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Emerging Nanostructured Materials for Musculoskeletal Tissue Engineering

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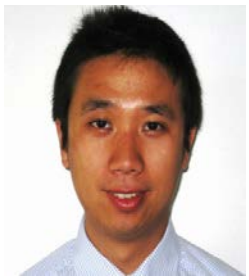

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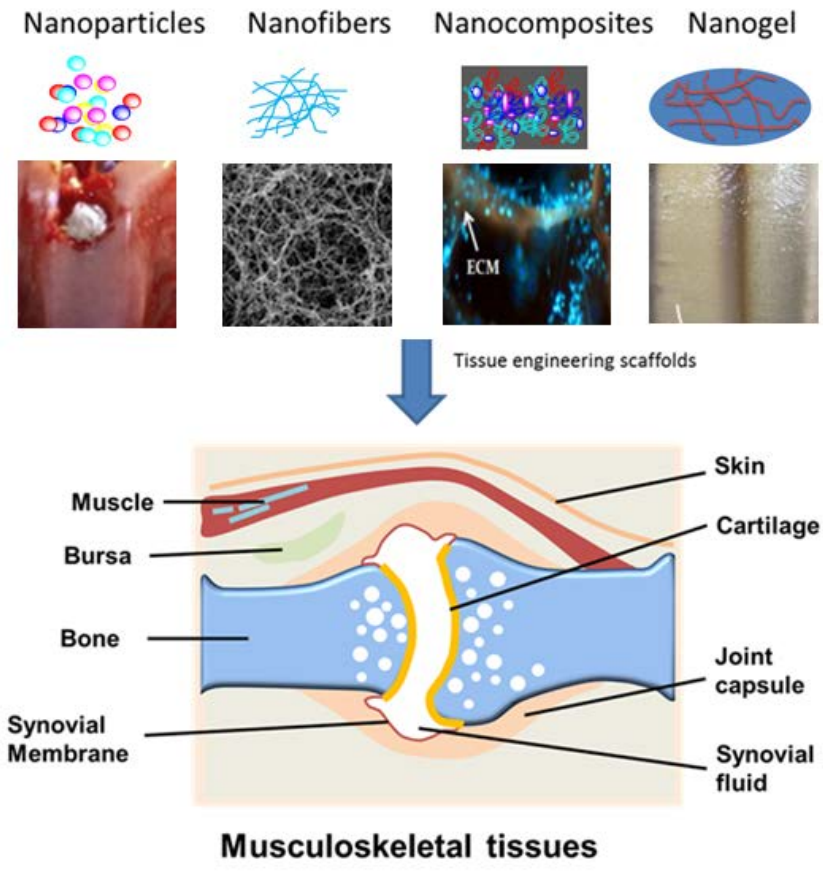

Graphic Abstract: this review summarizes the recent developments in the preparations and applications of nanostructured materials for musculoskeletal tissue engineering.

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Abstract: The musculoskeletal tissues are highly ordered nanostructured materials, and they have self-healing capability. However, when the tissue damage is beyond the capability, therapeutic approaches to repair or regenerate the tissues are needed. Nanomaterials have attracted much research attention to create novel tissue engineering scaffolds, because of their small size, large surface area, enhanced mechanical properties, tunable molecular and chemical structures, and various surface functionalities. With the development of nanotechnology, nanostructured materials with properties that more closely fulfill the requirement in the course of recovery of native tissues were designed, synthesized, characterized and utilized systematically. Here, we introduced the microenvironment of extracellular matrix in musculoskeletal tissues. We further summarized the current nanostructured materials used in musculoskeletal tissue engineering including natural polymers, synthetic polymers and inorganic materials. Specifically, the fabrications and applications of different nanomaterials in bone, cartilage, and muscle tissue engineering were discussed in details. The most recent research achievement in each category were presented and discussed. Overall, nanostructured materials can be synthesized with controlled composition, size, geometry, and morphology. In order to enhance biocompatibility, immune compatibility and cell adhesion, surface of these materials can be modified for different application in musculoskeletal tissue scaffolds. Although more tasks and challenges are need to be addressed and resolved in order to translate them into commercialized products, the nanostructured materials represent the very promising candidate in the development of musculoskeletal tissue engineering in the future.

Key Words: nanomaterials, musculoskeletal, tissue engineering

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1. Introduction

The musculoskeletal tissues (e.g. bone, cartilage, joint, muscle, etc.) are highly ordered nanostructured materials consisting of mainly nanofibers embedded in a matrix of different composition.[1-3] These tissues share very similar structures, although they exhibit different appearances. In a simplified model, a network of collagen fibrils with diameters of approximately 100 nm is embedded within a characteristic tissue matrix. The composition and structures of the matrix as well as the interaction of the fibers with the matrix determine the mechanical and biological properties of musculoskeletal tissues. The cellular components located within or on the surface of those composite materials are necessities for maintaining integrity of the tissues. For example, bone tissue has highly nano-hierarchical structure consisting mainly of collagen type I fibers and nano-hydroxyapatite crystals as the matrix, which combinedly contribute to the mechanical properties of bone materials, such as high compressive and tensional strength.[1, 4]

The musculoskeletal tissues have self-healing capability under certain damage degree. For example, small damages of bone tissue can be self-recovered from its constant dynamic remodeling and self-healing. However, when the damage is beyond the self-healing capability of the tissues and therefore severely impacts life quality, therapeutic approaches are desired to repair or regenerate the tissues.[5-7] Every year, total healthcare costs of approximately $15 billion are spent on over 2 million bone grafts worldwide.[8] With the recent development of nanotechnology and nanostructured materials, tissue engineering provides new opportunities for repair and regeneration of damaged and diseased musculoskeletal tissues.

The conventional materials used for tissue engineering have many challenges, such as infection, inflammation and implant loosening. It is very crucial to generate alternative materials with excellent mechanical and biocompatible properties for musculoskeletal tissue engineering. An ideal biomaterial should mimic the natural tissue formation process, possessing a hierarchically organized structure with different length scales and adequately promotes healing. Generally, appropriate material candidates for

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musculoskeletal tissue engineering should possess the following properties: 1) biocompatibility; 2) biodegradability or capability of being remolded; 3) optimized micro-environmental matrix, such as the abilities to promote cell attachment, ECM formation and carry growth factors; 4) proper mechanics strength; and 5) porous structures for cell growth, etc.[9]

Natural tissues possess certain mechanical properties from hierarchical architectures that are precisely controlled from nano to macroscale. To mimic the natural tissues, nanotechnology and nanomaterials have become a focus in the field of musculoskeletal tissue repair.[10, 11] Nanostructured materials have great properties, such as the small size, large surface area, enhanced mechanical properties, and surface functionality, which make them suitable for creating novel tissue engineering scaffolds.[1, 12-16] Nanostructured scaffolds can be tailored at the molecular level, so scaffold morphology can excellently mimic the features of ECM in terms of porosity, framing and biofunctionalities. Therefore, both mechanical properties of scaffold microenvironments and biomaterial-tissue interactions can be tuned.[17] Research results have also shown that nanostructured materials can be tuned to control cell behaviors at multiple levels, including adhesion, migration, proliferation, signaling, genetic expression and stem cell fate.[18-20] However, it has also become obvious that nanostructured materials had a longer history in the repair and regeneration of musculoskeletal tissues than initially thought and there were challenges and even failures, which are important to summarize at this point in order to guide future design with new ideas.[1]

Although there are a number of review articles discussing different kinds of nanostructured materials in musculoskeletal tissue engineering application, they just focused on either a single type of material or tissue. Comprehensive review and discussion of the applications of nanomaterials in musculoskeletal tissue engineering is rare. In this review, we will explore and discuss the major classes of current nanomaterials being used for musculoskeletal tissue engineering, such as natural biomolecules, artificial synthesized polymers, non-metal materials such as silicon and carbon, and metal materials including titanium, platinum, gold, and silver, along with their corresponding fabrication methods and properties. Due to the difference of the structure and composition, distinct designs and applications for

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nanostructured materials have been used in the bone, cartilage, and muscle tissue engineering, based on which we will discuss each part in details as illustrated in the following scheme (Figure 1).

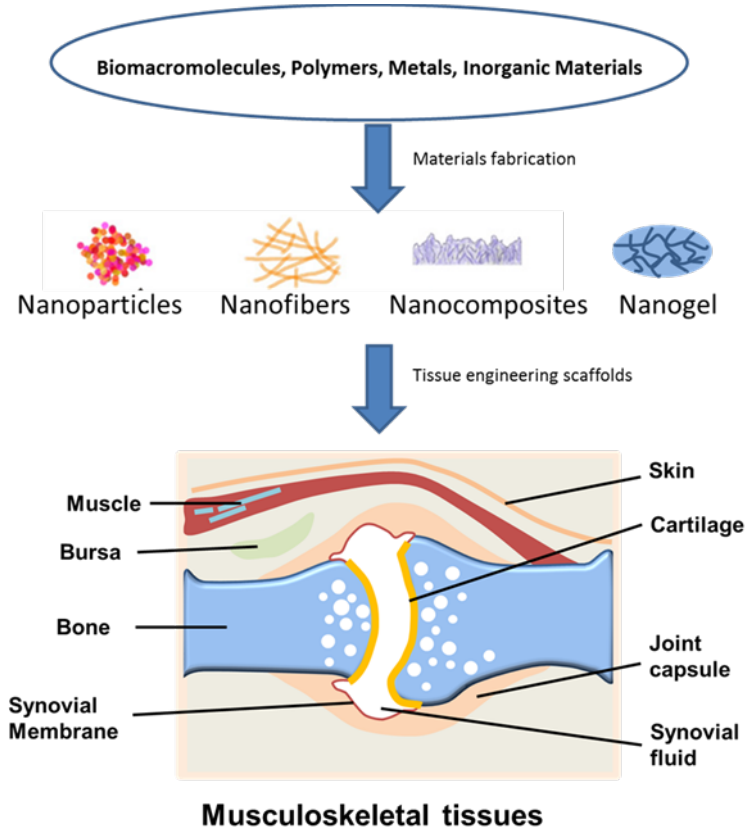

Figure 1. Materials and their scaffold structures used for musculoskeletal tissue repair and regeneration.

2. Nanostructured materials for bone tissue engineering

2.1 Bone and associated microenvironment clues

As the major component of musculoskeletal tissues, bone's primary functions include supporting and protecting the mammalian body. It is a type of hierarchically structured composite material, which is composed of organic and inorganic phases.[21] As shown by Fig. 2, the organic and inorganic phase is primarily type I collagen and hydroxyapatite (HAp) nanocrystals, respectively.[5, 22, 23] The stiffness of bone is provided by the thin carbonated HAp nanocrystals embedded in the collagen organic matrix.[24, 25, 26]

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The organic matrix, or extracellular matrix (ECM), mostly composed of collagen, forms a major part of the organic phase.[27] Cells make up 2-5% of the organic part, 95% of which are osteoblasts and osteocytes.[28] The organic phase also includes various growth factors, proteolytic enzymes, and inhibitors.[29, 30] These chemical factors can either promote or inhibit the activity of osteoblasts and osteoclasts cells, and thus, the rate at which bone is made, destroyed, repaired, or changed can be regulated.

Type I collagen has fibrous nature. The fundamental subunit is mineralized collagen fibril that consists of self-assembled triple helices of collagen molecules. The cylindrically shaped triple-helix has an average diameter of about 1.5 nm with lengths of 300 nm, and the fibril contains three polypeptide chains with about 1000 amino acids.[23] HAp nanocrystals grow on these self-assembled fibrils, with the crystal c-axes aligned with the fibril long axes.[31] It is still not entirely understood whether the HAp crystals are directly nucleated on the collagen fibrils, or if the HAp mineralization is directed by other charged macromolecules, which may be associated with the collagen fibrils. Although collagen is believed to be the most important component in controlling bone formation and repair, it should not be the only factor that in the controlling process since collagenous tissues that never mineralize are widely existed in the body. Therefore, the non-collagenous proteins associated with the bone are considered to play an important role in either inhibiting or promoting interactions during crystal nucleation and growth. There are some common features of these proteins, such as highly acidic properties.[21]

The nanocrystalline HAp counts 70% in weight of the bone matrix.[32] The basic building blocks are the extremely small plate-shaped crystals, just hundreds of angstroms long and wide with 20-30 angstroms thickness. They are arranged in parallel layers within the collagenous framework. In many bones these layers are organized into larger highly ordered structures from the molecular level to the macroscopic materials.[18, 33]

Water is another component in the bone materials. It is very important to maintain the mechanical functioning of bone. Mechanical measurements show difference between dry bone and wet bone. The

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water exists almost everywhere in the bone.[23]

Understanding the biomolecules which are responsible for supporting the growth of tissues is crucial for bone regeneration. Growth factors help in tissue growth and have been tested in different concentrations to seek appropriate method, concentration and correct factors. Otherwise, side effect may happen.[34] Growth factors that have been reported to regulate bone tissue including vascular endothelial growth factor (VEGF),[35, 36] transforming growth factor beta (TGF-β),[37] bone morphogenetic protein (BMP),[38-40] and fibroblast growth factors (FGFs).[41-45] Growth factors are usually incorporated to different kinds of bone tissue engineering scaffolds to deliver their functions.[31, 46-49]

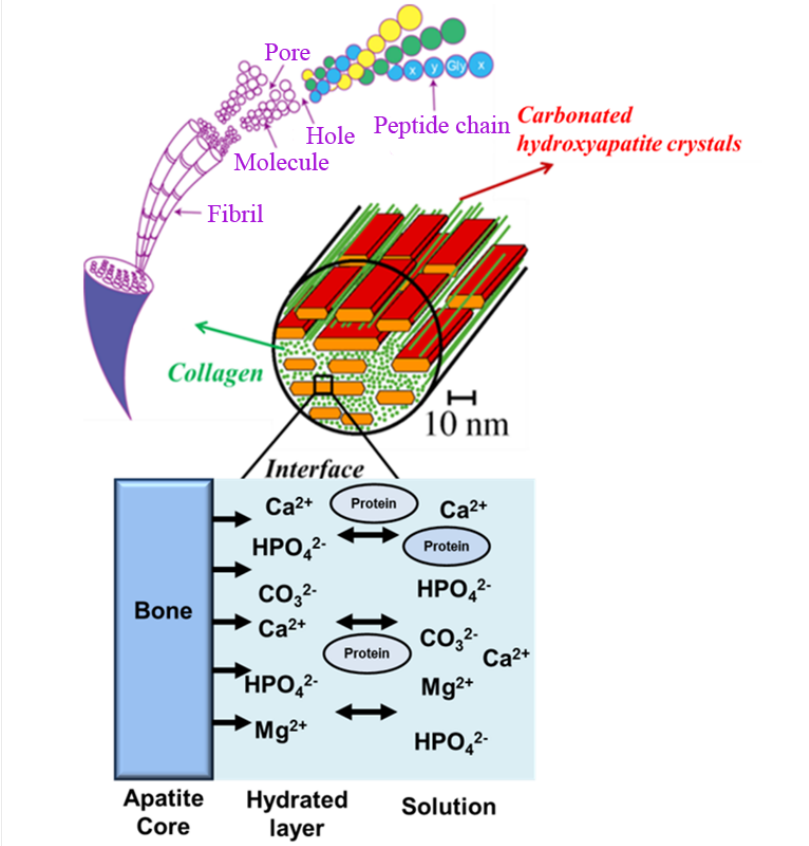

Figure 2. Bone structure shows carbonated HAp and collagen, and the surface of HAp.

2.2 Nanostrucuted materials for bone tissue engineering

Bone tissue engineering scaffolds are new emerging approaches for bone regeneration compared to

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the traditional orthopaedic implants.[50-54] It contains a complex mixture of molecules in three-dimension pattern.[55, 56] Cell differentiation, proliferation and growth as well as growth factors delivery can be achieved simultaneously to meet the requirements of bone regeneration. Temporary frameworks to support bone regeneration and controlled release growth factors to regulate bone formation are also desired properties for the ideal scaffold. It also needs to have macro and micro porosity, biodegradable or biocompatible property, and good mechanical strength for safe handling, as well as keeping functionality in physical conditions *in vivo*.[57-60] However, most current conventional tissue scaffolds cannot meet these ideal conditions, and suffer from limitations in terms of insufficient fulfillment for requirements of mechanical strength, cell growth promotion, and growth factors release.[16, 61-63] Compared with conventional materials, nanostructured materials present properties in the aspect of overcoming the current materials limitations. For instance, the presence of nanotubes or nanocrystals in composite materials can improve the mechanical properties for bone tissue engineering materials. Cellular responses to nanomaterials, such as cell attachment, proliferation, and differentiation, can be regulated by the presence of nanostructures. Nanostructured surfaces with chemical modification have shown increased surface energy and wettability for specific purposes, such as enhancement for cell response.[64] Many studies have also shown that nanostructured surfaces promote inorganic phase mineralization and enhance *in vitro* osteogenesis.[65] Many research efforts have been made to develop nanostructured bone tissue engineering materials. We will briefly discuss the different materials used for these bone tissue engineering scaffolds.

2.2.1 Natural polymers

Natural polymers are materials obtained from natural sources, such as from animal or vegetal sources. Collagen, fibrinogen, chitosan, starch, hyaluronic acid, and poly(hydroxybutyrate) are commonly used natural polymers for tissue engineering. The main advantages of these materials include low immunogenic potential, the bioactive behavior, chemical versatility, and easy availability.[61, 66-69] Type I collagen is often used in various kinds of musculoskeletal tissue engineering scaffolds to enhance the

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bioactivity, cell response and make the scaffolds more closely mimic the nature tissue's properties. However, other nanostructured materials may need to be added to the collagen scaffolds to further promote the mechanical properties, as well as facilitate other tissue functions.[70-76]

Recently, numerous research efforts have been dedicated to applying chitosan based materials into bone tissue engineering. It was found that such natural biomaterials has great biocompatibility not only causes less cytotoxicity, but can be facilely processed into various geometries with delicate nanostructures so that cell can growth and form osteoconduction.[77-85] For example, HAp/chitosan–pectin (nHCP) composites were synthesized by in situ mineralization of HAp in chitosan–pectin polyelectrolyte complex (PEC) network. Figure 3 shows the nHCP synthesis process. The pH and the chitosan/pectin ratio play an important role in the formation nano-HAp crystals. Results demonstrated site nucleation and the nano-HAp crystals growth along the c-axis, which was probably due to the interfacial interactions between nano-HAp crystal and chitosan–pectin network.[86] The nano-hydrpxyapatite crystals synthesized with this method have similar size and morphology as that in the nature bone.

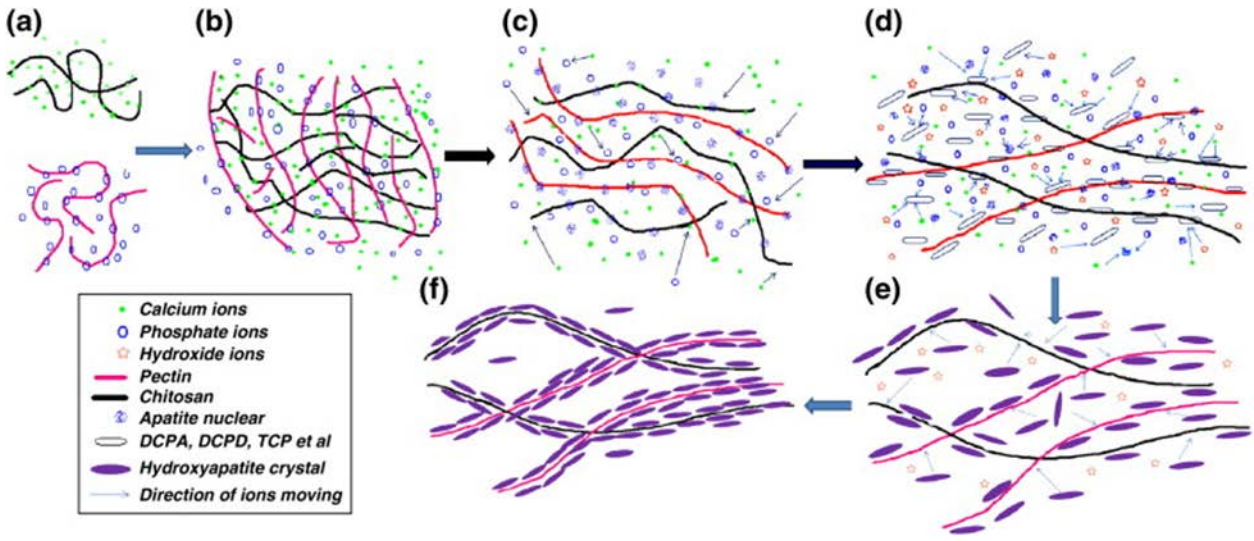

Figure 3. Schematic model of nano-HAp crystals synthesis process in the presence of chitosan–pectin PECs network. (reprinted with permission from reference 86). [86]

2.2.2 Synthetic polymers

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Synthetic polymers have more advantages than natural polymers because of tunable biodegradation speed, predictable properties, better consistency between batches and they could be easily designed and fabricated accordingly.[87-90] Various polymers have been tested for materials design and fabrication.[91-100] Based on the requirement of the bone tissue engineering materials, synthetic polymer materials used as bone scaffolds should meet the following criteria: biocompatibility or biodegradable, suitable chemical and mechanical properties, good stability under physical conditions for bioapplication.[101]

One of the most frequently used polymer materials in the field of bone tissue engineering are saturated aliphatic polymer materials, including poly (lactic acid) (PLA) and poly (glycolic acid) (PGA), poly (lactic-coglycolide) (PLGA) and their copolymers. They are mostly synthesized by the condensation polymerization method to achieve high molecular weight. [7, 101]

Other synthetic polymers utilized for nanostructured bone materials include poly (propylene fumarate) (PPF),[101] polyalkylcyanoacrylate,[102] poly (3-hydroxybutanoicacid)(PHB),[103] poly(organophosphazene)(POP),[104] poly(ethylene glycol)(PEG),[105] poly(caprolactone)(PCL),[106, 107] poly(ethylene oxide)(PEO),[108] polyanhydrides,[101] and copolymers such as PLA-PEG.[109, 110]

However, the bond between polymers and bone is usually weak, and therefore, hard to form integrated bone tissue eventually. Weak mechanical properties, such as low elastic moduli and a deformation mechanism called creep, is another major drawback that limits synthetic polymers' application in bone tissue engineering. Hence, other materials, usually ceramics, are often used as reinforce agent with polymers, and form composites for bone tissue engineering.[87, 111, 112]

2.2.3 Metallic nanostructured materials

As the orthopaedic biomaterials currently in use, metals have advantages due to their properties such as strong anti-corrosive, strong strength, and toughness.[113] In the actual production of joint prostheses, titanium, stainless steel based and cobalt alloys are three major types of metal materials that have been used.[114] Among which, Ti and its alloys are the most used metallic implant materials.[6] New generation of alloys, such as Ti15Zr4Nb4Ta and Ti29Nb13Ta4.6Zr, have improved corrosion resistance,

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mechanical properties, and cytocompatibility, and do not contain the potentially cytotoxic elements V and Al.[115]

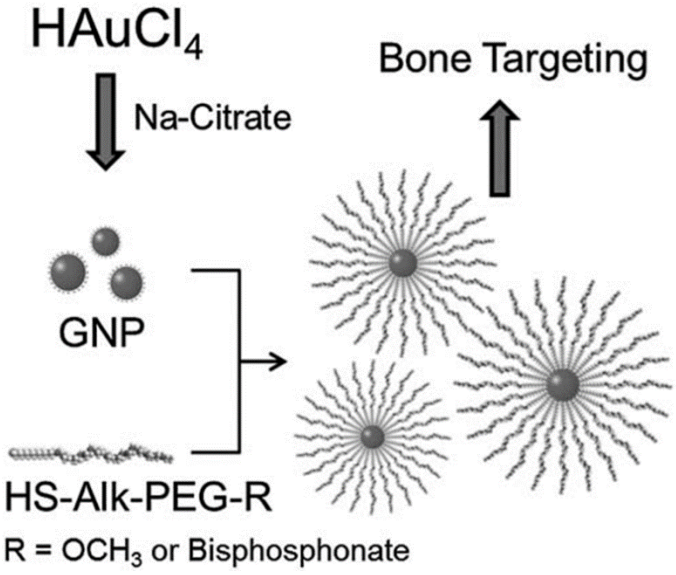

Figure 4. AuNPs modified with heterobifunctional PEG derivatives bone mineral targeting (reprinted with permission from reference 116).[116]

Gold-containing nanoparticles (AuNPs) attracted great attention because of many various biomedical applications, including imaging, diagnostics and drug delivery, especially in cancer.[117] Gold nanoparticles can also been used in tissue engineering recently. Damaged bone tissue can be targeted by functionalizing AuNPs with molecules exhibiting affinity for calcium.[116, 118] For example, in Figure 4, AuNPs were modified with heterobifunctional PEG derivatives for bone mineral targeting. One end of the PEG was modified with thiol group to form stable self-assembled monolayers on gold. On the other end of the PEG molecule, a bisphosphonate was attached to allow the targeting of HAp rich tissues, such as bone. In another similar study, Ross et al. reported using AuNPs functionalized with either carboxylate (l-glutamic acid), phosphonate (2-aminoethylphosphonic acid), or bisphosphonate (alendronate) for targeted labeling of damaged bone tissue *in vitro*.[119] The surface density of functionalized AuNPs was significantly greater within damaged tissue compared to undamaged tissue. Biris et al. designed a new nanocomposite material containing graphenes layers and Au/HAp nanoparticles by chemical vapor deposition.[120] The resulting multicomponent nanocomposite material had good biocompatibility and could induce excellent

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bone cellular proliferation. This composite material can potentially be used as bone tissue engineering scaffolds considering the excellent biocompatibility, 3D structure, and composition.

Silver is a broad spectrum antibacterial agent and it is used in both ionic and metallic forms as nanoparticles in colloidal solutions. The antimicrobial activity is achieved by disruption of bacterial cell membranes and inhibition of many cell activities, such as enzymatic activities, ATP production within ion transport processes and DNA replication. Silver nanoparticles (AgNPs) have better antibacterial activity due to the huge relative surface. AgNPs have been reported about their antimicrobial effects on different bacterial strains of clinical relevance.[121] Nanocomposites including silver nanoparticles as the antimicrobial nanomaterial have been developed.[122-126] HAp and AgNPs nanocomposite combines the bioactivity of the ceramic matrix with the antibacterial activity of the AgNPs, makes this material an excellent candidate for bone replacement, bone filling and bone repair surgery applications.[127] For example, an alginate/HAp composite scaffold with AgNPs was prepared with average pore sizes of 341.5 μm and porosity of 80%. The incorporation of AgNPs was indicated by the color change from white to yellow-brown color. *In vitro* biological tests demonstrated that silver did not affect the ability of the scaffolds to promote osteoblasts proliferation and that at the same time it exerted a strong bactericidal effect against bacterial strains.[121] Stanic et al synthesized silver doped HAp NPs, and antimicrobial studies on theses NPs showed that the silver-doped HAp samples exhibited better antimicrobial activity *in vitro* than the HAp NPs alone.[125] As shown in Figure 5, AgNPs significantly inhibited the growth of *E. coli* after the microbial contacted the silver-doped HAp scaffolds.

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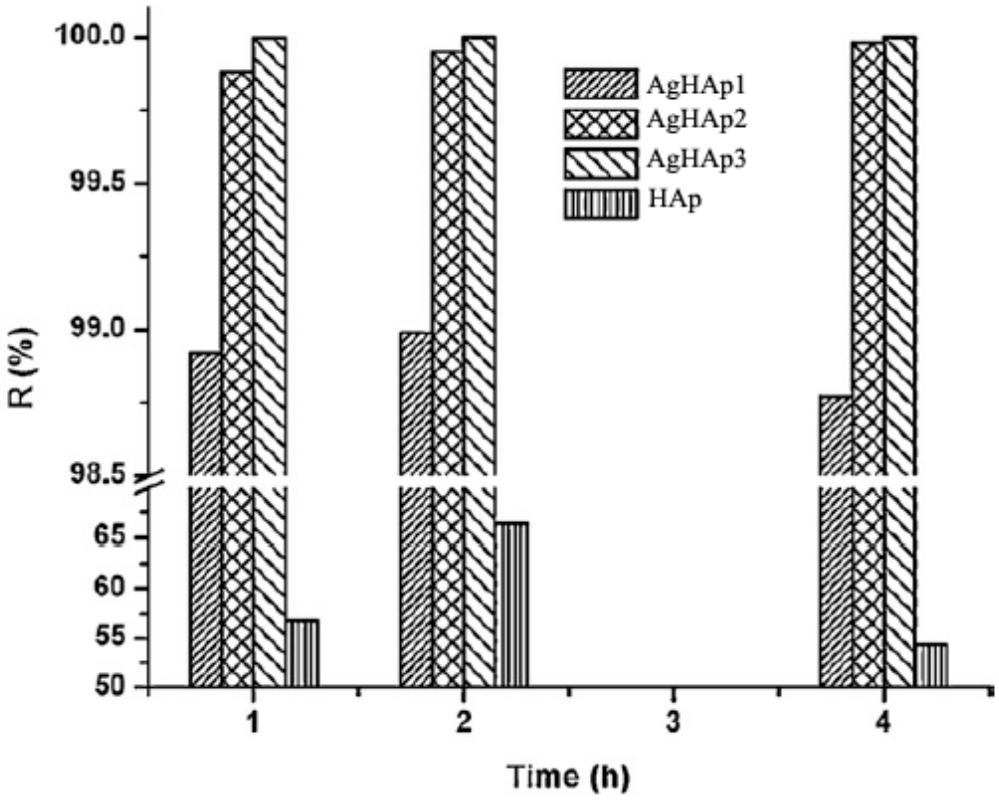

Figure 5. The percentage of microorganism (E. coli) reduction (R) 1h, 2h, 3, and 4h after contact. Silver: Ag; Synthetic hydroxyapatite, HAp; The ratios of Ca+Ag and HAp in AgHAp1, AgHAp2, and AgHAp3 scaffold were 1.64, 1.62 and 1.63, respectively. (reprinted with permission from reference 125). [125]

There are two major concerns in the using of the metallic materials for orthopaedics. The first one is that the release of the metals over long term may cause toxicity issues; and the other is that the metallic materials are not bioactive.[113, 128] Two methods can be applied to improve the bioactivity of metals for bone tissue engineering. The first one is to coat the metal surface of the implant with a bioactive ceramic (such as HAp), and the second one is to chemically modify the surface of the material, so that the metallic materials would more easily induce cell adhesion or protein attachment *in vivo*.[114, 129]

2.2.4 Inorganic nanomaterials

Calcium phosphates are the main inorganic phase in nature bone. Therefore, they are the major inorganic materials that have been considered to fabricate tissue scaffolds for bone substitution and regeneration.[81, 130-136] The advantages of the calcium phosphates in bone tissue engineering include non-

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toxicity, biocompatibility, and bioactivity, and can form strong bond with nature bone.[137, 138] The bioactive behavior of calcium phosphates was reflected by their osteoconductive activity and temporary framework for bone cell growth, as well as induce of osteoblasts adhesion and proliferation.[139] Calcium phosphate cements (CPCs) are one of the most developed bone repair and regeneration materials, due to their self-setting, easily-shaped capability, biocompatibility, osteoconductivity and osteoinductivity properties.[140] Loading bone morphogenetic protein-2 (BMP-2) into scaffold has been largely reported as another efficient way to obtain osteoinductivity and enhance bone repair and reconstruction.[140] In the future, the criteria for improving CPCs include modification of the chemical and physical properties, as well as enhancement of the bioactivity by loading growth factors.[141]

Silica-based materials have been of particular interest due to their bio-inertness and better biocompatibility.[80, 82, 142-145] Mesoporous silica with their reactive surface functional (Si–OH) groups can serve as nucleation sites and help initial cell adherence. The surface roughness, porosity, and geometry of the scaffolds are considered as key determinants in promoting the phenotypic and genotypic expression of cells. This property is invaluable in bone tissue engineering. Thus far, mesoporous silica scaffolds for bone tissue engineering have been fabricated using evaporation-induced, self-assembly, and sol–gel techniques.[146]

Another family of inorganic materials used for bone tissue engineering is bioactive glasses, which are composed mainly of silica, and modified by the Ca, Na, or P additives.[21, 117, 147] These materials are suitable for bone tissue engineering because of their high biocompatibility, positive biological effects, low toxicity, and good integration with nature bone. However, the insufficient mechanical properties of bioglasses limit their further application in the bone tissue engineering scaffolds. Nowadays, the bioglasses are usually reinforced with mechanically strength materials and osteogenic agents to form bone tissue engineering scaffolds, which would present a physically strong and bioactive bone tissue replacement and regeneration candidate material.

Carbon nanotubes (CNTs) have attracted much research attention because of their excellent physical, electrical and chemical properties. CNTs have been widely used in biomedical engineering

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fields.[148-152] They have been reported to have the functions of improving mechanical properties; interacting with growth factors; enhancing cell adhesion and cell shape regulation; and controlling stem cell differentiation. One particular property of CNTs is the electrical property, which can be used to investigate the electrical stimulation effects of cells. The tubular structure and the nano-scale features of CNTs make them the good substitution of the fibrillar collagen proteins in the ECM. Figure 6 showed the influences of carbon nanotube with various diameter and type on osteogenic or proliferative outcomes.[18] Significant studies of the potential use of CNTs in bone tissue engineering have been discussed in Newman's review article.[18] The chemical properties of CNTs can be chemically modified by adding functional groups. For example, adding carboxyl or alcohol groups onto the walls of CNTs have been reported. The chemically modified CNTs have better dispersion in water and enhanced affinity with calcium and thus easier to form bone mineral.

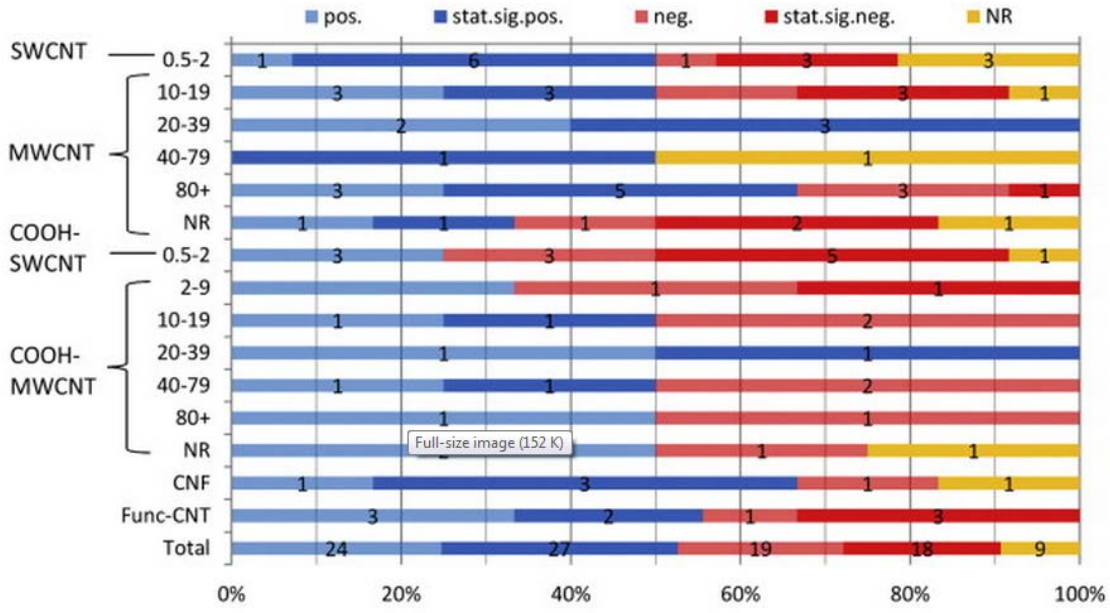

Figure 6. The influences of carbon nanotube with various diameter and type on osteogenic or proliferative outcomes. The data represented positive (pos.), negative (neg.), positive $P < 0.05$ (stat.sig.pos.), negative $P < 0.05$ (stat.sig.neg.), and neutral or not reported (NR). SWCNT: Single-walled carbon nanotube; MWCNT: Multi-walled carbon nanotube. Carboxyl groups (COOH) was used to conjugate with other active groups. (reprinted with permission from reference 18).[18]

2.2.5 Nanocomposite materials

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The natural bone is a composite material with both inorganic and organic phases. The organic phase possesses bioactivity, and the inorganic materials enhance the mechanical properties of the organic materials. The desired bone composite scaffold should have the advantages from both organic and inorganic phases.[63] The most obvious choice of materials for a synthetic analogue of bone would be a collagen-HAp composite which mimics the natural bone matrix[71]. Both components render the necessary mechanical strength, and HAp would confer the necessary bioactivity to collagen.[21] The other biopolymers and synthetic polymers also used to form composite materials HAp nanocrystals.[85, 153-157]

Based on the component of nature bone, nature biopolymer and HAp composite materials are the most obvious option. For example, bacterial cellulose/HAp nanocomposites for bone healing applications were fabricated using a bio-inspred approach. Briefly, bacterial cellulose was negatively charged by the adsorption of carboxymethyl cellulose to initiate nucleation of calcium-deficient HAp (cdHAp), which resulted in increased cell attachment.[8] In recent years, chitosan included composite materials have been widely used in the field of bone tissue engineering, [149, 158, 159] especially of the chitosan and HAp composite, because of the excellent biodegradability of chitosan, and the bioactivity of HAp.[77] The other composite materials could be fabricated with polymer and carbon nanotubes, [160] polymer and HAp,[157, 161] polymer and bioactive glass,[162] etc.

2.3 Fabrication of nanostructured materials for bone tissues

2.3.1 Nanofiber

Nanofibrous scaffolds are one of the most used materials for bone tissue engineering.[56, 163, 164] Due to the architectural, functional and morphological similarities to the collagen fibrils in bone, nanofibrous scaffolds are one of the most reasonable design for bone tissue engineering.[165] Woo et al. designed a 3D PLA nanofibrous scaffold to improve protein absorption and enhance cell attachment on the scaffold.[166] Furthermore, cell shape and morphology and cell spreading can also be affected by the presence of the nanofibrous scaffold.[167]

Electrospining is one of the most used techniques to fabricate nanofibers. For instance, tetraethyl

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orthosilicate, polyvinyl pyrrolidone, and the tri-block copolymer P-123 were mixed to fabricate continuous ordered mesoporous silica nanofibers by electrospinning method. The resulting scaffolds had a combination of multi-level porous structures, from micro- to macro-scale. The porous nanofibrous morphology showed bioactivity as demonstrated by the proliferation of human osteoblast-like cells (MG63).[146] A PCL nanofiber was fabricated by electrospinning and blended with silica nanoparticles, which were used to improve the polymers' mechanical property and bioactivity.[168]

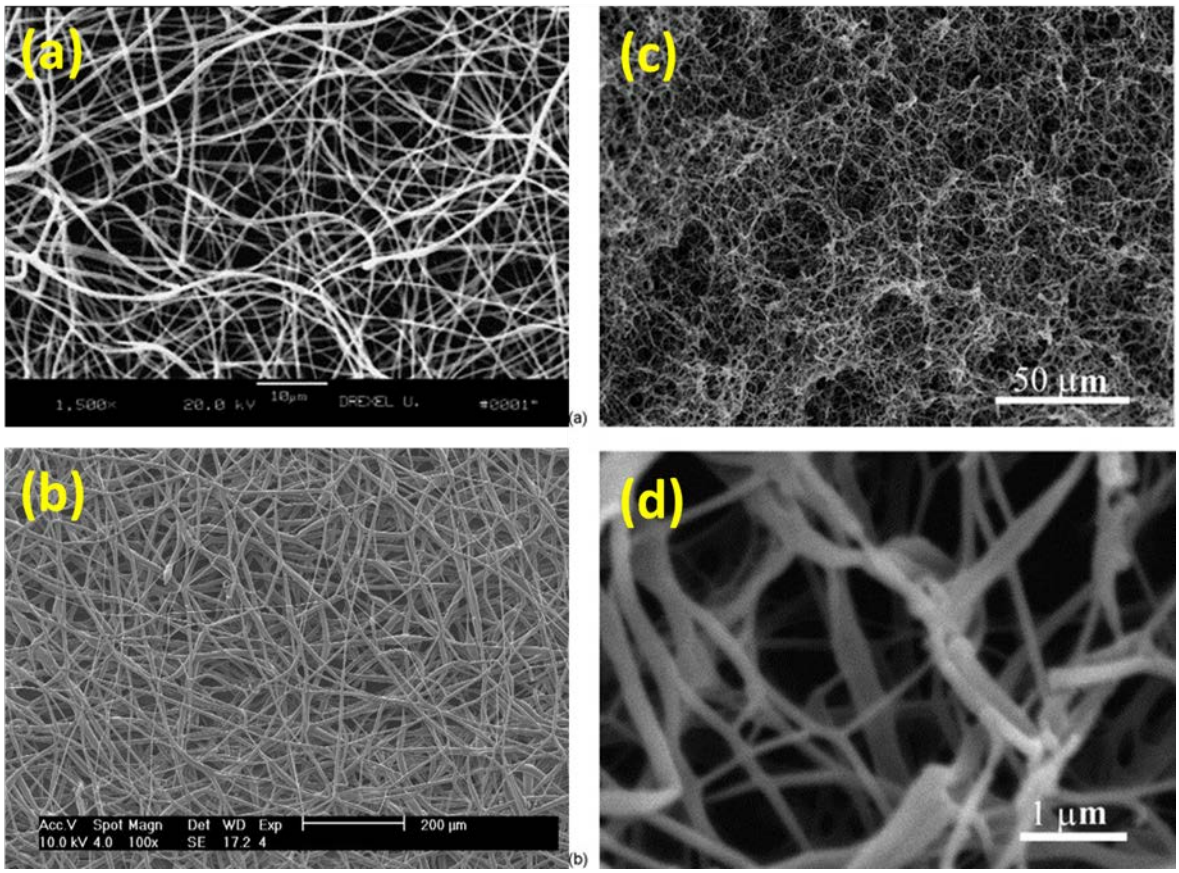

Figure 7. SEM micrographs of nano-fibrous scaffolds for bone tissue engineering. (a) the electrospun PLGA nano-fibrous structure. (b) electrospun PCL scaffolds. (c) and (d) a PLA fibrous matrix prepared by phase seperation technique. (c) 500× and (d) 20K× (reprinted with permission from reference 165). [165]

HAp/alginate nanocomposite fibrous scaffolds were fabricated via electrospinning[169] and in situ synthesis of HAp that mimics mineralized collagen fibrils in bone tissue. This novel process resulted in a uniform deposition of HAp nanocrystals on the nanofibers, overcoming the severe agglomeration of HAp

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nanoparticles processed by the conventional mechanical blending/electrospinning method. The nanofibrous topography combined with the hybridization of HAp and alginate can be advantageous in bone tissue regenerative medicine applications.[155] Self-assembly and phase separation are also used to fabricate nanofibers for bone tissue engineering.[165] Figure 7 shows fibrous nanomaterials for bone tissue engineering fabricated by electrospun and phase separation.

2.3.2 Nanoparticles

HAp nanocrystals are the main inorganic content in bone. Therefore, HAp nanoparticles are widely used in bone tissue engineering materials. It plays a very important role in supporting the bone structure and maintaining the mechanical strength. As a bioactive material, it extensively interacts and regulates the cell activity in bone.[170-173]

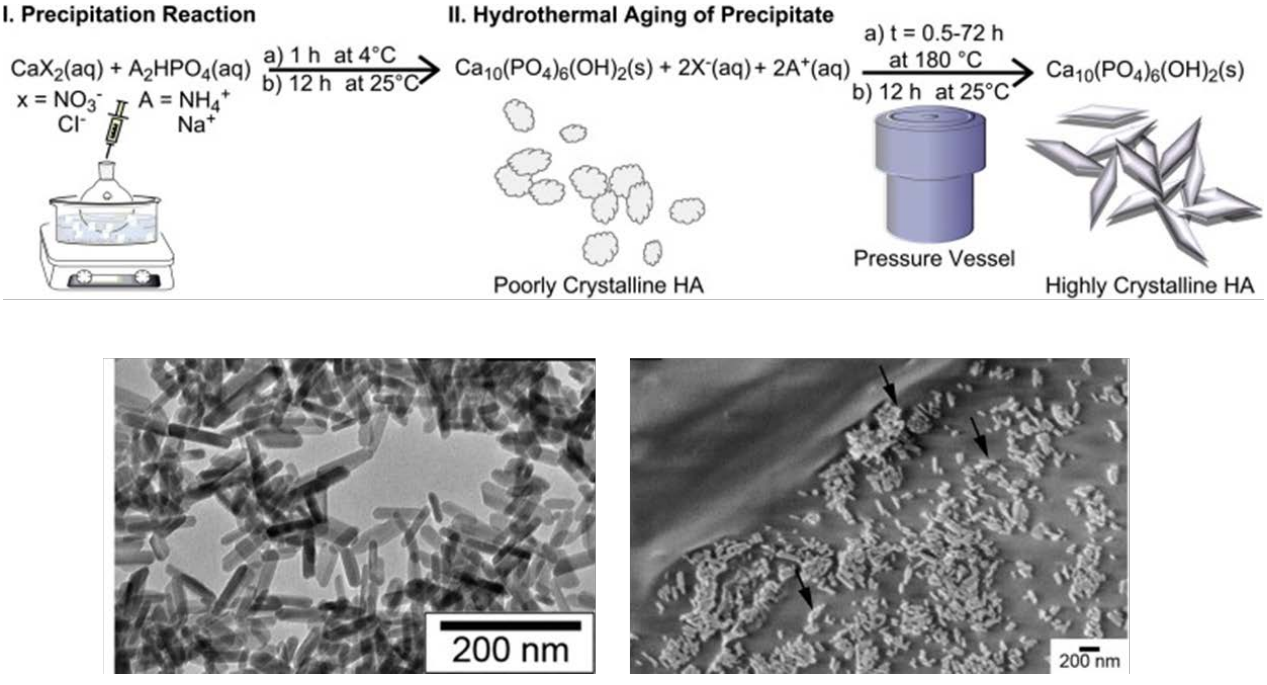

Figure 8. A two-step HAp nanoparticle synthesis process. TEM images (bottom left) of the HAp nanoparticles used to prepare mineral-containing scaffolds. SEM image (bottom right) showing the HAp nanoparticles on the scaffold surface, and arrows point to individual particles (reprinted with permission from reference 171). [171]

HAp nanoparticles were synthesized by a precipitation and hydrothermal aging process as shown by Figure 8. Briefly, the first step was a typical precipitation reaction between a calcium salt and a phosphate

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salt. Then a hydrothermal aging of the precipitate was used to obtain HAp particles with a narrow size distribution, as the TEM image showed. Porous mineral-containing scaffolds with PLGA and these HAp nanoparticles were fabricated by a gas-foaming/particulate leaching technique.[171] In another study, HAp nanoparticles were incorporated into PPF scaffolds, and showed improved the surface properties of PPF/ HAp composite scaffolds. The scaffolds had increased roughness and hydrophilicity, and showed significant protein adsorption and initial cell attachment.[170]

Other kinds of nanoparticles, such as gold,[117, 118] silver,[121, 124] titanium dioxide,[174-176] silica,[168, 177] and magnetic nanoparticles[79, 178] are also involved in bone tissue engineering materials, for targeting drug delivery, reinforced strength, and controlling purposes.

2.3.3 Nanogels

Cross-linking is one of the most used methods to create nanogels for bone tissue engineering.[179-183] A biodegradable nanogel containing cholesteryl group- and acryloyl group-bearing pullulan (CHPOA) and thiol-bearing PEG (PEG-SH) were fabricated to deliver two different growth factors, BMP2 and recombinant human FGF18. The CHPOA and PEG-SH are shown by Figure 9a, and they were cross-linked by thiol linkage. The nanogels were used for bone repair, which sustainably released two growth factors and strongly promoted the cell activities. Sustained release of multiple growth factors represent a future direction for the development of scaffolds for bone tissue engineering.[184]

A starch/N-vinylpyrrolidone nanogel has been synthesized by γ-radiation-induced graft copolymerization and crosslinking process. HAp nanocrystals were introduced to the nanogel via in situ deposition. The obtained hydrogel/HAp nanogel showed good bioactive and biocompatible properties in *in vitro* tests.[185]

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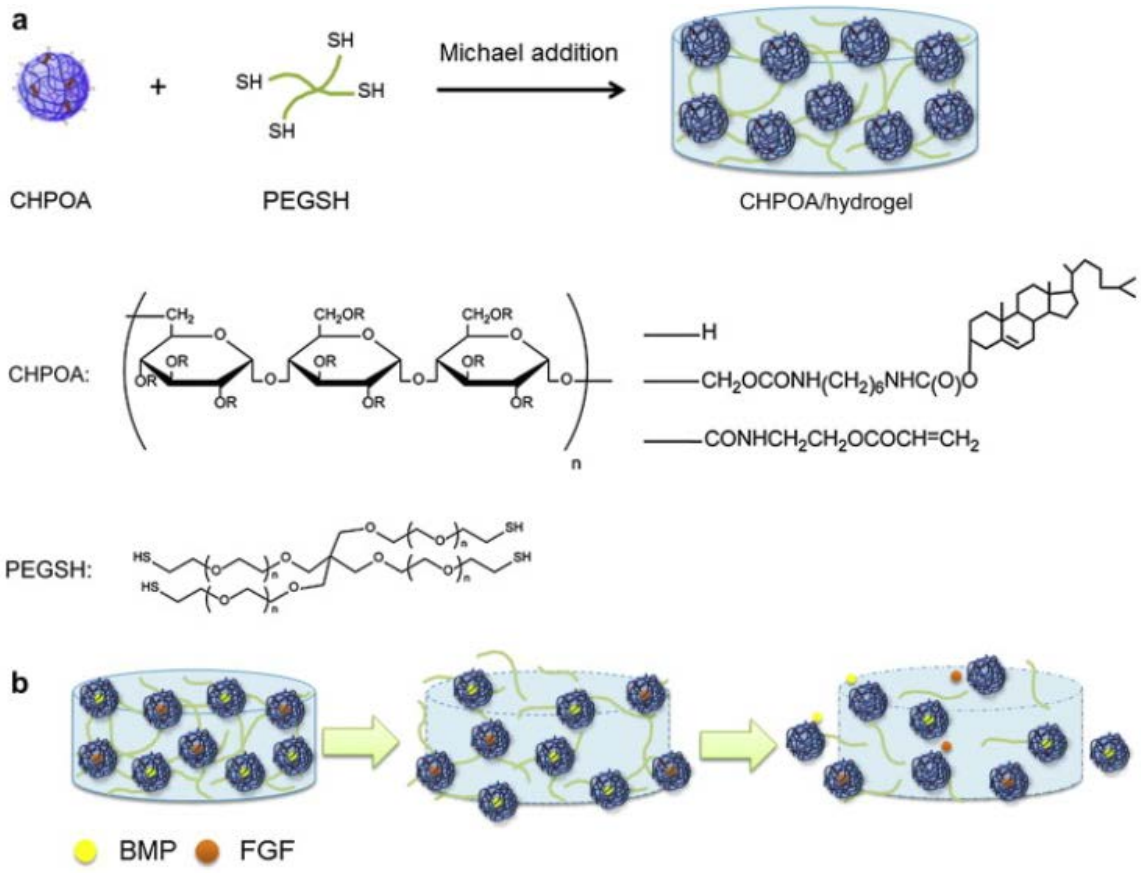

Figure 9. (a) Synthesis of the CHPOA/PEG-SH hydrogel and their chemical structure. (b) Releasing of the FGF18 and BMP2 as the hydrogel degrades. (reprinted with permission from reference 184).[184]

2.3.4 Nanocomposite scaffolds

In situ synthesis of the HAp nanocrystals is one of the most used methods to incorporate the HAp into the nanocomposites. For example, different kinds of HAp and chitosan nanocomposites have been synthesized by in situ precipitation process. [85, 86, 149, 169]

Many composites were prepared by sol-gel method.[5, 154] The HAp and polymer nanocomposites were prepared using the templates of self-assembling Pluronic F127.[5] The HAp precursor was mixed with the polymer solution, and precipitated in the polymer gelation induced by temperature change. As shown in Figure 10, citrate molecules were also used to control the nanocrystals' size in this process. The crystals' size would be reduced by increasing citrate concentration.

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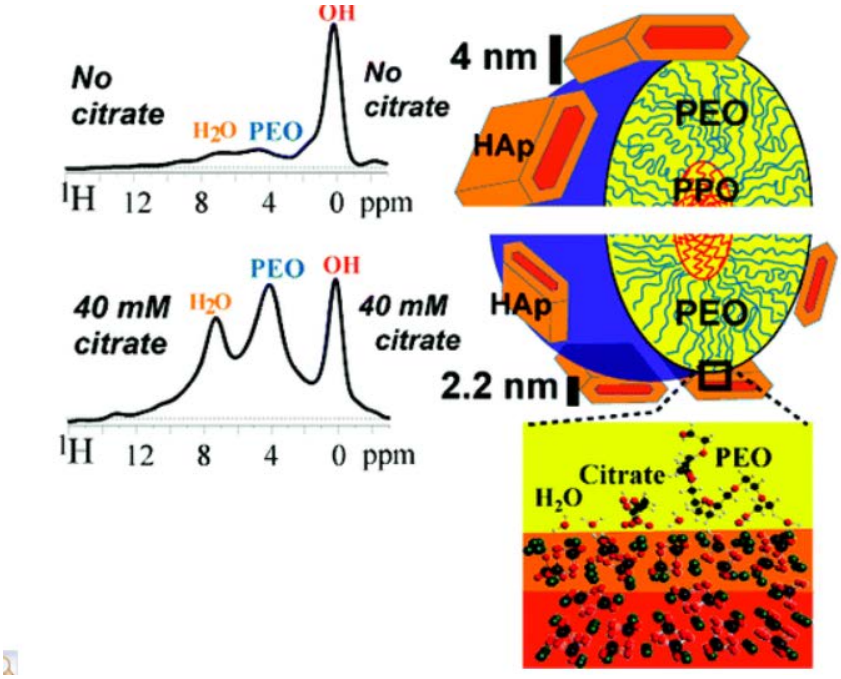

Figure 10. Nanocomposite formed by HAp nanocrystals precipitates on the self-assembled PEO-PPO block copolymers. NMR spectra showed that higher citrate concentration will reduced the size of HAp nanocrystal (reprinted with permission from reference 5). [5]

A biomimetic gelatin-amorphous calcium phosphate nanocomposite scaffold was fabricated by a double diffusion method under physiological condition; the experiment is shown in Figure 11. The resulting nanocomposite scaffolds had a 3D microporous structure, and nanocrystalline calcium phosphates were distributed among the gelatin matrix evenly. After incubation at 37°C for 5 days, the calcium phosphate was converted to HAp nanocrystal. [186]

Solution casting followed by particle leaching is the conventional method frequently used to fabricate polymer/bioceramic composites.[153] For example, nanocomposite scaffolds of PCL and forsterite nanopowder were fabricated by this method, with improved mechanical properties, bioactivity, biodegradability, and cytotoxicity.[187]

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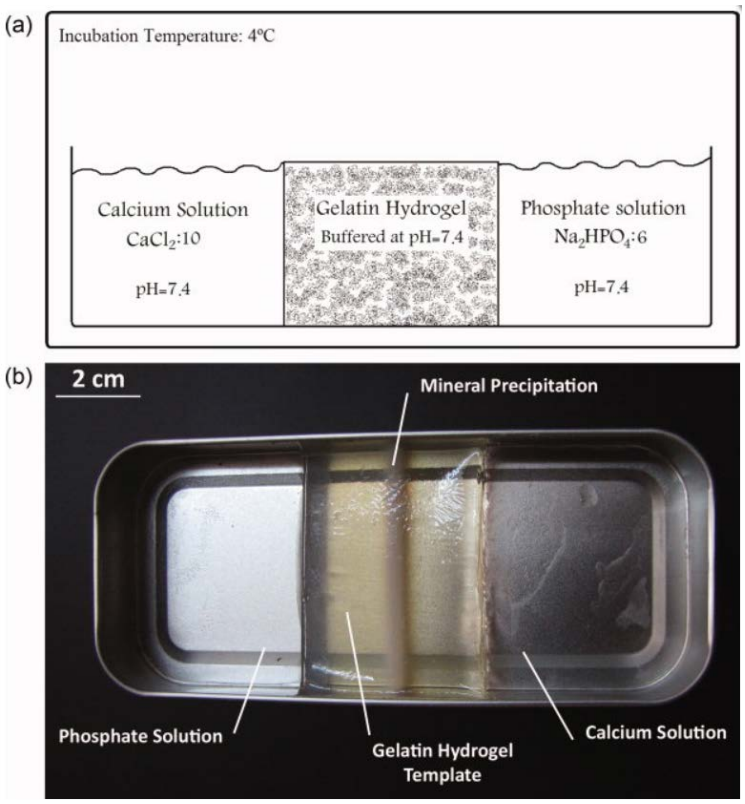

Figure 11. (a) Schematic view of the double diffusion method for calcium phosphate formation in a gelatin gel (b) formation of white calcium phosphate precipitate within gelatin matrix (reprinted with permission from reference 186).[186]

Cross-linking can also be used to fabricate tissue engineering nanocomposites.[188] Two 3-D nanocomposite porous materials were reported, based on collagen type I and beta-tricalcium phosphate nanopowder and the selection of the best-method for their chemical cross-linking using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride.[189]

2.4 Clinical trials of nanostructured materials in bone tissue engineering

Clinical translation of nanostructured bone tissue engineering scaffolds is the ultimate purposes of the materials development. Currently, the clinical challenges are the insufficient vascularization, weak mechanical strength, and bone infection management.[87] Therefore, the current research attention is mainly focused on improving the scaffolds bioactivity, biocompatibility and mechanical properties.

However, there are developments in the clinical trials. For example, the clinical trial using

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autologous bone marrow stromal cells showed an exciting outcome of functional bone recovery after 6-7 years of the implantation. The host bone maintained well and the recovered bone had good osseointegration. The only concern is the possibility of long term infection. As one of the mostly used materials for bone tissue engineering, collagen based scaffolds, such as BMP-2 or BMP-7-loaded InFuse bone graft (Medtronic Sofamor Danek), and OSIGRAFT (Stryker Biotech) have been reported with successful early stage in clinical trial.[87] The clinical trials of nano-HAp/polymer composites have been reported with successful material-cell interactions.[190] A type I collagen–HAp composite that mimics the nature bone was obtained by precipitating HAp nanoparticles on the collagen fibrils. In the clinical study, MRI evaluation has confirmed good early stability of the implanted biomaterial after 6 months of implantation. The further systematic evaluation is necessary to determine the clinical and morphological outcome.[191]

Overall, there is a great progress as well as great potential in the clinical trial of nanostructured materials for bone tissue engineering. There are also many unanswered questions and unsolved problems waiting in the future. Further fundamental understandings in both the life sciences and materials sciences are needed to develop successful bone regeneration materials.[190]

2.5 Summary

As the major part of musculoskeletal tissues, bone supports and protects the other organs of the body. The osteogenic and osteoclastic balance of bone determines the complexities and difficulties of bone tissue engineering. Many scaffolds with adequate mechanic strength fail in mimicking the natural functions of bone. Although these scaffolds possess good mechanic properties, they can’t achieve refreshment of nutrients and metabolites before vascularization. The thickness of wall and deepness of pore in the scaffolds influence the exchange of cells within the microenvironment, resulting in weak adherence, low proliferation, premature differentiation of seeded cell, even the death of the cells.

Although there have been substantial developments in using nanostructured materials for bone tissue engineering in recent years, current and new challenges need to be further evaluated and overcome

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to develop a commercial tissue engineered bone eventually. The first priority is to further understand the bone biology, such as the growth factors interactions between each other and within cells. Researchers should further investigate the growth mechanism of natural bone, especially signal regulation of bone. With better understanding of bone biology, the better biomimetic scaffolds for bone tissue engineering could be designed and achieved. Materials science and engineering are the second aspect that needs to be improved for developing new generation of bone replacement materials. New materials with biocompability, biodegradability, osteoinductivity, mechanical properties, microarchitecture including porosity and surface properties are favorable for bone tissue engineering.[87] Finally, the scaffold processing techniques need to be improved.[61] Nanotechnology and nanomaterials alone may not be sufficient to improve all the aspects. An optimized scaffold could be a comprehensive product based on combination of several materials and techniques in the future.[190]

3. Nanostructured materials for cartilage tissue engineering

3.1 Cartilage and associated microenvironment clues

Cartilage is a flexible but strong supportive connective tissue including the joints between bones, the rib cage, the bronchial tubes, and the intervertebral discs. Cartilage is composed of specialized cells called chondroblasts that can produce a large amount of extracellular matrix, such as collagen fibers and elastic fibers, embedded in a rubbery ground substance rich in proteoglycan. According to the different fiber composition in cartilage tissues, cartilage can be divided into three different categories: hyaline cartilage, fibrocartilage and elastic fibers. Among them, the hyaline cartilage has a wide distribution and typical structure. The surface of the cartilage in the body is coated by perichondrium which is a membrane of dense irregular connective tissue. It can be transformed into bone periosteum when it is replaced by cartilage. Cartilage cells can continuously produce new cartilage matrix, all cells are surrounded by cartilage capsule respectively. Cartilage mucin, mainly composed of acid glycosamine glycan, is the primary component in transparent cartilage matrix. Although there are no blood vessels in the cartilage,

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the cartilage matrix contains enough water. Therefore, nutrients can easily penetrate into matrix. Collagen accounts for about 40% of the organic composition of cartilage. Cartilage capsule contains less collagen, but it has more chondroitin sulfate because the matrix is rich in collagen fiber. Perichondrium can protect the cartilage and provide the nutrient to it. Therefore, it plays an important role in the growth of cartilage.

There are many cartilage seed cells which can produce cytokines including as cartilage derived morphogenetic protein 1 and 2 (CDMP-1 and -2), BMP, TGF-β, insulin like factor (IGF), FGF as well as the newly-discovered growth factors in cartilage tissue. The studies have confirmed that growth factors such as IGF-I, TGF-β1, BMP-7, and platelet-derived growth factor AB (PDGF-AB), can regulate the metabolism and growth of chondrocyte. The inclusion of serum, IGF-I, and BMP-7 can stimulate proteoglycan deposition and decrease tensile integrity of cartilage. The combination of various cytokines may further improve the formation of cartilage when they are mixed in the scaffold materials.

3.2 Materials for cartilage tissue engineering

3.2.1 Natural polymeric nanomaterials

As a major component of connective tissue, collagen gives connective tissue strength and flexibility. Yuan *et al*. constructed scaffold of artificial cartilage using collagen type I.[192] After cultured *in vitro* for 7, 14, and 28 days, they implanted neonatal rabbit chrondrocytes into hydrogel matrix. They found that collagen type I may play a role as immunomodulatory factor in the formation of cartilage. In recent years, the chitin nanocomposite has been widely used in cartilage tissue engineering. When chitin composite scaffolds have been placed into tissue defect, the scaffolds only lead to negligible foreign body rejection. Many neutrophils gathered around graft, but they dissipated quickly without chronic inflammation and fibrous tissue hyperplasia. Gilbert's group evaluated the potential use of chitosan, a natural derivatives of chitin, as scaffold material for cartilage tissue engineering. The porcine chondrocytes were cultured *in vitro* up to 28 days; the cells adhered on the surface of chitosan scaffolds and maintained their normal morphology. After 18-day culture, the chondrocytes began to synthesize extracellular matrix such as proteoglycan and collagen type II.[193] Jiang *et al*. designed a novel silk-fibroin peptide

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(SGGAGGAGGAGGAGGS) hydrogel for 3D culture of chondrocytes *in vitro*.[194] The studies included the morphology characteristic of cells, DNA content analysis, and histological and immune-histochemistry staining. The data confirmed that the fibroin hydrogel induced collagen type II and glycosaminoglycans (GAGs) in the chondrocytes. Deena et al. tried to regulate the mechanical performance of PEG and agarose interpenetrating network (IPN) hydrogels for cartilage tissue scaffolds.[195] Their results supported that the viability of encapsulated chondrocytes was not significantly affected by IPN formulation, compared to the scaffolds with just agarose (Figure 12).

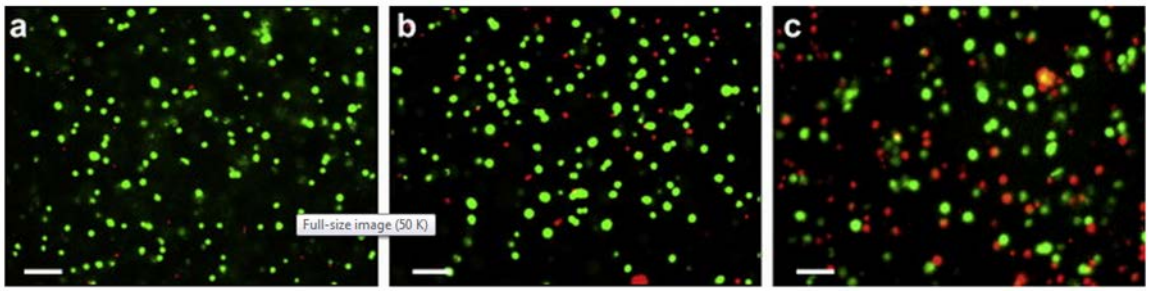

Figure 12. Images of (a) agarose and (b) IPN with a ration of agarose and PEG-diacrylate (1:10) 24 h after encapsulation, and (c) an IPN 3 weeks after encapsulation. Live cells were stained green with Calcein AM (green), while dead cells were stained red with ethidium homodimer-1. Scale bars = 50 μm (reprinted with permission from reference 195). [195]

3.2.2 Synthetic polymeric nanomaterials

The poly-α-hydroxy esters family, including PLA, PGA and their copolymers PLGA, are widely used materials in cartilage tissue engineering, due to their excellent biodegradability and biocompatibility.[196-204] Scaffolds made of these polymers are easy to modify their properties. A major drawback of synthetic polymers is the insufficient bioactivity. Yoo *et al*. found that addition of the hyaluronic acid (HA) into PLGA can increase the cartilage cell adhesion and synthesis of glycosaminoglycans (GAG) and collagen.[201] Wu *et al*. confirmed that appropriate proportion of porous poly (acrylic ester/chitosan) composite scaffolds has better biological characteristics than pure porous poly propylene carbonate.[202] It can not only enhance cell adhesion, proliferation, but promote the generation of type II collagen. Sarasam et al. reported that chitosan/PCL nanocomposite has good

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mechanical properties.[203] PHB originally isolated from bacteria has piezoelectric effect. However PHB is fragile and unstable when it is heated. Other properties such as long degradation time, bad plasticity and mechanical strength limited its clinical application. Some researchers modified the PHB in principal chain with the pentanoic acid (PHV) to form the PHBV copolymer and improve its performance.[203]

3.2.3 Composite nanomaterials

Composite materials, including natural biological polymers, artificial synthetic polymers and their combinations, may possess good optimized structure and biological features and may be more feasible to develop artificial cartilage. Yamane *et al*. found that the hydrogel made with chitosan and hyaluronic acid can maintain cell phenotype when inoculated with chondrocyte.[205] The composite hydrogel can also generate type Ⅱ collagen and proteoglycan. After that, Hsu *et al*. implanted chondrocyte into chitosan/alginate/sodium hyaluronate composite scaffolds which were modified with arginyl-glycyl-aspartate peptide, it was reported that cartilage cells grew well on the scaffolds and excreted redundant glycosaminoglycans and type Ⅱ collagen.[206]

Nečas's group has studied the mechanical response of porous scaffold for cartilage engineering. They investigated the effect of hyaluronic acid, HAp nanoparticles or chitosan nanofibers on the mechanical response of the lyophilized scaffold of cross-linked type I collagen. The data supported that hyaluronic acid significantly reduced the tensile elastic modulus and increase the strength whereas HAp nanoparticles and chitosan nanoparticles increased the elastic modulus of scaffold. In addition, chitosan also increased cell growth comparing to the type I collagen alone *in vitro* and extended its resorption more than 10 weeks.[207] Kuo *et al*. designed polyethylene oxide (PEO)/chitin/chitosan composite scaffold with different ratios, and analyzed the effect of components changes on the porosity of scaffolds.[208] As shown in ternary phase diagrams (Fig. 13), it was found that the composite scaffold had better bioactivity and elasticity than single-composition scaffold.

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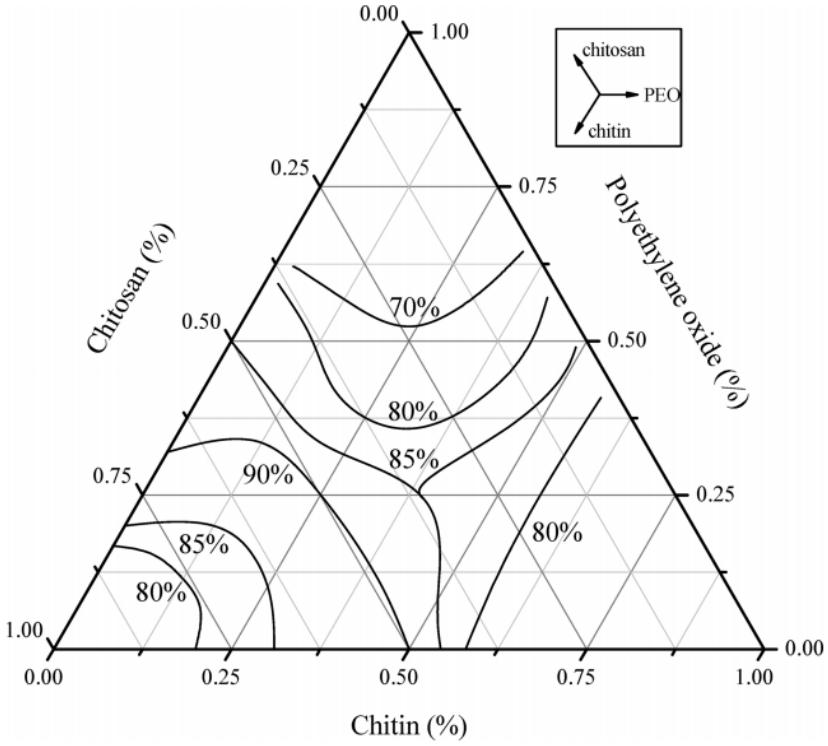

Figure 13. Porosity changes of the chitosan/PEO/chitin scaffolds in ternary phase diagrams. Note: the data were calculated by mean value of all compositions (n=3), and the axes indicated the weight percentage of the various components (reprinted with permission from reference 208).[208]

Iwasaki *et al* designed an artificial scaffold by mixing polysaccharide hybrid materials containing ploying complex with alginate and chitosan.[209] The studies have been confirmed that alginated-based chitosan was excellent material for chondrocytes adhesion. In addition, morphologic studies indicated that the round morphology of chondrocytes was maintained and the dense fiber of the type II collagen was excreted in the hybrid polymer (Figure 14).

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Figure 14. Adherences of rabbit articular chondrocytes on alginate/chitosan nanofibers after 14 days of culture. The typical round shape of the chondrocyte and the dense fiber of the type II collagen were showed on the nanofiberous scaffolds (reprinted with permission from reference 209).[209]

Gu *et al*. mixed CDMP-2 and TGF- β into PLGA scaffolds and observed the influence of scaffold on the excretion of collagen type I and II by the myoblasts.[210] Twelve weeks after implantation, the compressive moduli of artificial implant reached 85.7% of the health meniscus with a high level of glycoaminoglycan content. Jung *et al*. observed the effects of local BMP-7 release from PLGA scaffold on the repair of osteochondral defects in rabbits.[211] Their results also confirmed that the composite material containing BMP-7 in the scaffold of PLGA matrix might be a potential candidate for osteochondral repair. Ab-Rahim *et al*. compared the difference of extracellular matrix expression between superior alginate matrix with or without TGF-β1 and monolayer culture system.[212] The results demonstrated the alginate construct can significantly increase the level of glycosaminoglycan/mg protein in the matrix than monolayer cultures.

3.3 Fabrication of nanostructured materials for cartilage tissue engineering

3.3.1 Nanofiberous materials

There are three methods mainly used to fabricate nanofiberous materials for cartilage tissue engineering: electrospinning, phase separation, and self-assembly.[213-216] Electrospinning is a classical technique used widely for nanofibers fabrication (Figure 15).[209, 217] Nanofibers for cartilage tissue

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engineering can be easily produced from various polymers solution.[218, 219] Generally, synthetic polymers are more convenient for electrospinning fabrication.[220] Polymer composition, such as ratios of different polymers, and salt additives are used to regulate the electrospinning process.[221] In alginate/chitosan composite fibers, the added poly (ethylene oxide) (PEO) enhanced the chain entanglements and reduced the conductivity of the charged polysaccharide solution. PEO can be removed using water clearance after the fibrous structure formed.[222] Gelatin and collagen were electrospun to form nanofibrous structure with diameters of 100nm, which are similar to native cartilage.[223] Other synthetic polymers, including PCL, PLA, copolymers of P(EG-CL), P(LA-CL), and PLGA, have also been used in the electrospinning process for cartilage tissue engineering.

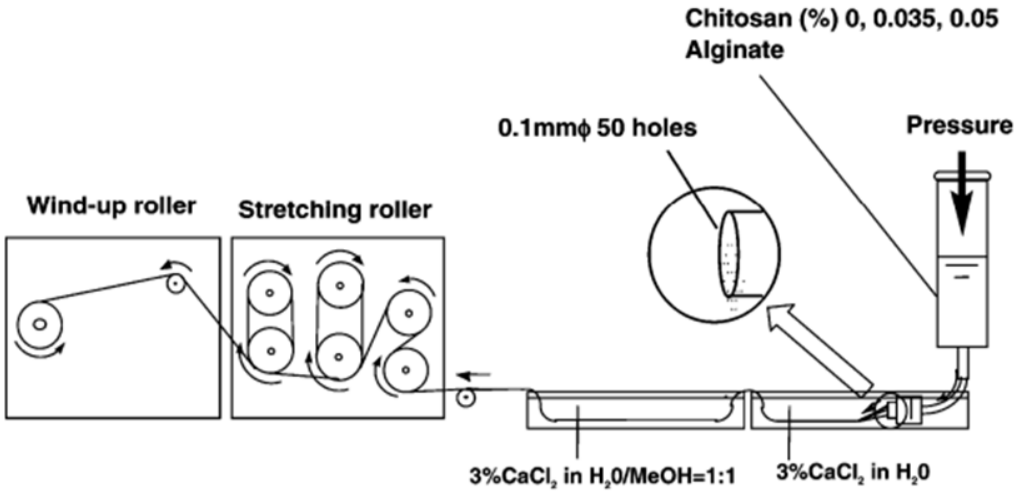

Figure 15. The spinning process of alginate fiber coated with chitosan (reprinted with permission from reference 209).[209]

Casper et al. seeded periosteal cells into ploy-ε-caprolactone nanofiber scaffolds to prepare artificial cartilage tissue.[224] Results confirmed that cell can infiltrate into entire scaffolds. They also found that chitosan significantly decreased the production of GAG and cartilage yield. There was no statistically difference in GAG content and cartilage yield between TGF-β-injected matrix and TGF-β free matrix. However, TGF-β was benefit to the mineral deposition but chitosan decreased the level of that.

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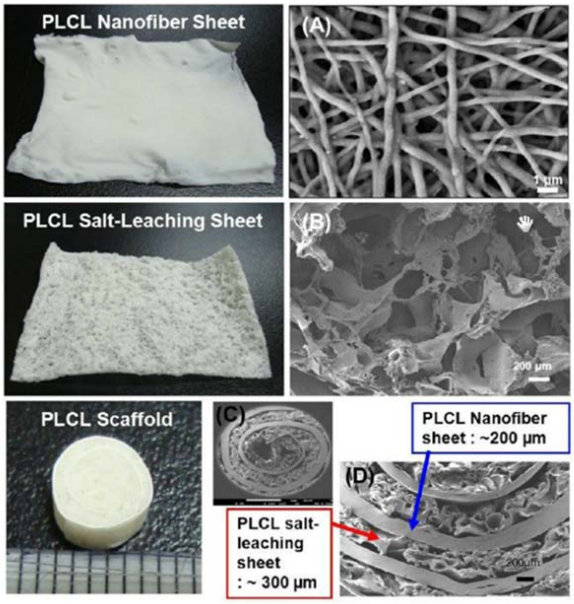

Figure 16. SEM images and photographs of PLCL scaffolds. PLCL nanofiber sheet by electrospinning(A), followed salt-leaching (B), PLCL scaffold by rolling (5 mm in diameter and 3 mm in thickness in left section) (C) and PLCL scaffold with a high magnification(D) (reprinted with permission from reference 225). [225]

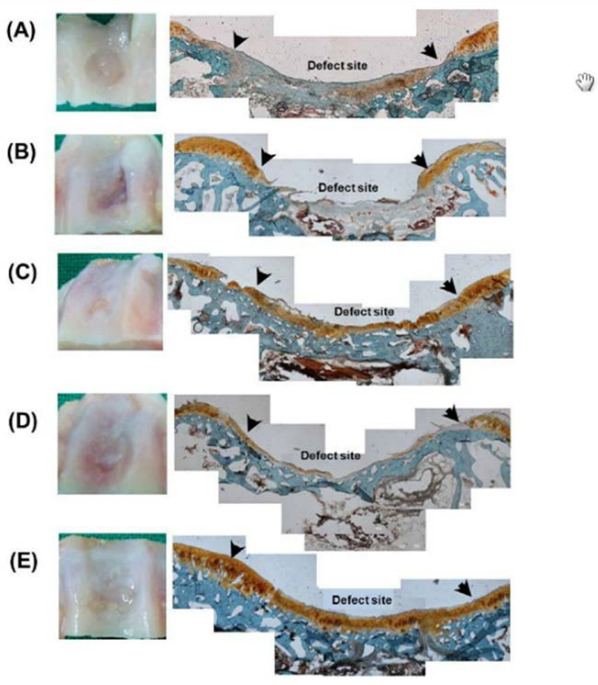

Figure 17. Macroscopic appearance of the defect site and histological staining of cryo-section in rabbit knee tissue four months after implantation. (A) Control group (partial defects), (B) only PLCL scaffold, (C) PLCL scaffold with chondrocytes, (D) only PLCL scaffold/hydrogel, and (E) PLCL scaffold/hydrogel/ chondrocytes. The arrows point to the borders of initial defect sites (reprinted with permission from reference 225). [225]

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Kim et al. prepared a composite system with poly (L-lactide- co-ε-carprolactone) (PLCL) scaffolds and heparin-based hydrogel to combine the advantages of nanofibrous and hydrogel to mimic cartilage microenvironment for seeded cells.[225] Gelatin-mixed PLCL was similar to natural cartilage while heparin-based gel provided chondracytes survival conditions (Figure 16 and 17).

3.3.2 Nanoparticles materials

Recently, nanoparticles system has been extensively used to control the release of growth factors in the cartilage scaffolds.[92, 226-229] Particle size, charge, morphology and release behavior of loaded molecules can be regulated for different applications.[230] Nanoparticles fabricated from both natural and synthetic polymers have been used for cartilage tissue engineering.

Weber *et al*. proved that the porous PLGA particles could slowly release the loaded-BMP, resulting in enhancement of regeneration of cartilage *in vivo*.[231] Nanoparticles made from natural polymer gelatin also showed a sustained release of the loaded TGF-β over an experimental period of 28 days.[229] Surface modification of nanoparticles can be achieved by covalent binding between surface and functional molecules or polymers and layer-by-layer assembly.[232] Ertan *et al*. controlled the release of IGF-1 and TGF-β1 to promote the growth of BMSCs and induce the cells differentiation into chondrocytes in the PLGA and poly(N-isopropylacrylamine) (ONIPAM) matrix.[233] On polystyrene tissue culture system, TGF- β1 promoted the proliferation while IGF-1 induced differentiation. The combination of two growth factors yielded the improved results such as collagen type II and aggrecan. In another study, Wang's group investigated the effects of exogenous bFGF on the repair of full-thickness articular cartilage defects in rabbits. The results demonstrated that fast release of bFGF stimulated the production of TGF-β2, VEGF, BMP-2, 3, 4 and bFGF. In addition, the existence of chondrocytes-like cells was observed at early stage of cartilage regeneration (Figure 18). [234]

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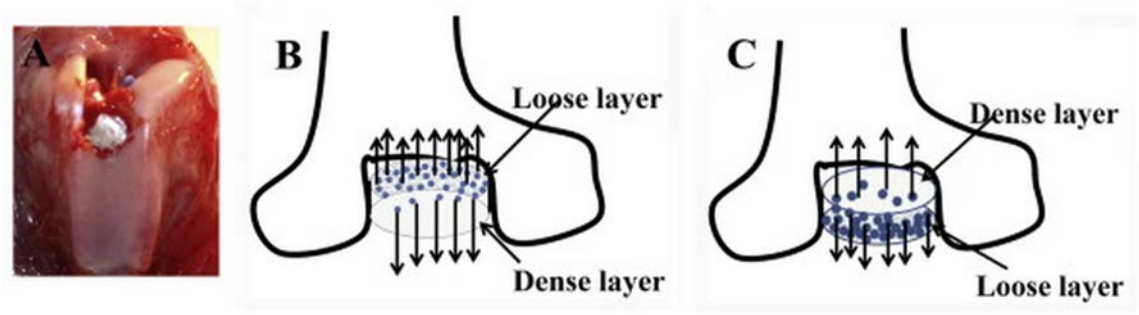

Figure 18. The graft of the membrane and schematic summarization of the grafting position and bFGF release from the membranes in two implantation direction. (A) graft of the double-layered collagen membrane. (B and C) Schematic summarization of the membrane graft and bFGF release profiles. (B) The loose layer or (C) The dense layer facing the subchondral bone. The blus dots indicate nanoaprticles loaded with bFGF (reprinted with permission from reference 234). [234]

3.3.3 Nanogels

Cross-linking by regulation of composition and reaction condition is the main method used to fabricate nanogels of artificial cartilage tissue. Photopolymerization by ultraviolet or visible light were also initiated to form hydrogels.[235] PEG can promote chondrogenesis after crosslinking into hydrogels.[236] Recently, degradable PLA added with PEG improved cell growth and ECM excretion.[236-239] Similarly, alginate can prepare stable ionically crosslinked nanogels through crosslinking. Researchers have modified alginate gels with synthetic adhesion peptides [240] or conjugated alginate with other materials to produce hybrid cartilage scaffolds. [209, 241, 242] Although the modified alginate nanogels have many advantages for chondrogenesis, they also have limitations such as low mechanical strength and slow degradation. Furthermore, the exchange of growth factors and metabolites from hydrogel into microenvironment still impact its clinical trials. Small molecules can easily penetrate through the network of hydrogels and consequently interact with cells in hydrogel. Therefore, nanoparticles loading with growth factors can be tethered for a sustained release when they were encapsulated in hydrogel.[228, 243, 244]

3.3.4 Nanocomposite scaffolds

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Hydrogels have been widely used in cartilage tissue engineering due to their biological similarity with highly hydrated natural tissues. However, the feasibility of hydrogel in the clinical applications is not existed due to its poor mechanical properties. Composite scaffolds can overcome the disadvantages of hydrogel to mimic natural biological tissue structures. Some composite scaffolds fabricated with biomacromolecules such as alginate and collagen with polymer meshes have been developed in cartilage tissue engineering and shown positive results.[245] The most popular method for creating a nanocomposite cartilage tissue scaffold is incorporation of fibers within a bulk hydrogel system and layering fibers within gels. Tan *et al*. fabricated a porous scaffold composed of gelatin/chitosan/hyaluronan ternary complex by the freeze-drying technique.[246] Other researchers found that the emulsion coating technique could delay the TGF-β release by changing the copolymer composition of the coating.[247] Nela Buchtova *et al.* designed mesoporous silica nanofibers interlinked with siloxane modified polysaccharide for cartilage tissue engineering.[248] Recently, sponge-like scaffolds fabricated by collagen, glycosaminoglycan, or native cartilage extracellular matrix components have been reported to induce the chondrogenic differentiation of mesenchymal stem cells (MSCs). [249, 250]

3.4 Clinical trials of nanomaterials in cartilage tissue engineering

The clinical translation and feasibility of scaffold-based cartilage tissue engineering need to be considered in many aspects. Currently, the clinical use of these materials is limited by the risk of disease transmission and immunological repulsion. Future research should mainly focus on investigation and evaluation of tissue-engineering approaches to design artificial cartilage.

At present, the nanostructured scaffolds have made steady progress in the development of cartilage tissue engineering. Collagen- and hyaluronan-based matrices are the most popular natural scaffolds in clinical trials.[251-253] Peterson *et al*. reported that autologous chondrocyte transplantation in dissecans lesions of the knee produced an integrated repair tissue with successful clinical results few years later.[254] Cherubio *et al.* also suggested that autologous chondrocyte implantation using collagen nanomatrix had the advantages of easy operation and lower immunogenisity.[255] In some random clinical trials, Gudas *et*

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al. demonstrated that autologous osteochondral transplantation has good clinical results 10 years after implantation.[256] Schaefer *et al.* seeded bovine articular chondrocytes into a PGA mesh scaffold, which transformed well-developed cartilaginous-like tissues.[257] In addition, the seeded cells maintained their individual phenotypes during the composite culture and formed a well-defined cartilage-bone interface.

3.5 Summary

Overall, nanostructured materials used for cartilage tissue engineering have made great progress in the past decades and hold tremendous impact for future clinical applications. Compared with bone, cartilage is more flexible connective tissue and does not need the vascularization after implantation. The thickness of cartilage is also thinner than that of bone, which is easier to refresh the nutrients and metabolites for cell survival by diffusion mechanisms. Artificial cartilage should contain more proteoglycan and elastin fibers, and induce the excretion of these compositions by seeded cell. Nanocomposites scaffolds may mimic the composition and structure of natural cartilage. Due to the recent developments, nanomaterials have the ability to guide cartilage tissue cells. Surface of the nanostructured material is similar to natural ECM, which can further improve cell adhesion and biocompatibility of the material.

However, there are shortcomings of nanostructured cartilage scaffolds, either natural or synthetic. The flow mechanics in the matrix is real challenge. Despite the remarkable progress in nanomaterials, current available techniques are not able to fully restore particular cartilage. As we have mentioned above, it is essential to combine several suitable materials and techniques to fabricate scaffolds. Through the combination of new techniques, such as 3D printing, the scaffold properties can be greatly improved. The imaging technique might also help us to monitor the refreshment of humor liquid in the nanostructured cartilage matrix. Smarter software of computer simulation might calculate the dynamic behavior of solid, liquid substance in the matrix, and help us to generate an ideal artificial cartilage replacement tissue.

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4. Nanostructured materials for muscle tissue engineering

4.1 Muscle and associated microenvironment clues

As the largest part of the human body, skeletal muscle tissue is of great importance.[258] It controls voluntary movement, and maintains the structural contours.[259, 260] Muscle is composed of bundles of muscle fibers, which are multinucleated organization of cells derived from myoblasts. Muscle fibers consist of a longitudinal arrangement of myofilaments with actin and myosin as major components.[261, 262] The cells employed in skeletal muscle tissue engineering research mainly include muscle satellite cells, muscle cells, embryonic stem cells and bone marrow derived stem cell. The self-renewal of skeletal muscle tissue arises from satellite cells residing beneath the basal lamina. The proportion of these cells (up to 1-5 %) in the total skeletal muscle changes with age and muscle fiber types.[263] These cells may maintain a quiescent and undifferentiated state. When cells are stimulated by specific factors, they will enter the mitotic circle.[264] When injuries occurs, satellite cells migrate and proliferate in the injured area.[264, 265] The primary skeletal muscle cells can be harvested from adult muscle.[266] Skeletal muscle tissue engineering mainly depends on the regenerative properties of the satellite cells and their potential for proliferation and differentiation.[267, 268] The satellite cells without proliferation ability can't promote regeneration and will form connective tissue. Therefore, it becomes an urgent goal to generate new muscle fibers via satellite cells when muscle structure is irreversibly compromised or individual muscles (or part of them) have been ablated by surgical procedures or injuries. The following schematic shows the concepts of muscle tissues engineering to treat related diseases *in vitro* and *in vivo* (Figure 19).[269]

Currently, there are many different growth factors associated with muscle tissue engineering growth reported in the literatures. They are muscle differentiation factor (MyoD), myogenic regulatory factor (Myf5), IGF, FGF, epidermal growth factor (EGF), PDGF, TGF, hepatocyte growth factor (HGF), and leukemia inhibitory factor (LIF).[270]

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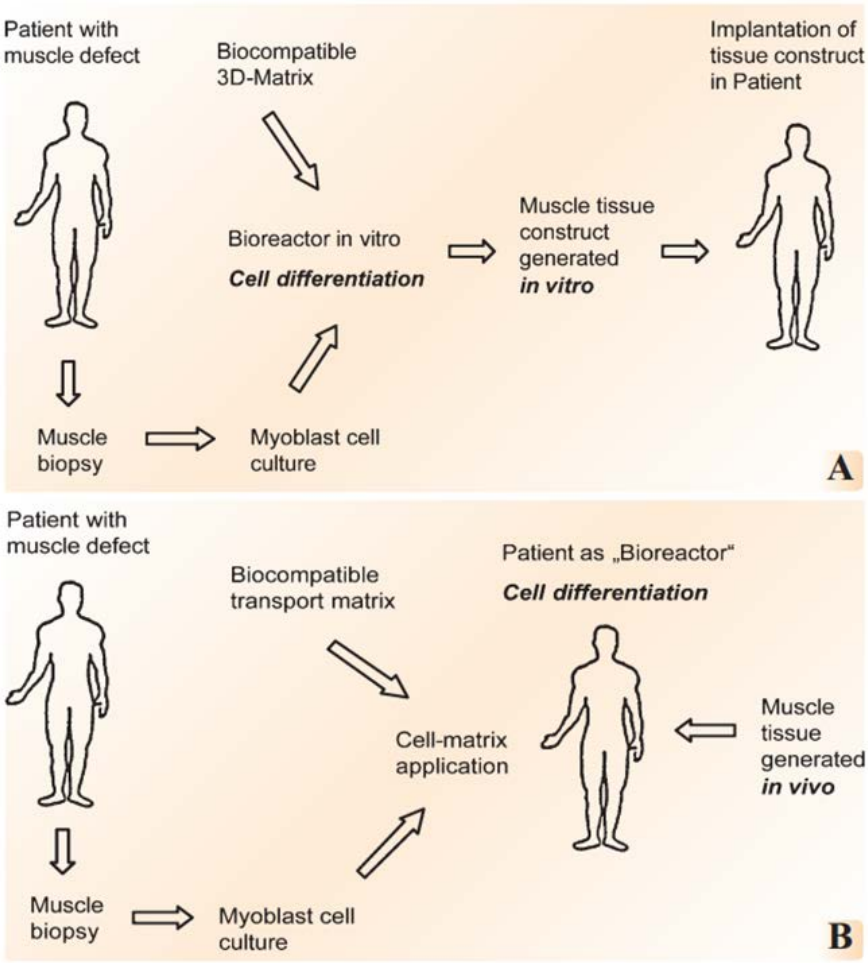

Figure 19. The concept of tissue engineering approach *in vitro* and *in vivo* (reprinted with permission from reference 269). [269]

4.2 Nanostructured materials used for muscle tissue engineering

4.2.1 Natural polymeric nanomaterials

Currently, the natural polymeric scaffolds using in muscle tissue engineering mainly include collagen, chitosan, and acellular matrix (ACM). Kroehne *et al*. utilized a novel collagen matrix with oriented pore structure to induce muscle cell differentiation *in vitro*.[271] They investigated the adherence of permanent myogenic cell line C2C12 on the surface of collagen sponge (CS) scaffolds in a highly-density cell suspension and observed the differentiation and formation of multinucleated myotubes. CS with either proliferating cells or myotubes was transplanted into the beds of excised anterior tibial muscles of

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immunodeficient host mice. The results showed that biodegradable CS with parallel pores was able to orient muscle fibres compatible of force generation in regenerated muscle.

Recently, it gathered attention in the surface modification of scaffolds for muscle tissue engineering. ACM with negligible antigenicity and good biocompatibility can provide desirable characteristics required for cell growth. In addition, the residual neural pathways and microcirculation system of ACM can promote the vascularization and neurotization of tissue engineered muscle.[272, 273] Therefore, ACM has become the excellent muscle tissue engineering scaffolds. Furthermore, matrix components have the advantages of clinical applicability and are more attractive regarding regulatory issues comparing to Matrigel™.[274] The drawback of ACM of scaffold is the extreme fragility and the associated difficulties of handling. They usually cause batch-to-batch variations during isolations, have low mechanical strength and involve high cost.[275]

4.2.2 Synthetic polymeric materials

Poly(1,8-octanediol-co-citrate) (POC) and PLCL are biodegradable polymers suitable for muscle tissue engineering.[276, 277] Huang *et al*. fabricated the nanofiber scaffolds using PLLA by electrospinning.[278] The results confirmed that the scaffolds regulated muscle cell adhesion, growth, and proliferation at multiple levels. Figure 20 showed myotube striation and myoblast proliferation on aligned nanofibers various days after cell seeding.

Levenberg *et al*. co-cultured endothelial cells (HUVEC or hES cell-derived endothelial cell) with skeletal myoblasts on the PLA and PLGA scaffolds and observed the vascularizaiton in the skeletal muscle tissue matrix *in vitro*.[279] Furthermore, they studied the survival and vascularization of the engineered muscle engrafts *in vivo*. The data indicated that prevascularization was able to improve the vascular neogenesis, blood perfusion and survival of the muscle tissue constructs after transplantation.

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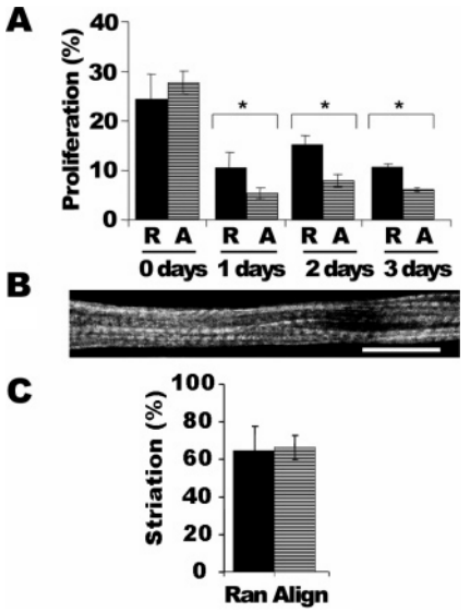

Figure 20. Statistical analysis of myotube striation and myoblast proliferation on aligned nanofibers. (A) Cell proliferation test by BrdU incorporation (R, random; A, aligned). (B) Fluorescence imaging of striated myotube on aligned nanofibers by anti-MHC staining (scale bar = 20 μm). (C) The comparison of striated cells 7 days after seeded. * $P<0.05$. (reprinted with permission from reference 278). [278]

4.2.3 Nanocomposite materials

Sole biomaterial is difficult to satisfy all the requirements of muscle tissue engineering. Therefore, the preparation of composite scaffolds with desired characteristics is the focus of future research in muscle tissue engineering.[280-282] Choi *et al*. have made fibrous scaffolds combined with PCL and collagen using electrostatic spinning technology.[280] They found that myoblast can keep stable adhesion and generation after inoculating with the composite scaffold.

Based on similar properties to muscle tissues, Hajiabbas *et al*. designed chitosan-gelatin sheets as scaffolds for muscle tissue engineering.[283] They evaluated the effect of polymer concentration and scaffold stiffness on the behavior of seeded cells on the sheets. Chitosan concentration could be used to regulate the elastic characteristics for surgical purposes, while intermediate stiffness possessed the best cell attachment, expansion, and proliferation. Bhat *et al*. mixed chitosan, agarose, and gelatin (CAG) into

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cryogel scaffolds in optimized ration using the cryogelation technique.[284] They studied the feasibility of the matrix for cardiac tissue engineering (Figure 21). To mimicking hollow organ such as bladder, Horst *et al*. developed a bilayered hybrid microfibrous PLGA-acellular matrix scaffold and evaluated the feasibility of hollow organ regeneration in rat bladder model.[285] The data confirmed that the produced 3D scaffolds provided good support for growth, attachment and proliferation of primary bladder muscle cells. Importantly, the hybrid scaffold featured with normal bladder capacity and held potential for engineered bladder and other hollow organs.

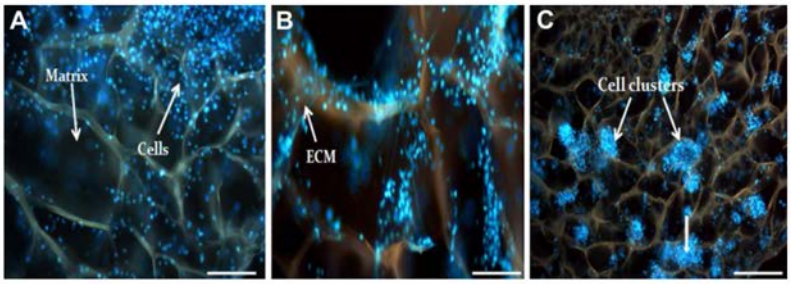

Figure 21. The proliferation of C2C12 cells on the CAG scaffolds (200 μm) stained with DAPI (A, B). Proliferation of HL1 cells on cryogel section (200 nm) stained by DAPI (C). Scale bar: 100 μm. initial cell density for seeding: 1 $\times 10^6$ cells/mL (reprinted with permission from reference 284). [284]

4.3 Fabrication of nanoscale materials for muscle tissue engineering

4.3.1 Nanofibers

The parallel aligned fibers was a favorable design for muscle tissue engineered.[286] For example, collagen-1 nanofibers have been fabricated for muscle tissue engineering. Electrospinning is a simple and effective technique to create nanofibers.[165, 287] PCL nanofibrous meshes was made to support colonies of myocardiocytes which began spontaneously contracting after 3 days of culture.[288] Various other electrospun fiber scaffolds have been designed, fabricated, and applied for muscle regeneration, and the results showed good adhesion and proliferation properties.[289-294] Zhong *et al.* created an unorganized collection of fibers using the standard electrospinning technique.[295] The resulting fibers were well aligned and induced cell organization and growth along the fiber's direction. Liquid-liqiud phase separation was

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used to produce nanofibrous PLA scaffolds for nerve generation.[296] Self-assembled nanofibrous scaffolds use engineered materials which undergo self-assembly to form a matrix of nanofibers. To make the self-assembly happen, the engineered materials are usually modified with amphiphilic peptide sequences.[297]

4.3.2 Nanoparticles

There has been a great interest in application of nanoparticles as scaffolds for muscle tissue engineering.[298] Freeman' group observed the characteristics of electrospun PLA and gold nanoparticle composite scaffold for muscle tissue engineering. They designed a scaffold with PLA and gold nanoparticles to regulate muscle cell elongation, orientation, fusion, and striation.[299] In another study, C2C12 cells labeled with magnetite nanoparticles were adhered and oriented on the matrix of scaffold to form multilayered muscle cell sheets under magnetic force direction.[300]

4.3.3 Nanogels

The naturally derived hydrogels have some properties that make them well-suited for muscle tissue engineering. For example, the hydrogels are usually compacted materials with designed geometry for the alignment of muscle cells with high cell density.[301-305] The designed geometry and cell favorable conditions are critical for their application as muscle tissue engineering scaffolds. Photolithographic patterning of hydrogels enable a fast layer-by-layer assembly of cells into 3D structures with controllable geometry and size.[306] Synthetic[307] or natural hydrogels[308] have been both investigated by this method. The major concern of this method is from photosensitive cross-linkers and ultraviolet radiation for the hydrogel formation, which may cause side effects on cell activities.[309] Bian *et al*. designed mesoscopic hydrogel to control the 3D geometry of artificial muscle tissue.[310] They provided a protocol to precisely and reproducibly mimic the 3D muscle tissue architectures through soft lithography technique *in vitro*. A several square centimeter large and few hundred micron-thick biomimetic muscle tissues formed after 2 weeks of cell cultures on this 3D hydrogel (Figure 22).

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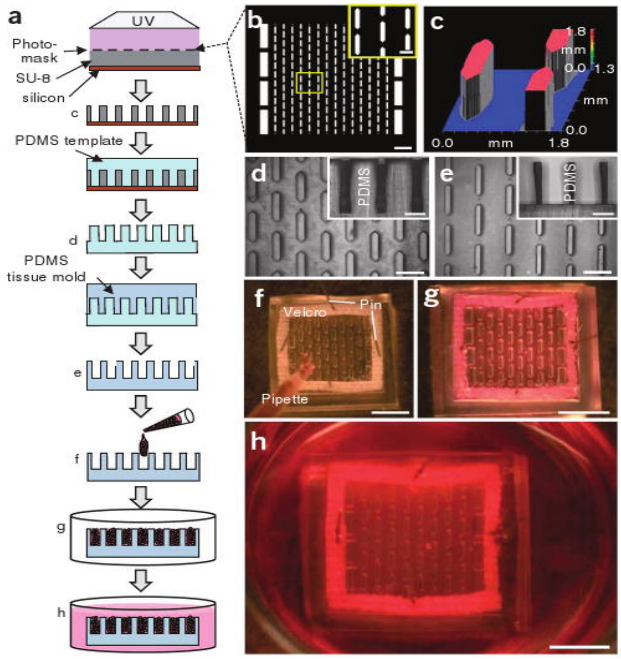

Figure 22. Fabrication of bioartificial muscle tissue scaffolds. (**a**) The SU-8 photoresist-coated silicon wafer; (**b**) a predesigned photomask. Scale bar, 2 mm (inset, 500 μm). (**c**) Optical profile of the resulting master wafer after UV light exposure. (**d**) Negative replica PDMS mold without the master wafer. Scale bar, 1 mm. (inset: vertical cross-section, scale bar, 500 μm). (**e**) PDMS tissue mold without the negative replica PDMS. Scale bar, 1 mm (inset: vertical cross-section, scale bar, 500 μm). (**f**) Cell–hydrogel solution into the PDMS tissue mold and incubation at 37 °C. (**g**) hydrogel polymerization and (**h**) submerged in culture medium. A pinned Velcro frame served as an anchor for hydrogel. Scale bars in **f**–**h**, 5 mm (reprinted with permission from reference 310). [310]

4.3.4 Nanocomposite scaffolds

Composite scaffolds have shown significant improve for muscle tissue engineering applications, especially for the considerations of mechanical properties. Song *et al*. have prepared chitosan/PGA nanocomposite scaffold using layer by layer self-assembly technique.[282] Results demonstrated that the composite scaffold could significantly promote the adhesion and growth of C2C12 cell *in vitro*. Jun *et al*. prepared three kinds of PLCL/polyaniline 3D nanocomposite scaffolds by electrostatic spinning technology.[281] They tested the mechanical properties and conductivity of the scaffold. The three scaffolds have showed to promote the adhesion and proliferation of C2C12 cells *in vitro*. Beier *et al*. compared different scaffolds in the application of muscle tissue engineering, such as hybrid collagen-I-fibrin gels,

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collagen nanofibres, collagen sponges, and open-cell PLA (OPLA) scaffolds.[311] The data confirmed that pure collagen sponges and OPLA-scaffolds were less benefit to cell proliferation while the former matrix promoted apoptosis of cells on the scaffolds. Therefore, the nanocomposite scaffold is a better candidate for muscle tissue engineering.

4.4 Clinical trials of nanomaterials in muscle tissue engineering

At present, more and more nanomaterials have been put into clinical trials for muscle tissue engineering. For example, transplantation using autologous skeletal myoblast for the treatment of heart failure[312-314] and stress urinary incontinence[315, 316] are on the way. Powell *et al*. developed a muscle-based delivery system by using transduced bioartificial muscles tissues.[317] This method has many advantages, such as high survival rate, good fusion efficiency, and the possible retrievability of transplanted muscle constructs through simple myoblast injections. However, muscle tissue engineering still has many limitations and challenges to solve. It requires myogenic progenitor cells and scaffolds which can support growth and differentiation of progenitor cells. Although tissue engineering undergoes steady progress in clinical practice, the engineering of muscle tissue is still a scientific challenge. Hopefully, the nanotechnology-mediated functional scaffolds could further throw light on skeletal muscle tissue engineering.[311, 318-320]

4.5 Summary

Muscle is a soft tissue which can produce force and motion under the direction of the nerve. Therefore, muscle tissue engineering is involved in three aspects, including structure and function of muscle, vascularization for the survival condition for seeded cells, and nerve network to control the muscle motion. Replacement of artificial muscle scaffolds should not only fill the room, but recover the lost function. Recovery of muscle functionality needs the combination of systematic engineering and complicated techniques. It is more difficult to design the functional muscle scaffold than artificial bone or cartilage. That is the possible reason that there is no muscle scaffold available in the market until now.

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Nanotechnology makes it possible to construct devices interacting at nanoscale level. The application of nanotechnology and nanostructured materials in muscle tissue engineering is a new and fast developing field. Although the nanostructured materials have made quick progress in muscle tissue engineering, there are hurdles to overcome. Currently, research mainly focuses on cell adherence and proliferation after cell seeding in nanostructured muscle scaffolds. Some group studied the cell elongation, orientation, fusion, and striation in the scaffolds. In order to create ideal nanostructured muscle scaffolds to regenerate muscle layers with appropriate tissue organization, successful integration, and robust mechanical properties, future work should focus on the selection of appropriate expanding materials and fabrication techniques.

5. Conclusions and Outlook

The development of nanotechnology has brought a remarkable progress in the field of using nanostructured materials for musculoskeletal tissue engineering, which provide opportunity to design and tailor the materials at the molecular level; therefore, the scaffold fabricated by nanomaterials can be tuned to excellently mimic the naturally hierarchical tissue structures and ECM components in terms of morphology and biofunctionalities. Natural biopolymers, synthetic polymers, metals, and inorganic materials in the form of various nanocomposite scaffolds, nanogels, nanofibers, and nanoparticles containing different kinds of seeded cells or growth factors have been designed and synthesized for potential use in bone, skin and muscle tissue engineering. Table 1 summarizes the similarities and differences of various nanostructured materials for musculoskeletal tissue engineering in terms of growth factors, compositions, and mechanical properties.

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Table 1. Comparision of Nanostructured Materials for Musculoskeletal Tissue Engineering

	Growth factors		Compositions			Mechanic properties
	Same	Different	Polymers		Other compositions	
			Same	Different		
Nanostructured materials for bone	Transforming growth factor (TGF); fibroblast growth factor (FGF)	Vascular endothelial growth factor (VEGF); bone morphogenetic protein (BMP)	Natural: collagen; chitosan	Natural: starch; hyaluronic acid; fibrinogen; poly(hydroxybutyrate)	Metal: gold; silver	Stiffness (37-2900 MPa)
			Synthetic: poly (lactic acid) (PLA); poly (lactic-coglycolide) (PLGA); poly(carprolactone)(PCL)	Synthetic: poly (glycolic acid) (PGA); polypropylene fumarate (PPF); polyalkylcyanoacrylate; poly (3-hydroxybutanoicacid)(PHB); poly(organophosphazene)(POP); poly(ethylene glycol)(PEG); poly(ethylene oxide)(PEO); polyanhydrides	Organic: calcium phosphates; Carbon nanotubes	
Nanostructured materials for cartilage	TGF; FGF	BMP; insulin-like factor (IGF); platelet-derived growth factor (PDGF-AB)	Natural: collagen; chitosan	Natural: gelatin; fibrin; hyaluronic acid; alginate; agarose; and fibroin	None	Middle (0.133-14 MPa)
			Synthetic: PLA; PLGA; PCL;	Synthetic: PGA; poly (acrylic ester); PEO; poly (L-lactide-co-ε-carprolactone) (PLCL); poly(N-isopropylacrylamine) (ONIPAM)		
Nanostructured materials for muscle	TGF; FGF	Muscle differentiation factor (MyoD); myogenic factor 5 (Myf5); IGF; epidermal growth factor (EGF); PDGF; hepatocyte growth factor (HGF); and leukemia inhibitory factor (LIF)	Natural: chitosan; collagen	Natural: gelatin; and acellular matrix	None	Elastic (0.17-2.10 MPa)*
			Synthetic: PLA; PLGA; PCL	Synthetic: Poly(1,8-octanediol-co-citrate) (POC); PLCL; poly (L-lactide) (PLLA); PLGA		

*: Mechanics results are tested when stress at break.

Compared with conventional materials, the nanomaterials have improved mechanical properties, tunable chemical and bioactive-functions, organized structures, enhanced protein adsorption, cell adhesion, proliferation and differentiation of various cell types. However, most of the current studies are limited to laboratory investigations, either *in vitro* or *in vivo*, clinical studies are needed. As the candidate for tissue engineering, nanomaterials are also needed to be evaluated for *in vivo* studies to provide more solid evidence, and eventually move the lab investigation forward to clinical trials.[321] In order to achieve successful clinical translation of nanomaterials' application in musculoskeletal tissue engineering, the following challenges and tasks need to be considered and resolved, including the lack of strong bioactivity, insufficient mechanical strength, issues relating to the infection management, as well as non-biodegradability and long term toxicity. The influence of nanomaterials on human health and the environment is needed to be more understood.[322] For example, it is reported that surface functionalization

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can reduce the toxicity of CNTs *in vivo*, but long-term safety of CNTs in the body has not yet been systemically studied yet. Similarly, for other nanomaterials, a systematic investigation of the potential health risks is critically needed before clinical trials.[323]

The most promising design of a nanostructured biomaterial for musculoskeletal tissue engineering is to create multi-component and multi-functional material that promotes tissue regeneration on multiple levels. It demands comprehensive fundamental understandings in both life and materials sciences to achieve such ideal regeneration technologies.[324] New materials with biocompability, biodegradability, mechanical properties, microarchitecture, porosity, surface properties are favorable for musculoskeletal tissue engineering. Further understanding of tissue biology, such as the interaction between the organic and inorganic materials in different tissues, and the function of growth factors, is also very crucial for new design of nanostructured materials for musculoskeletal tissue engineering. Finally, the scaffold processing engineering techniques need to be optimized. There is no single method or kind of material so far that can produce the perfect ideal scaffold for tissue engineering. Each technique or material has advantages and drawbacks. Therefore, the successful development of nanostructured materials for musculoskeletal tissue engineering depends on more understanding in tissue biology, discovery of new nanomaterials and new materials processing methods, as well as combining nanotechnology with other techniques.

Acknowledgments

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