a magnetic field, torque or pressure is generated toward the mechanical force receptors (such as actin fibers, adhesion sites, and integrins) in the cell (Winklhofer and Kirschvink, 2010), accompanied by actin redistribution or integrin-mediated MAPK signaling pathway activation; as a result, mechanical signals can be transmitted to the nucleus to affect the expression of osteogenic genes (Wang, 2009).

In summary, many magnetic biomaterials have been exploited in recent years. MNPs impart magnetic responsiveness and remarkable mechanical performance when combined with hydrogels. The magnetic and mechanical properties of magnetic hydrogels largely depend on the type of magnetic particles incorporated, their content, and their particle size. Wang et al. (2018b) designed a magnetic chitosan hydrogel embedded with MNPs that showed a magnetic response and improved morphological and mechanical features, and the mechanical properties (such as compression strength, yield strength, and probe displacement) of the magnetic hydrogel were enhanced with the rising MNP concentration from 0 to 15 wt %, which was attributed to the cross-linking role of MNPs in the process. Furthermore, the mechanical properties of the nanocomposite hydrogels increased with increasing size of the NPs. Smaller Fe₃O₄ NPs show inevitable aggregation, which results in fewer linking points on NP surfaces than larger Fe₃O₄ NPs (Jaiswal et al., 2016). Because of their intrinsic magnetic performance, these materials are capable of improving cell adhesion and promoting osteogenesis (Filippi et al., 2019) and thus show great potential as bone substitutes. Aldebs et al. reported a 3D hydrogel scaffold based on self-assembled RADA16 peptides containing IONPs that could promote bone regeneration (Aldebs et al., 2020). Magnetic hydrogels respond to changes in the magnetic field of the external environment, which induce vibrational and rotational displacement of SPIONs, and the surface of the gels exhibits micropores and unevenness that are conducive to cell adhesion and growth. Based on this property, Siu Hong et al. designed a platform composed of a hydrogel substrate conjugated with arginylglycylaspartic acid (RGD)-bearing MNPs that can dynamically and reversibly modulate cell-matrix interactions (Wong et al., 2020) (Figure 5). The magnetic hydrogels had a small swelling ratio, allowing them to better support cell survival, eventually facilitating the formation of new tissues. The osteogenesis promotion effect of IONPs or SMFs depends on the particle magnetism and the strength of the magnetic field. An SMF of moderate intensity (1 mT-1 T) can stimulate proliferation and osteoblastic differentiation of human BMSCs (Feng et al., 2010).
Magnetic nanocomposite hydrogels can be used to create a new platform with enhanced angiogenesis induction capacity for bone regeneration. The activity of cells inside implanted constructs is impaired if the distance from the cells to the nearest capillary network exceeds the diffusion limit of nutrient materials and oxygen (100–200 μm) (Liu et al., 2017). Thus, the regenerative potential of engineered tissues is greatly limited by insufficient angiogenesis.

Filippi et al. (2019) designed magnetized nanocomposite hydrogels consisting of PEG-based hydrogels with IONPs and stromal vascular component cells incorporated in human adipose tissue. The constructs not only promoted matrix mineralization and bone tissue formation but also prompted robust vascularization of the tissue compared with the effect of MNPs or an SMF alone. Furthermore, magnetic nanocomposite hydrogels can achieve time- and space-controlled release of loaded drugs because the IONPs inside the hydrogels have a tendency to rotate to align with the EMF, resulting in mechanical vibration of the polymeric chains in the matrix, thus promoting drug release (Mahdavinia et al., 2015). For example, Madani et al. designed a magnetically responsive hydrogel system (Madani et al., 2020) that contained an inner ferrogel compartment enabling electromagnetically triggered release of BMP-2. The timing of the BMP-2 release could be remotely controlled, ranging from immediate release to a delay of up to 11 days.

Notably, IONPs tend to aggregate in hydrogels due to their anisotropic dipolar attractions and high surface energy. A high aggregation rate can impede blood vessels under physiological conditions.
Functionalization is considered to be effective in improving NP dispersion. For example, IONPs can be coated with an amphiphilic layer comprising natural or artificial materials, such as polyethylene glycol, chitosan, and dextran. IONPs can also bind with complex biological molecules, such as peptides, antibodies, or hormones (Sadeghiani et al., 2005). Bare NPs are generally considered to be more toxic than coated NPs, and thus, functionalization of IONPs also reduces their cytotoxicity. In addition, IONPs might be ingested by cells and cause impaired mitochondrial activity, morphological changes, and membrane leakage. IONPs physically coated on scaffolds might leak into the surrounding environment, which would weaken the inductive coupling magnetic effect. One solution is to chemically connect the embedded IONPs to the hydrogel so that they are less likely to diffuse into the surrounding environment and be ingested by surrounding cells (Huang et al., 2020).

**BP-Based Hydrogels**

In recent years, BP has emerged as a new 2D material, with each layer composed of phosphorene. Black phosphorus nanosheets (BPNs) can be easily oxidized and degraded in aqueous media. In addition, the degradation product is nontoxic phosphate. Indeed, phosphorus is a primary constituent of bone and constitutes approximately 1% of the entire human body weight. BP-based hydrogels have shown excellent mechanical strength and high intrinsic bioactivity, with great potential for use in bone repair. After BP-based hydrogels are implanted into bone defect regions, BPNs first recruit bone-forming cells to the region and then absorb signaling molecules that are secreted by the recruited cells to initiate bone formation. At the same time, BPNs continually release phosphate acid to extract Ca\(^{2+}\) from the physiological surroundings, thus generating new CaP nanoparticles (Figure 6) (Wang et al., 2019). In addition, osteoclasts and osteoblasts are capable of engulfing and degrading CaP to promote osteointegration by remodeling of newly formed bones (LeGeros, 2008). The deposited signaling molecules and biomineralized CaP can mimic some functions of the ECM of bone in terms of chemical structure and bioactivity. This process also provides a surface for cell adhesion and differentiation. Wang et al. incorporated methacrylate gelatin, polysaccharides (alginate and chitosan), inorganic BP nanocrystallites, and mineralized CaP nanocrystallites into hydrogels, and the results showed that the biologically active components combined with the high mechanical performance of the BP-based hydrogels improved osteoblast activity and promoted bone formation (Wang et al., 2019). A BP-based hydrogel platform developed by Huang et al. was fabricated via photocrosslinking.
of cationic arginine-based unsaturated poly(ester amide)s, BPNs, and GelMA. The introduction of BPNs improved the mechanical properties of the hydrogels, facilitated photoresponsive release of phosphate, and accelerated mineralization in vitro. In vivo experiments showed that the BPNs promoted bone regeneration. In a PVA/BP composite hydrogel, the addition of 0.02, 0.05, and 0.1 w/v % BPNs led to an enhancement in the modulus to 4.4 ± 0.8, 5.3 ± 1.0 and 7.3 ± 1.4 kPa, respectively (Yang et al., 2018b). All these studies suggest that a continuous supply of calcium-free phosphate and BP-based hydrogels are promising for bone regeneration.

BP-based hydrogels show good printability. For example, Miao et al. accurately controlled the deposition head and substrate temperature to print control hydrogels and BP/gel nanocomposite hydrogels. The printing process for the control hydrogels required a high solid concentration. The control hydrogels had difficulty withstanding gravity. In contrast, the BP/gel nanocomposite hydrogels printed well and showed better stability with an accurately designed geometry. This result suggests the great potential of BP-based hydrogels as bioinks for repairing tissue defects. In addition to promoting bone regeneration, BPNs also have antimicrobial functions. Thus, BP-based hydrogels can act as photothermal agents and repair tumor-induced bone defects. Yang et al. produced scaffolds made from BPNs combined with BioGlass (BG) via 3D printing, and the survival rate of osteosarcoma cells on BP-BG scaffolds decreased significantly after near-infrared (NIR) irradiation (Figure 7). The results demonstrated that BP-BG scaffolds have high photothermal therapeutic efficacy in vivo, making in vivo oncology applications feasible (Yang et al., 2018a). Together, these studies reveal that 3D printing with biomaterials doped or coated with BP could be useful and provide a stepwise countermeasure to improve the therapeutic effects of postoperative defects in osteosarcoma.

BPNs are excellent drug carriers due to their high specific surface area and can achieve controlled release of drugs due to the photothermal effect. The drug loading efficiency of BPNs can reach 950%, and when a drug is adsorbed to BPNs through nonchemical bonds, the activity and impurities of the drug are ensured. BP can accelerate the release of drugs under irradiation with NIR light. Yang et al. manufactured a new NIR photothermal-responsive PVA/BP nanocomposite hydrogel (Yang et al., 2018b). The 3D physically cross-linked networks (hydrogen bonds between PVA crystallites and PVA chains and hydrogen bonds between Congo red [CR, chosen as a model drug molecule] and the hydrogels) remained stable without NIR irradiation. Thus, relatively low mobility of CR molecules was observed. Under NIR irradiation, the networks of the nanocomposite hydrogel were destroyed, resulting in enhanced mobility of CR molecules, inevitably leading to an increase in CR release. This is because the photothermal conversion induced by BPNs can generate heat at the nanocomposite hydrogel, causing PVA crystallites to partially melt and destroy hydrogen bonds in the networks. After withdrawal of NIR irradiation, the heat dissipates, and the nanocomposite hydrogels gradually cool. The destroyed hydrogen bonds recoccur in the nanocomposite hydrogels, allowing the destroyed networks to be reconstructed and again limiting the mobility of the CR molecules. Thus, the PVA/BP hydrogels again show a slower rate of CR release. Therefore, the PVA/BP synthetic hydrogel shows potential as an on-demand drug release system. The NIR-triggered osteogenesis-related drug release system can be applied in bone regeneration. Wang et al. reported BP-SrCl2/PLGA microspheres, from which Sr2+ release could be photothermally controlled (Wang et al., 2018a). The results showed that the BP-SrCl2/PLGA microspheres had good cell viability, biodegradability, and bone regeneration capacity.

We can conclude that BPNs can endow hydrogels with multiple functions, including improved mechanical performance, enhanced bone regeneration, and photothermal performance, which shows great potential in bone tissue engineering applications.

**Graphene-Containing Hydrogels**

Graphene was first separated by Novoselov and Geim in 2004 and is a monoatomic 2D nanoscale crystal formed by carbon atoms interconnected by triangular hybridized (sp2 hybridized) orbitals (Cheng et al., 2018). Graphene is currently known as the 2D material with the largest strength and the smallest thickness. GO is obtained by chemical exfoliation of graphene and has many oxygen-containing functional groups. GO has many excellent properties, such as good biocompatibility, biodegradability, antibacterial properties, adsorption capacity, and low toxicity (Schinwald et al., 2014). Since their discovery, graphene and GO have set off research booms in many fields, and research on GO has gradually extended to the field of bone regeneration. Reports have shown that GO can promote adhesion, proliferation, and differentiation of
BMSCs, MSCs, and other osteoblast-related cells (Nishida et al., 2016). The osteogenesis-promoting effect of GO is due to multiple mechanisms. First, GO provides a microenvironment suitable for long-term cell survival and regulates cell behavior through “cell-substrate” interactions. Second, GO can significantly adsorb extracellular proteins and osteoinductive factors because it has a large surface area and can provide a large number of active sites for interactions between the material and proteins; oxygen-containing groups, such as negatively charged carboxyl groups and hydroxyl groups, on the surface of graphene derivatives can react with positively charged proteins; and the π electron cloud in the core region can interact with the hydrophobic core of proteins (Shen et al., 2020). Kumar et al. and Lee et al. found that the enrichment effect of GO on osteoinductive factors accelerated the osteogenic differentiation process, and the osteogenic differentiation time was reduced from the original 21 days–12 days (Lee et al., 2011). Third, GO can strengthen tissue mineralization. GO can be used as an active site to gather mineral ions, thereby improving the nucleation and growth of apatite (Gorainov et al., 2014), and apatite is the basic component of mineralization. Last but not least, GO can regulate signaling pathways: graphene derivatives can enter the cytoplasm through receptor- or nonreceptor-mediated endocytosis, triggering different signaling cascades (Foroutan et al., 2018), thus playing a role in promoting bone formation.

When GO is incorporated into hydrogels, it can improve the mechanical strength of the hydrogels, and the resultant nanocomposite hydrogels are conducive to protein adsorption, cell adhesion and apatite deposition and can achieve higher quality and faster bone regeneration. For example, graphene incorporation within sericin methacryloyl (SerMA) hydrogels (SMHs) led to improvements in the compressive moduli of...
SMH/GO-0.02% (34 kPa) and SMH/GO-0.04% (43 kPa) compared with that of pure SMH (17 kPa) (Qi et al., 2020), showing that the addition of GO improved the mechanical properties of hydrogels in a GO-concentration-dependent manner (Qi et al., 2020). Similarly, GO, regenerated cellulose, and polyvinyl alcohol hydrogel composites were prepared by Rui Hong et al., and their elongation at break and tensile strength were greatly increased (Rui-Hong et al., 2016). In another approach, Elkhenany et al. implanted an agar stent containing GO NPs and MSCs into an SD rat tibial defect model for 45 days. Compared with scaffolds containing only MSCs, the GO + MSC scaffolds presented more vasculature and mineralized bone tissue, increased bone healing area, and no obvious complications (Foroutan et al., 2018).

The effect of graphene derivatives on bone regeneration is affected by many factors, including the concentration, number of layers, and chemical composition of the graphene derivatives. GO promotes bone formation in a concentration-dependent manner; however, at low concentration (0.1 μg/mL), GO can effectively promote MSC proliferation and osteogenic differentiation, whereas a high concentration of GO (10 μg/mL) induces abnormal cell morphology and inhibits MSC proliferation (Wei et al., 2017). Qiu et al. found that properly increasing the number of graphene layers can enhance its ability to promote bone differentiation but can slightly inhibit the initial adhesion and proliferation of MSCs in rats (Qiu et al., 2017). Graphene materials with different chemical compositions also have different effects on cells. GO has more oxygen-containing functional groups than rGO and a stronger effect on promoting MSC adhesion, stretching, and osteogenic differentiation, whereas GO modified with amino groups is more conducive to mineral deposition (Kumar et al., 2015).

GO can not only efficiently adsorb proteins and protect them from enzymatic hydrolysis but also achieve continuous release. Thus, GO can be applied as a nanoplatform for adsorbing and transporting serum, growth factors, and inductive molecules and realizing controlled release through noncovalent interactions, such as hydrophobic interactions, π-π stacking, and electrostatic interactions. For instance, GO-doped nanofibers and GO substrates promoted MSC proliferation and osteogenic differentiation, primarily due to pre-enrichment of serum, β-glycerophosphate, and Dex in the culture medium by GO (Lee et al., 2011).

Hydrogel bioinks containing GO can improve the printing performance of cells, including their printability, structural integrity, and osteoinductivity (Choe et al., 2019), and thus, such bioinks are very suitable for bio-3D printing in bone tissue engineering. For example, Choe et al. explored the use of alginate/GO composites as bioinks (Choe et al., 2019). They added GO (0.05–1.0 mg/mL) to 3% alginate to form a nanocomposite hydrogel, which showed significantly improved viscosity, shear-thinning performance, and shear recovery ability. In addition, MSCs printed with alginate/GO showed good proliferation ability, high bioactivity, and a high survival rate under oxidative stress. Compared with the 3D scaffold printed with MSCs and alginate without GO addition, the 3D scaffold printed with MSCs and alginate/GO had a stronger ability to promote bone formation. Among groups with different concentrations, the bioink with 3% alginate and 0.5 mg/mL GO showed the most balanced characteristics.

In addition to a direct effect on osteoblast-related cells, graphene derivatives also have antibacterial, vascular regeneration, and antitumor effects, which indirectly promote new bone formation. Graphene derivatives can effectively inhibit the adhesion of Streptococcus mutans and Enterococcus faecalis on the surface of titanium and the formation of biofilms, reducing the risk of postoperative infection and bone resorption (Zhang et al., 2016). Graphene derivatives can also increase the expression of angiogenesis-related factors, thereby promoting angiogenesis and providing nutrition for new bone (Zhang et al., 2016). Furthermore, due to their antitumor effect and osteogenesis effect, GO-containing hydrogels can be an effective treatment for large tumor-related bone defects that may have a significant risk of locoregional relapse after surgical curettage (Li et al., 2018).

Graphene has poor biodegradability and tends to accumulate in the body. In vivo experiments have shown that unmodified graphene accumulates in the lung, liver, spleen, and kidney and has chronic toxicity. Zhang et al. intravenously injected GO into mice and found that GO primarily accumulated in the lungs of the mice, causing inflammation when its concentration reached 10 mg/kg (Zhang et al., 2011a). However, after injection of GO modified with PEG into mice, the material did not cause obvious damage or inflammation within 3 months after aggregation in the liver and spleen (Yang et al., 2011). In addition, covalent bond-modified dextrose on a graphene sheet layer was traced via 125I, and the material was not found to cause obvious toxicity (Yang et al., 2011). In addition to improving the biosafety of graphene through modification
methods and changing its size and structure, at present, we hope that graphene materials can be bio-
degraded in vivo after being applied and will not stay in the tissue for a long time, which would be beneficial
to clinical applications related to in vivo transplantation. Therefore, the biodegradability of graphene has
become an extremely important research direction in the current biomedical field.

Gold Nanoparticle Hydrogels

Recently, GNPs have emerged as a new breed of osteogenic agents. After uptake in cells, GNPs are
capable of promoting MSC differentiation into osteoblasts through activation of the MAPK pathway.
GNPs with diameters ranging from 20 to 40 nm can improve the osteogenic differentiation rate of
MC3T3-E1 osteoblast-like cells (Liu et al., 2010). Heo et al. confirmed the osteogenic effect of GNP hydro-
gels on ADSCs. In addition, GNPs can affect the formation of osteoclasts. Sul et al. reported that GNPs can
promote osteoclast formation by inhibiting the receptor activator of the nuclear factor-κB ligand pathway
in bone marrow-derived macrophages (BMMs) (Sul et al., 2010). At the laboratory scale, synthesis of various
types of GNPs from gold chloride is a simple procedure (Dykman and Khlebtsov, 2012). In addition, GNPs
do not show obvious toxicity in vivo. After intraperitoneal injection, the accumulation of 30-nm GNPs in the
liver, kidney, and spleen caused no obvious damage to organisms (Zhang et al., 2011b). The antibacterial
activity of GNPs was reported recently (Badwaik et al., 2012). The mechanism by which GNPs inhibit bac-
terial growth might involve their ability to damage the bacterial wall and induce bacterial cell death (Regiel-
Futyra et al., 2015). Ribeiro et al. reported that silk fibroin/nHA hydrogel mixed with all concentrations of
AuNPs inhibited the growth of gram-positive bacteria and gram-negative bacteria but had no toxicity to
osteoblasts (Ribeiro et al., 2017).

Classification of Nanocomposite Hydrogels Based on the Hydrogel

In addition, we can divide nanocomposite hydrogels into two main categories according to the nature of the
polymers used: synthetic polymer-based nanocomposite hydrogels and natural polymer-based nanocomposite
hydrogels (Tutar et al., 2019). The advantage of synthetic polymers is that they can be chemically functionalized
so that their properties can be adjusted. Therefore, synthetic polymers enable better control over hydrogel
properties, are more predictable than natural polymers, and can be designed for specific uses. In addition,
because of their covalent cross-linking, nanocomposite hydrogels made from synthetic polymers are mechani-
cally stronger than nanocomposite hydrogels generated from natural polymers. Natural polymers, such as car-
bohydrates, proteins, and glycoproteins of natural origin, have good biocompatibility and biodegradability.
They can provide functional sites that can interact with proteins and biochemical signals to promote cell inter-
action to form tissues. However, natural polymers have limitations, such as being difficult to extract from natural
sources and being difficult to separate from proteins and other materials, resulting in variations in product qual-
ity, which may lead to immunogenicity and elicit an innate immune response with a constructive remodeling
phenotype (Morriss et al., 2017) and induce macrophage polarization. In addition, due to the physical cross-link-
ing of natural polymers, nanocomposite hydrogels prepared from natural polymers exhibit poor mechanical
properties and rapid hydrolysis and/or enzymatic degradation.

FABRICATION PROCESSES FOR INORGANIC NANOCOMPOSITE HYDROGELS

The characteristics of inorganic nanocomposite hydrogels depend on several factors, including the com-
ponents of the NPs and hydrogels, the concentration of the NPs and hydrogels, and the size and uniformity
of the NPs in the hydrogels. Four main methods are used to fabricate inorganic nanocomposite hydrogels:
the blending method, in situ precipitation method, freeze/thawing method, and grafting-onto method.

Blending Method

In the blending process, NPs and hydrogels are prepared separately, and the NPs are mixed and cross-
linked with the hydrogel solution (Liu et al., 2020). Filippi mixed PEG-functionalized iron oxide (II, III) NPs
with an average particle size of 15 nm (1 mg/mL in an aqueous suspension) and cells in sterile Tris-buffered
saline (TBS; 50 mM, pH 7.6) containing 50 mM calcium chloride and 1.5% v/v PEG. Then, 10 U/mL thrombin
activating factor XIIIa was added to cross-link and form a hydrogel network (Filippi et al., 2019). The ob-
tained hydrogel had a smooth inner gel texture, slow relaxation kinetics, and a high elastic modulus.

The blending method is the easiest preparation approach and has many advantages (Liu et al., 2020). First,
inorganic NPs with a uniform particle size can be obtained by changing the stirring speed, material concen-
tration, and fabrication period. Second, as the preparation and cross-linking of NPs are carried out
separately, the preparation process is easy. However, the asymmetric distribution of NPs in nanocomposite hydrogels and the diffusion of nanocomposite hydrogels in solution need to be further studied.

**In Situ Precipitation Method**

In the *in situ* polymerization method, inorganic NPs are first uniformly dispersed in polymer monomers, and then, the monomers are polymerized to form a nanocomposite hydrogel. In this method, the surface of the inorganic NPs must be modified before the reaction to ensure that the NPs are uniformly dispersed. This method for preparing nanocomposite hydrogels can avoid polymer degradation during heating. Moreover, this synthesis approach is straightforward and economical. However, this method is only appropriate for limited hydrogels that possess stable networks. This is because the alkali solution used in the process might destroy the hydrogel network and limit cell encapsulation (Wang et al., 2018b).

Wang et al. reported an *in situ* precipitation method to construct MNP hydrogels. Briefly, 4 g chitosan was dissolved in 100 mL acetic acid solution (2%, v/v) to obtain a chitosan solution (4%, wt/wt). Next, 10 mL magnetite precursor containing 7 mmol iron (I) and 3.5 mmol iron (ID) was added to the chitosan solution, which was then stirred for 2 h to obtain a homogeneous solution. The mixture was soaked in NaOH solution (1.25 mol/L) for 4 h to form a magnetic chitosan hydrogel. Finally, the MCH was rinsed with deionized water to achieve a neutral pH. In the process, the network of the hydrogel acts as a chemically reactive substance, within which the iron ions from inorganic salts react with alkali solutions (e.g., NaOH) to prepare the magnetic particles (Arias et al., 2018; Liu et al., 2019). A chelating effect between chitosan and iron ions ensures the uniform dispersion of MNPs in the chitosan hydrogel and prevents oxidation of the MNPs synthesized *in situ*. In addition, after hybridization, a weak interaction is formed between chitosan and the MNPs, thereby enhancing the mechanical properties of the MCH and giving the MNPs an *in situ* chitosan layer (Wang et al., 2018b). The resulting magnetic hydrogel showed a magnetic response and improved morphological and mechanical features, including homogeneous distribution of the MNPs and excellent wettability. Moreover, the mechanical properties (such as compression strength, yield strength, and probe displacement) of the magnetic hydrogel were enhanced with an increasing concentration of MNPs from 0 to 15 wt %, which was attributed to the cross-linking role of MNPs in the process.

**Freeze/Thawing Method**

The freeze/thawing method is a preparation method based on physical cross-linking. The hydrogel solution is repeatedly frozen and thawed at low temperature and room temperature. When the aqueous solution freezes and solidifies, polymer aggregation areas and nonaggregation areas are formed inside the gel. During the thawing process, polymer aggregation areas are used as physical cross-linking points to form a cross-linked network. A common feature of hydrogels formed by the physical cross-linking method is that the molecular chains form a 3D network through hydrogen bonds and microcrystalline regions, that is, physical cross-linking points. These cross-linking points change with changes in external conditions, such as temperature. For example, when the temperature is increased to a certain level, the gel will melt and return to the original aqueous solution state; thus, the physical cross-linking process is reversible. A hydrogel obtained by the physical cross-linking method has a cross-linked network structure that is not very strong and is greatly affected by the outside world, and the cross-linking distribution of the polymer is uneven. Without additives, the resulting hydrogel generally has poor optical transparency, and the degree of cross-linking is difficult to control. Huang et al. used the ultrasonic dispersion method and the freeze/thawing method to form a magnetic nanocomposite hydrogel composed of n-HA, poly(vinyl alcohol), and MNPs (Huang et al., 2018). They weighed a certain amount of PVA, placed it in distilled water, and heated and stirred at 90°C for 1 h to form an aqueous PVA solution. A certain amount of n-HA particles and Fe₂O₃ was added to the PVA aqueous solution, with the n-HA, Fe₂O₃, and PVA mixed at a ratio of 1:0.5:10. After ultrasonic treatment, the solution was stirred at 60°C until uniformly mixed. After ultrasonic mixing, the bubbles were removed, and the solution was poured into a mold, incubated in a refrigerator (−20°C) overnight for 16 h, and then removed and thawed at room temperature for 4 h. Freezing and thawing cycles were repeated seven times to obtain an n-HA/Fe₂O₃/PVA composite hydrogel. Finally, the prepared n-HA/Fe₂O₃/PVA composite hydrogel was soaked in distilled water for 1 day to remove uncross-linked monomers. The obtained hydrogel showed good mechanical properties, and BMSCs grew uniformly on the hydrogel and had a high proliferation rate.
**Grafting-Onto Method**

The grafting-onto method induces the formation of covalent linkages between hydrogel networks and NPs. Specifically, the surface of NPs is grafted with specific functional groups that act as micro- or nano-cross-linking agents, thus covalently bonding with hydrogel monomers. Hu et al. used the grafting-onto method to fabricate a magnetic hydrogel consisting of a nontoxic polyacrylamide (PAAm) hydrogel and methacrylic acid 3-(trimethoxy silyl) propyl ester-coated Fe$_3$O$_4$ (Hu et al., 2019). First, they dissolved the AAm powder in deionized water, where the total amount of AAm was 14 wt %, and then dissolved the cross-linking agent N,N’-Methylenebisacrylamide (MBAA) (AAm is 0.0608 wt%) into the solution. Then, 20%, 40%, and 60% (relative to the total weight of polymer and water) coated Fe$_3$O$_4$ NPs were added to the solution, which was stirred manually for 30 s. After that, 2% (relative to the total weight of polymer and water) PAAm as a thickener and 1% (relative to the total weight of polymer and water) dispersant (ammonium citrate) were mixed into the dispersion with mechanical stirring for 2 h, and the PAAm powder was completely dissolved in the solution. After that, the thermal initiator APS (0.029% by weight of AAm) and the accelerator TEMED (0.199% by weight of AAm) were added to the mixture. This new type of magnetic hydrogel exhibited high mechanical performance properties, such as fracture toughness and tensile strength. One advantage of this method is that covalent bonds can encapsulate NPs in hydrogels and enhance the stability of nanocomposite hydrogels. However, its long production time, high cost, and complex manufacturing process limit its wide application in the biomedical field.

**SUMMARY AND OUTLOOK**

Introducing nanomaterials into 3D polymer networks alters the original properties of the nanomaterials and adds enhanced and/or synergistic and novel features to the formed nanocomposite hydrogels. The final 3D nanocomposite hydrogel constructs have improved mechanical strength, porosity, interconnectivity, nano/micro topography, stimuli-responsiveness, and bioactivity compared with nanocomposite-free hydrogels and show great potential in bone tissue engineering. At the same time, these parameters are crucial for tissue regeneration applications: for example, mechanically strong nanocomposite hydrogels are needed to replace or substitute bone and must resist in vivo mechanical compression and/or stretching. Porous and interconnected nanocomposite hydrogels have been fabricated to promote cell migration, oxygen and nutrition transport, and stem cell differentiation.

Despite the significant benefits of nanocomposites in the fabrication of advanced 3D biomaterials, there are still many disadvantages. The instability of NP dispersion increases the difficulty of preparing nanocomposite hydrogels, and a more scientific manufacturing method is needed. The physicochemical characteristics of nanomaterials that change with time under different environmental conditions and thus their potential risks over time and the impact of contamination on the properties of nanomaterials are still not well studied. The properties of nanomaterials change with time and with even small differences in the synthesis and storage conditions. For example, two types of nanomaterials with the same size, shape, and chemical composition may have different properties when there is a subtle difference in their preparation method, storage, or preparation time. Thus, it is difficult to predict the bioavailability of functional nanomaterials-based biomaterials with time and their impact on the health of living systems and the environment. Therefore, additional techniques for reproducible nanomaterials synthesis, a combination of various complementary characterization techniques for reliable characterization, and accurate monitoring of changes in the physical and chemical characteristics of nanomaterials over time under different environmental conditions will help overcome the current challenges. In addition, as nano(bio)technology merges biology, chemistry, physics, materials science, and bioengineering, an interdisciplinary approach is needed for further progress in nanotechnological techniques.

The future development direction of nanocomposites for tissue repair and regeneration is to clearly understand the interactions between nanocomposites and tissues and optimize their long-term use, composition, hierarchical structure, and related mechanical strength properties, especially the fatigue limit under cyclic external stress. Another important factor is achieving the proper surface chemistry, which can induce selective cell adhesion, spreading, migration, and differentiation; these considerations are key for enhanced tissue regeneration and thus will be important for clinical utilization of nanocomposite hydrogels (Ringer et al., 2017). Nanocomposite hydrogels composed of functional nanomaterials are suitable platforms to study the impact of the mechanical and chemical cues of a biomaterial on cell behavior. Chemical modification of nanomaterial surfaces with cell adhesive ligands and growth factors; regulation of mechanical stiffness, topography, nanostructure surface, and the gradient properties of hydrogels and substrate
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