HHS Public Access

Author manuscript

Mater Sci Eng R Rep. Author manuscript; available in PMC 2018 January 01.

Published in final edited form as:

Mater Sci Eng R Rep. 2017 January ; 111: 1–26. doi:10.1016/j.mser.2016.11.001.

Injectable scaffolds: Preparation and application in dental and craniofacial regeneration

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Abstract

Injectable scaffolds are appealing for tissue regeneration because they offer many advantages over pre-formed scaffolds. This article provides a comprehensive review of the injectable scaffolds currently being investigated for dental and craniofacial tissue regeneration. First, we provide an overview of injectable scaffolding materials, including natural, synthetic, and composite biomaterials. Next, we discuss a variety of characteristic parameters and gelation mechanisms of the injectable scaffolds. The advanced injectable scaffolding systems developed in recent years are then illustrated. Furthermore, we summarize the applications of the injectable scaffolds for the regeneration of dental and craniofacial tissues that include pulp, dentin, periodontal ligament, temporomandibular joint, and alveolar bone. Finally, our perspectives on the injectable scaffolds for dental and craniofacial tissue regeneration are offered as signposts for the future advancement of this field.

Keywords

scaffold; injectable; dental; craniofacial; tissue regeneration

1. Introduction

Tooth is an important organ for our daily life. However, a tooth is susceptible to losing part or even all of its structures due to bacterial invasion, trauma, or congenital anomalies. From a report of the Centers for Disease Control and Prevention, dental caries or cavities, is one of the most common chronic diseases of young children and adolescents (6 to 19 years old) [1]. Dental caries also affects adults, with more than 90% of all the population over the age of 20 having some degree of tooth decay. Meanwhile, nearly half of the U.S. adult population aged 30 years and older has mild, moderate or severe periodontitis, and 64% of adults over the age of 65 have moderate to severe forms of periodontal disease, which is the major cause for tooth loss [2]. The loss of tooth can cause immediate problems with eating and speech and

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subsequent bone resorption, leading to physical and mental suffering that compromises an individual's self-esteem and quality of life.

Clinically, if dental caries progresses and severely inflames the pulp tissues inside the tooth, a root canal procedure is often performed to remove the necrotic dental tissues, clean the pulp chamber, and seal it with bio-inert materials. While this therapy has been used for many years with high success rates, the repaired tooth is not a living organ and loses a significant amount of the tooth structure, which weakens the strength of the tooth. Similarly, an artificial prosthetic dental implant is used to replace the lost tooth. Despite its clinical success, dental implant failure is also well documented in the literature including peri-implant bone loss, infections, and allergic reactions [3]. Another example is temporomandibular joint disorders (TMDs), which are a heterogeneous group of diseases that cause orofacial pain, affecting a patient population of more than 10 million in the United States [4]. Treatment options for TMDs are few and have limited success rates. For patients with severe TMDs, a surgical procedure called a "discectomy" is often performed to remove the diseased temporomandibular joint (TMJ) disc that compromises the normal physiological function. Therefore, there is a need to develop an alternative to traditional dental and craniofacial clinical treatments.

Tissue engineering is a promising approach to replacing damaged/missing dental and craniofacial structures and restoring their biological functions; a number of publications have shown the success of regenerating dental and craniofacial tissues using this strategy [5–11]. Typically, the tissue engineering strategy involves three critical elements: stem cells or progenitor cells, signaling molecules (e.g., growth factors), and scaffolds. The scaffold is an artificial extracellular matrix (ECM) and serves as a template for cell growth and tissue regeneration. Ideally, the scaffold should be biocompatible and biodegradable, possess proper mechanical and physical properties, and mimic the *in vivo* microenvironment (niche) to facilitate cell adhesion, proliferation, differentiation, and neo tissue formation [12]. Based on when a scaffold is shaped, it can be considered a pre-formed or an injectable scaffold. A pre-formed scaffold has a definite shape prior to its application, while an injectable scaffold forms the shape *in situ*. Compared to the pre-formed scaffold, the injectable scaffold has several advantages, including (1) it is performed in a minimally invasive manner, therefore decreasing the risk of infection and improving comfort; (2) it can easily fill any irregularly-shaped defects; and (3) it overcomes the difficulties of cell seeding and adhesion, and the delivery of bioactive molecules, as these factors can be simply mixed with the material solution before being injected *in situ*. Considering the size, morphology, and complicated structure of dental and craniofacial tissues, an injectable scaffold is more appealing than a pre-formed one. For example, the root canal is a long, narrow channel with an average total volume of approximately 20 μl [13]. With such a small volume and unique anatomical structure, it is a challenge to implant a pre-formed scaffold into the root canal and seamlessly cover the entire space of the canal; however, an injectable scaffold can easily achieve this. Another example is the maxillary sinus lift, which is a surgical procedure in which natural or synthetic bone graft materials are added to the upper jaw to induce bone formation. During the surgery, a surgeon cuts the gum and bone tissues and opens a small oval window to introduce bone-graft materials into the sinus space. Obviously, the adoption of injectable materials is a better choice to reduce the surgical wound size and decrease the

risk of infection. Because of the above reasons, injectable scaffolds have received more and more attention in recent years. However, there is no comprehensive summary on the recent advancement of injectable scaffolds for dental and craniofacial tissue regeneration.

In this paper, we first present an overview of various injectable scaffolding materials, including natural and synthetic polymers, inorganic and composite materials, and self-assembled materials. Next, we discuss the characteristic parameters and mechanisms for injectable scaffold formation. Among the various fabrication techniques, we highlight the development of advanced injectable scaffolding systems that have appeared in recent years. These systems include new approaches to incorporating bioactive molecules in injectable scaffolds, the development of cell-instructive biomaterials, novel self-assembly peptides, and bio-inspired nanofibrous microspheres. Finally, we summarize the clinical applications of the injectable scaffolds for the regeneration of dental and craniofacial tissues, including pulp, dentin, periodontal ligament (PDL), TMJ, and alveolar bone. We expect that this review article will give a full perspective vision of the injectable biomaterials to our readers, and further stimulate the increasing interest in the development of better injectable biomaterials for dental and craniofacial tissue regeneration as well as for other applications of translational medicine.

2. Biomaterials used as injectable scaffolds

A variety of biomaterials have been proposed for use as injectable scaffolds. According to the source of origins, they can be classified as natural and synthetic biomaterials. Natural biomaterials are derived from natural resources and have the advantage of biological recognition that may positively support cell adhesion and growth. These materials usually are biocompatible and biodegradable, and do not cause inflammatory or immune responses. However, there are concerns with natural materials regarding potential pathogen transmission and the variability of quality from batch to batch. These concerns have led to a vast amount of research in the development of synthetic biomaterials as substitutes for naturally derived ones for tissue engineering. Synthetic biomaterials can be manufactured on a large scale with well-controlled properties of strength, degradation rate and microstructure. Furthermore, synthetic biomaterials avoid the risk of pathogen transmission associated with natural biomaterials. However, the synthetic biomaterials usually do not provide biological cues in their molecular chains, and the conjugation of specific cell-recognizable signal molecules (motifs) is often needed to facilitate cell-material interaction. Another way to improve the overall performance of a biomaterial is the use of a composite biomaterial, which combines the advantages of its individual components. In this section, we will first introduce the well-documented natural and synthetic injectable biomaterials, followed by a discussion of the injectable composite biomaterials. A new class of peptide-based injectable biomaterials called “self-assembling peptides” have gained great interest in recent years and are discussed separately in the last subsection.

2.1 Natural polymers

2.1.1 Collagen—Collagen is one of the most widely used injectable natural biomaterials. As the main organic component of many mammalian tissues such as dentin, bone, and PDL,

collagen has a triple-stranded helical structure. Currently, 29 distinct collagen types have been identified, among which Type I collagen is the most abundant [14]. Collagen possesses motifs (e.g., Arg-Gly-Asp) that can be recognized by receptors on the plasma membrane to initiate cell adhesion and subsequent cellular response; therefore, it has excellent biocompatibility [15]. Collagen can also act as a reservoir for growth factor delivery in the ECM. The degradation of collagen *in vivo* is modulated by collagenases such as the matrix metalloproteinases (MMPs).

Collagen is obtained by decellularization and demineralization of tissues through extraction and purification processes. A three-dimensional (3D) collagen hydrogel structure can be formed through self-assembly or crosslinking. In a neutral pH environment, the collagen fibrils self-assemble into bundled collagen fibers with a diameter of around 100 nm, which further aggregate and form a collagen hydrogel. Because a self-assembled collagen hydrogel has weak mechanical strength, a crosslinking step is often included to improve its mechanical property. Adding crosslinking agents (e.g., glutaraldehyde) significantly improves the mechanical strength. However, non-degradable crosslinkers may affect the degradation behavior and biosafety of the hydrogel. One approach to solving this problem is the incorporation of hydrolytically or enzymatically cleavable substrates that provide a more desirable way to modify the biodegradability of the collagen hydrogel. For example, a novel scaffold modified by poly(ethylene glycol) (PEG) and MMP7 exhibits increased mechanical and biological properties of collagen hydrogels and recreates a complex dynamics similar to natural ECM [16].

Collagen gels were first utilized as a source for skin replacement prior to its wide use in the regeneration of various tissues [17]. Studies showed that collagen hydrogels can facilitate angiogenesis [18]. Moreover, injectable collagen gels were shown to promote adipogenesis and osteogenesis of stem cells [19, 20]. Like other natural biomaterials, batch-to-batch variance and the risk of pathogen transmission are concerns of using collagen as an injectable material for tissue regeneration. Meanwhile, even though a crosslinking step is included during the collagen gel preparation, the mechanical strength of the collagen gel is still relatively low. Therefore, collagen is more suitable for use as an injectable matrix for soft tissue regeneration.

2.1.2 Gelatin—Gelatin is a denatured protein obtained by the partial hydrolysis of collagen. The degree of collagen conversion into gelatin is related to the pH, temperature and time of the extraction procedure. There are two types of gelatins called “type A” gelatin (isoelectric point at pH ~ 8–9) and “type B” gelatin (isoelectric point at pH~ 4–5), which are obtained under acidic and alkaline pre-treatment conditions, respectively. Compared to collagen, gelatin is available in a range of molecular weights, and its properties can be modulated by altering the molecular weights with different physical and chemical treatments [21]. Also, gelatin is a denatured biopolymer; the selection of gelatin as scaffolding material can circumvent the concerns of immunogenicity and pathogen transmission associated with collagen.

Gelatin is biodegradable and biocompatible both *in vitro* and *in vivo*. Additionally, as a collagen derivative, gelatin retains cell-binding motifs such as Arg-Gly-Asp (RGD) and

MMP-sensitive degradation sites, which is critical for successful cell adhesion and biodegradation. Gelatin has a sol-gel transition with a lower critical solution temperature (LCST) of approximately 37°C, which is close to human body temperature. Because of this characteristic, gelatin must be chemically crosslinked to stabilize its structure when used as an injectable biomaterial *in vivo*.

To improve its properties, the chemical modification of gelatin has been widely explored in recent years. One example is methacrylamide-functionalized gelatin (GelMA), which retains cell-binding motif RGD and MMP-sensitive degradation sites. In addition, GelMA can be photo-polymerized *in situ* using ultraviolet (UV) light [22]. GelMA can also be utilized in 3D printing techniques with the preservation of high cell viability [23]. Therefore, gelatin-based biomaterials are considered to be promising injectable materials for controlled drug delivery and tissue regeneration.

2.1.3 Chitosan—Chitosan is a deacetylated derivative of chitin and is composed of glucosamine and *N*-acetyl glucosamine. Chitosan is a positively charged polysaccharide; the free amino and hydroxyl groups on the chitosan chain provide flexibility to acidylate, carboxymethylate, and introduce other bioactive molecules into the polymer. Due to its excellent biocompatibility, biodegradability, low toxicity, and controlled degradation by enzymes such as lysozyme, chitosan is one of the most widely used biomaterials in tissue engineering.

Chitosan dissolves only in acidic solution and undergoes a sol-gel transition when the pH value of the solution changes from slightly acidic to neutral. The addition of glycerol-2-phosphate (β-GP) changes the pH value of the solution to neutral, so an injectable chitosan hydrogel can be formed by mixing chitosan with β-GP. The positively-charged polysaccharide chains in chitosan can form complexes with amino acids stretched in proteins, thereby decreasing the release of the protein from the system. Chitosan also has muco-adhesion capacity and can transiently open a tight junction between epithelial cells [24], mainly due to the electrostatic attraction between the positively charged $-NH_3^+$ of the chitosan and the negatively-charged mucosa.

Chitosan shows a high anti-microbial activity against many pathogenic and spoilage microorganisms. The most likely explanation is the interaction between the positively-charged chitosan and negatively-charged bacterial membrane leading to the leakage of cellular proteins and other intracellular constituents. Chitosan can also enhance the binding and retention of lentiviruses, prolonging and enhancing transgene expression within a PEG hydrogel scaffold [25]. A major drawback of chitosan in tissue engineering is its poor solubility in neutral aqueous solutions and organic solvents due to the presence of amino groups and its high crystallinity [26]; therefore, the modification of chitosan is necessary to expand its applications.

2.1.4 Alginate—In contrast to chitosan, alginate is anionic linear polysaccharide. Alginate has a wide distribution of brown algae in the cell walls and is comprised of repeating (1,4)-linked β-D-mannuronic sequences (M-blocks) and α-L-guluronic acid sequences (G-blocks)

interspersed with MG sequences (MG-blocks) (See Fig. 1). The composition, sequence and molecular weight of alginate vary with the source and species.

Alginate dissolves in water, and its solubility is governed by the pH and ionic strength of the solution. Alginate also can form hydrogels via ionic crosslinking. Alginate forms a hydrogel by the interaction between G-blocks that associate to form tightly held junctions in the presence of divalent cations such as Ca^{2+}, Sr^{2+} and Ba^{2+} [14]. The ionic crosslinked alginate hydrogel is usually weak and loses its mechanical integrity over time due to the reversible crosslinking and the outward flux of ions from the hydrogel.

In a neutral pH environment, alginate cannot be enzymatically broken down, but is susceptible to chain degradation in the presence of reducing compounds like hydroquinone, sodium sulfite, and sodium hydrogen sulfide [27]. Also, alginate can undergo hydrolytic cleavage in acidic conditions and β-elimination enzymatic degradation in strong alkaline environments [27].

As a biomaterial, alginate offers several advantages such as biocompatibility and non-immunogenicity; however, it also has some drawbacks including poor cell adhesion, low mechanical strength, and low degradability. Therefore, the modification of alginate for more desired properties is necessary. For instance, the incorporation of RGD or laminin into alginate resulted in enhanced cell adhesion, survival, and proliferation [15, 16].

2.1.5 Hyaluronic acid—Hyaluronic acid is a linear polysaccharide abundant in cartilage ECM and consists of two alternating units, β-1, 4-D-glucuronic acid and β-1, 3-N-acetyl-D-glucosamine. Its negative charge on the polymer chain attracts positive ions and results in an osmotic balance that brings in water, making it easy to form a hydrogel [28]. The concentration of gel solution, the degree of crosslinking, and many other processing procedures affect the mechanical properties of hyaluronic acid hydrogels [29, 30].

Hyaluronic acid usually has a molecular weight of over 10^6 Daltons. Because of its high molecular weight, hyaluronic acid has high viscoelasticity when dissolved in aqueous solution. Hyaluronic acid is a shear thinning gel that can be injected via a needle and forms a gel *in situ* [31]. Hyaluronic acid can be cleaved by the enzyme hyaluronidase to obtain lower molecular- weight molecules. Interestingly, high molecular-weight hyaluronic acid inhibits the proliferation, migration and angiogenesis of endothelial cells, while low molecular-weight hyaluronic acid promotes endothelial cell attachment and proliferation [29, 30]. Hyaluronic acid has excellent bioactivity, biocompatibility and biodegradability, and it can be further chemically modified to act as a reservoir of growth factors. For example, heparin was incorporated into a hyaluronic acid hydrogel using divinyl sulfone as a crosslinker, and the modified hyaluronic acid hydrogel showed higher bone morphogenetic protein 2-loading capacity and longer sustained release than the hyaluronic acid control [32]. Like other natural biomaterials, the disadvantages of hyaluronic acid include structural complexity, low mechanical strength and possible immunogenicity.

2.1.6 Fibrinogen—Fibrinogen is a blood-borne glycoprotein comprised of three pairs of non-identical polypeptide chains. Following vascular injury, fibrinogen is cleaved by

thrombin to form fibrin, the most abundant component of blood clots. Fibrin gel can form within a few seconds when blood coagulates due to an injury. Fibrinogen can interact with cells through both integrin receptors including two RGD sequences and non-RGD sequence integrin binding sites and non-integrin receptors [33, 34], giving the fibrinogen hydrogel excellent biocompatibility. Fibrin gel is readily formed under physiological conditions and is suitable for *in situ* delivery by a simple injection at the site of interest [35]. Since fibrin can be obtained from a patient's own plasma, the risk of immune rejection is avoided. The cleavage products of fibrinogen and the degradation products of fibrin show no toxicity and exhibit multiple regulation effects on cell proliferation and differentiation. Specifically, they promote angiogenetic and vasoactive activities and collagen deposition during tissue restoration [36]. These advantages have led to a widespread use of fibrin as scaffolds in tissue engineering.

Fibrinogen binds to other ECM molecules and can act as a reservoir for growth factors, proteases and protease inhibitors. This binding not only enhances enzymatic conjugation to the matrix and the cell-controlled cleavage of fibrinogen, but also enables the release of bioactive molecules in the local microenvironment [37–39]. Therefore, fibrinogen hydrogel can also be used as an efficient and biocompatible delivery carrier for both cells and proteins.

As a natural biomaterial, fibrinogen hydrogel has disadvantages such as low mechanical strength and less controllable biodegradability. Copolymerization with synthetic biomaterials offers some degree of control over the structural properties and the biodegradation of the fibrinogen hydrogel while maintaining its inherent biocompatibility [40].

2.2 Synthetic polymers

2.2.1 Polyethylene glycol—PEG is a biomaterial approved by the United States Food and Drug Administration (FDA) for certain pharmacological applications. It has also received considerable attention in tissue engineering. PEG shows several prominent characteristics such as low toxicity, great hydrophilicity and solubility in organic solvents, excellent biocompatibility, non-immunogenicity, and anti-fouling property [41]. In addition, the end hydroxyl group of a PEG molecule can be easily modified by various functional groups, such as carboxyl, thiol and acrylate, or some bioactive molecules.

Because of its great hydrophilicity, it is easy for PEG to form a hydrogel using chemical, physical, or ionic crosslinking methods. Chemically crosslinked PEG hydrogel has a more stable structure and higher mechanical strength compared to its physically and ionically crosslinked counterpart.

The anti-fouling property (also known as the "stealth characteristic") is a unique feature of PEG and has been used to hinder the adsorption of molecules and bacteria on the PEG surface. However, this property also prevents cell-PEG interaction, and therefore inhibits the adhesion of cells onto the PEG hydrogel. To solve this problem, bioactive motifs, such as the RGD peptide, have been incorporated into the PEG molecule to improve its cellular affinity [42, 43]. Copolymerization of the PEG framework with natural polymers, such as collagen

or hyaluronic acid, can also improve its inert bioactivity [44, 45]. Furthermore, the copolymerization of PEG with certain synthetic biomaterials, such as poly(ε-caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), and poly(*N*-isopropylacrylamide), endows the copolymer with a thermo-sensitive transition feature and enhanced bioactivity, which is preferable for *in situ* soft tissue engineering [46–48].

One disadvantage of using PEG as an injectable hydrogel is its non-biodegradability. To control its degradation, biodegradable groups or enzymatic proteins like MMPs have to be introduced into the PEG chains.

2.2.2 Poly(α-hydrxoy esters)—Poly(α-hydrxoy esters), which include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), their copolymers PLGA, and PCL, are the most widely used synthetic biomaterials in tissue engineering because they are well characterized and approved by the FDA for certain clinical use. PGA has a high degree of crystallization. Therefore, it exhibits high strength and modulus and is not soluble in most organic solvents. PLA has as two optical isomers, D-lactide and L-lactide. The L-lactide is the naturally occurring isomer, and the D, L-lactide is the synthetic blend of the D-lactide and L-lactide. PLLA exhibits the semi-crystalline structure, leading to a low solubility and high mechanical strength. PDLLA has an amorphous structure and lower mechanical strength. PLGA are the copolymer of PGA and PLA, and the GA/LA ratio governs the hydrophilicity and degradation rate of the copolymer. PCL is soluble in a wide range of organic solvents and shows good biocompatibility. However, it has a slower degradation rate compared to PGA, PLA, and PLGA. Poly(α-hydrxoy esters) are degraded by hydrolysis, and their degradation products are usually nontoxic.

Poly(α-hydrxoy esters) do not dissolve in water; therefore, they usually cannot form hydrogels. However, when a poly(α-hydrxoy ester) is modified with other hydrophilic components, an injectable hydrogel can be formed. For example, a PEG–PLGA–PEG triblock copolymer with a specific composition is a sol at room temperature, but becomes a transparent gel at body temperature [49]. Besides hydrogels, poly(α-hydrxoy esters) can also be fabricated into injectable microspheres as cell carriers and drug delivery vehicles for tissue regeneration.

2.2.3 Poly(*N*-isopropyl acrylamide)—Poly(*N*-isopropyl acrylamide) (PNIPAM) is a thermo-responsive polymer that is widely used in biomedical engineering. PNIPAM has a LCST of approximately 32°C, which means that PNIPAM remains soluble in aqueous solution below the LCST, while it aggregates when the temperature is above the LCST. This sol-gel transition is reversible, making PNIPAM a suitable injectable biomaterial for *in situ* hydrogel formation. However, the LCST of PNIPAM is several degrees Celsius below human physiological temperature (37°C), and a chemical modification of the polymer is often needed to adjust the LCST close to the physiological temperature. Generally, the addition of hydrophilic monomers increases the LCST of PNIPAM, whereas the incorporation of hydrophobic units shows the opposite effect [50].

Like PEG, PNIPAM is non-biodegradable, and its monomer and crosslinker may lead to toxic, carcinogenic, and teratogenic effects [51]. Chemical modification such as photo-

copolymerization with 2-methylene-1,3-dioxepane and polycaprolactone dimethacrylate has been adopted to increase the PNIPAM biodegradability and biosafety [52].

2.2.4 Pluronic block copolymers—Pluronics® are triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO). The arrangement of the PEO-PPO-PEO sequence results in an amphiphilic copolymer, in which the number of hydrophilic (PEO) and hydrophobic (PPO) repeat units can be changed to adjust the hydrophilic and hydrophobic balance of the copolymer. Pluronics® exhibit a unique thermo-sensitive sol-gel transition behavior in aqueous solution. At low temperatures, the system dissolves in solution due to the high PEO content. As the temperature increases to the critical micelle temperature, water progressively becomes a poor solvent for the PPO segment, forcing the PEO-PPO-PEO polymer to self-assemble into physically crosslinked micelles, and finally a gel. A number of parameters including the PEO/PPO ratio, the molecular weight, the block size, and the block sequence, affect the sol-gel transition [53]. The PEO-PPO-PEO hydrogel is physically stable and easy to handle.

As injectable biomaterials, Pluronic block copolymers also have several drawbacks, including non-biodegradability and rapid dissolution [54]. Copolymerization with natural biomaterials can improve its biodegradability as well as bioactivity [55].

2.3 Inorganic materials

Although most scaffolding materials for tissue engineering are produced from natural and synthetic polymers, inorganic materials such as bioceramics are also widely being used, especially for mineralized tissue engineering. Bioceramics are biocompatible inorganic materials, mainly composed of Ca^{2+} and PO_4^{3-} in varying proportions. Typical bioceramics include hydroxyapatite (HA), β-tricalciumphosphate (β-TCP), and calcium phosphate cements (CPC). Generally, bioceramics are highly biocompatible, non-immunogenic, and have the capability to form a chemical bond with the host bony tissues.

HA and β-TCP with Ca/P ratios in the range of 1.50–1.67 are known to promote bone ingrowth [56]. HA has a higher osteogenic potential than β-TCP. However, the degradation rate of HA is much slower than that of β-TCP [57]. Biphasic calcium phosphate (BCP) ceramics are combinations of different ratios of less soluble HA and more soluble β-TCP. The biodegradability of bioceramics can also be improved by creating porosity in the biomaterials. Because of their intrinsic properties, HA, β-TCP and BCP are difficult to inject; therefore, they are mostly used for making pre-formed scaffolds.

CPC, comprised of a mixture of tetracalcium phosphate and dicalcium phosphate anhydrous, can be molded and set *in situ* to provide intimate adaptation to the contours of defect surfaces. Therefore, CPC can be used as an injectable biomaterial. However, pure CPC is still difficult to inject from a syringe. To improve the injectability, some chemicals, such as polysaccharide xanthan, polymeric drugs, and glycerol were added to the CPC [58]. The addition of chemicals did improve the cement injectability; however, it also greatly increased the time it took for the cement to harden. Therefore, how to balance the setting time and the injectability is still a problem that needs to be addressed.

2.4 Composite materials (polymer/ceramic composites)

Bioceramics exhibit good osteo-conductivity and bone-bonding ability; however, they also have several limitations including inherent brittleness, low biodegradability, and difficulty of fabrication. Meanwhile, polymeric biomaterials are biocompatible and biodegradable, but have relatively weak mechanical properties. Composite biomaterials often show an excellent balance between strength and toughness and usually improved characteristics compared to their individual components. Therefore, ceramic/polymer composite scaffolds were developed and have gained wide popularity in tissue engineering.

Ceramic/polymer composite scaffolds can typically be classified into two types: ceramics with added polymers and ceramic particles encapsulated into a porous polymer carrier [59]. Some of the composite materials have been fabricated into injectable scaffolds, such as collagen/nano-bioactive glass and PLGA/nano-hydroxyapatite hydrogels [60, 61], and showed appropriate mechanical strength and improved osteogenesis. Parameters such as the composition and ratio of the composite material, porosity, and the introduction of bioactive factors, affect the mechanical strength and cellular response of the composite material.

2.5 Self-assembled peptides

Peptides are versatile building blocks for fabricating materials. Under proper conditions, some well-designed peptides can self-assemble into biomimetic nanofibrous supramolecular architecture, making them appealing injectable biomaterials for tissue regeneration [62]. One example is the peptide-amphiphile (PA) that was synthesized using standard solid phase chemistry [63]. The PA has five structural units, in which region 1 is a long alkyl tail that conveys the hydrophobic character to the peptide; region 2 is used to form disulfide bonds to polymerize the self-assembled structure; region 3 provides the hydrophilic head group; region 4 is designed to interact with calcium ions and help the direct mineralization of HA; and region 5 presents the cell adhesion ligand RGD, as illustrated in Fig. 2. As the pH value of the PA solution drops to below 4, the PA assembled into 3D nanofiber networks and formed a gel. The process is driven by the formation of a hydrophobic core composed of closely packed alkyl tails, whereby fibrous strands can be built because of the hydrogen bond formation between the amino acids of adjacent PA molecules. Another way to trigger the PA gelation process is the addition of polyvalent ions. The PAs remain as amorphous aggregates at a neutral pH due to the repulsive negative charge, and the addition of polyvalent ions eliminates the charge and allows self-assembly into cylindrical micelles that undergo physical crosslinking to form a gel.

Another example is PuraMatrix®, which is a synthetic, biologic hydrogel composed of repeating amino acid sequences of arginine-alanine-aspartic acid-alanine prepared in an aqueous solution [64]. Within the repeating units, there are positively charged residues (arginine) and negatively charged residues (aspartic acid) separated by hydrophobic residues (alanine). PuraMatrix® self-assembles into nanofibers when exposed to physiological levels of salt. PuraMatrix® possesses good plasticity, absorption, and biocompatibility properties and is devoid of animal-derived pathogens and antigens.

A major advantage of self-assembled peptides is that their functional properties can be tailored by altering the peptide sequences [65]. Also, since they do not need any additional crosslinkers, cells and proteins can be incorporated into the gel without exposing them to harsh chemicals. However, due to the lack of chemical crosslinking, the self-assembled peptide gels generally have very low mechanical strength. In addition, the low productivity of the self-assembled peptides is another limitation.

3. Fabrication of injectable scaffolds

3.1 Requirements of injectable scaffolds

3.1.1 Injectability—Compared to pre-formed scaffolds, the major advantage of injectable scaffolds is injectability, which means that the scaffold can be injected into the defect area using a syringe needle and then solidified *in situ*. Injectability is an essential requirement for a biomaterial when it is performed in a minimally invasive manner. Ideally, the material should remain flowable before injection and rapidly become immobile after the material diffuses within the defect. As for hydrogels, the transition from solution to gel can meet this requirement. This transition can be stimulated by changes in temperature, pH, light, enzyme, or the addition of a crosslinking agent. The injectability is generally related to the rheological properties of the monomers or pre-polymers [66], and factors such as the concentration, viscosity, gelation process and gelation rate all influence the injectability of a hydrogel. The concentration and viscosity of a pre-gelation solution should be appropriate to avoid premature gelation while retain easy operation. A mild gelation process is desired to maintain high cell viability and molecular bioactivity and avoid damage to the surrounding tissues. Also, an appropriate gelation rate is important; a fast rate may hinder the diffusion of the hydrogel polymer and the operation of surgery, while a slow gelation rate may impair the integrity of the hydrogel. As for other injectable scaffolds such as microspheres, the concentration, particle size, degree of aggregation, and surface charge, are critical factors to affect injectability.

3.1.2 Cytotoxicity—The injectable hydrogels should be non-toxic before, during, and after injection, and should have mild solidification conditions, such as a neutral pH and physiological temperature. The scaffolds used to encapsulate cells must be capable of being gelled without damaging the cells. Any initiators, crosslinkers, or other additives used in the injectable materials must not cause cellular toxicity. The polymerization process should be done without producing elevated temperatures that cause thermal necrosis at the injury site. The scaffolds should show no toxic degradation or inflammatory reactions.

A challenge specifically related to hydrogels is the fact that oligomers and prepolymers are directly injected into the body before gelation, risking a greater possibility of cytotoxicity. Therefore, caution is required to ensure that all hydrogel components are safe and reasonably non-toxic. As to hydrogels from natural polymers, they are usually considered non-toxic, while the chemicals used as crosslinkers may possess toxicity to some extent. For synthetic hydrogels, however, cytotoxicity is a major concern. To date, only PLA, PEG, and PLGA have been approved by the FDA for clinical applications.

Other components involved in the gelation process, including the initiators, organic solvents, stabilizers, and emulsifiers, may also be hazardous to host cells and tissues if they are not thoroughly removed or if they seep out into the tissues or encapsulated cells. Therefore, the toxicity of the injectable hydrogels should be monitored closely prior to their applications.

3.1.3 Host responses—Host responses towards a biomaterial is pivotal for a biomaterial, which means that the biomaterial must not elicit an unresolved inflammatory response nor demonstrate extreme immunogenicity [56]. A biocompatible material would support cell growth and proliferation without causing any toxicity or immunological response. One major index of biocompatibility is the status of cell adhesion on biomaterials, which is necessary not only for cell attachment, but also for the activation of various downstream pathways that regulate cell activities.

Physicochemical properties including the components, stiffness, porosity, surface charge, hydrophilicity or hydrophobicity all influence how host responds to hydrogels [67]. Since most natural polymers have monomeric units similar to natural ECM and the cell-adhesion sites are usually retained during fabrication procedures, hydrogels from natural polymers are generally considered as biocompatible materials. However, most natural hydrogels are derived from animal origins; thus, care must be taken when they are used in tissue regeneration so that the risk of disease transmission, cross-reactivity, and immunological responses is reduced.

Synthetic hydrogels often lack cell adhesion sites; therefore, modifications are usually necessary to achieve improved host responses. Typically, incorporation of RGD peptide is useful for synthetic biomaterials, while other modifications also include the incorporation of fibronectin segments or surface receptors like CD44 and CD168 [34, 68]. In addition, copolymer hydrogel acquired by natural polymers and synthetic polymers show enhanced biocompatibility.

3.1.4 Mechanical properties—Appropriate mechanical strength is another critical consideration for materials used as scaffolds in tissue engineering. Hydrogels should possess sufficient mechanical strength after gelation *in situ* to withstand biomechanical loading and provide temporary support for the cells [66]. Different tissues exhibit a wide range of matrix strength, from soft (e.g., brain ~0.1 kPa) to stiff (e.g., pre-calcified bone ~80 kPa), leading to various corresponding cellular responses [69] related to the remodeling of the cytoskeleton via pathways like the GTPase family. Therefore, scaffolds should mimic the mechanical properties of the native ECM.

The mechanical integrity of the scaffolds basically depends on the original rigidity of the polymer chains, types of crosslinking molecules, crosslinking density, and swelling as a result of the hydrophilic/hydrophobic balance [70–74]. Compared to other scaffolds, injectable hydrogels are soft and elastic due to their thermodynamic compatibility with water. Many factors such as biomaterial composition, concentration, fabrication process, porosity, and crosslinking density influence the mechanical strength of injectable hydrogels. Physically crosslinked hydrogels usually show weaker strength than chemically crosslinked hydrogels. Modification methods, such as the incorporation of lipophilic domains,

microspheres or ceramic components into the polymer, improve the mechanical properties of the injectable hydrogels [75–77].

Stiffness and toughness are two important mechanical parameters of the injectable hydrogels. A hydrogel should have sufficient stiffness to provide a relatively stable structure for cells and adequate toughness to prevent the hydrogel from becoming brittle. Increasing crosslinking density improves both the stiffness and toughness of the hydrogel. However, the degradation time of the hydrogel with high crosslinking density is also prolonged. Thus, a compromise between mechanical strength and degradability is necessary for the use of hydrogels as scaffolding materials for tissue engineering.

3.1.5 Degradation—The materials used for hydrogel formation must degrade over time in order to release the contents and allow space for newly formed tissues. Ideally, the degradation rate should match the rate of new tissue formation. Rapid degradation will cause scaffolds to lose their carrier function for cell growth, whereas a slow degradation rate can decrease the available space and impede new tissue formation [78]. In general, hydrogels can be degraded by three mechanisms: simple dissolution, hydrolysis, or enzymatic cleavage. Physically crosslinked hydrogels lose their shape and dissolve in a solution in response to the change in environmental conditions, such as temperature and the pH value. Ionic crosslinked hydrogels respond to the change in the ion strength in the solution and the hydrogel will dissolve as the ions diffuse out of the hydrogel. Strictly speaking, simple dissolution is not a degradation process since the materials of the hydrogel are not broken into small molecules.

Hydrolysis of labile ester linkages in the polymer backbone (e.g., lactide or glycolide segments in PLGA) is the most common mechanism of hydrogel degradation [79]. Factors determining the hydrolysis rate include crosslinking density, molecular weight, morphology, porosity, and amount of residual monomer. Increasing the crosslinking density, molecular weight, or hydrophobicity of the polymer decreases the degradation rate of the hydrogel. Other factors, such as the local pH and incorporation of filler, may also play a role [80, 81]. For example, inclusion of a ceramic filler (β-tricalcium phosphate) into a poly(propylene fumarate) (PPF)-crosslinked poly(propylene fumarate)-diacrylate (PPF/PPF-DA) matrix showed delayed degradation by acting as an internal buffer for acidic degradation products resulting from hydrolytic cleaving of the ester group [81]. Also, it was demonstrated that a PPF/PPF-DA matrix with a higher double bond ratio and a lower crosslinking density exhibited a faster degradation rate than the base formulation. Another degradation process occurs upon irradiation and can be controlled by the irradiation intensity and wavelength [82, 83].

Enzymatic degradation utilizes the sequence-specific cleavage of peptides such as MMPs incorporated into hydrogels via the Michael addition [84–86]. Proteolytic degradation by MMPs has been shown to play a role in cell migration, as it enables the cells to penetrate the dense matrix. Local cellular activity may also introduce additional degradation to the polymeric network [81].

While there are different degradation mechanisms, the degradation products of the hydrogel should be non-toxic and not trigger any immuno-reaction. Ideally, the degradation of hydrogels produces naturally molecules in the body that are recognized as biocompatible. For example, the hydrolysis of poly(α-hydroxy acids) generates lactic acid/glycolic acid, which enters the tricarboxylic acid cycle and is eventually excreted.

3.2 Types of injectable scaffolds

There are two types of injectable scaffolds: hydrogels and microspheres. Hydrogels are the most widely explored injectable scaffolds and can be classified based on the method of crosslinking into physically and chemically crosslinked hydrogels [87]. The physically crosslinked hydrogels are formed by the self-assembly of polymers upon a change in the environmental conditions, such as temperature, ionic concentration, pH value, or other condition having to do with the mixture of two components [87–89]. The physically crosslinked hydrogels can be further divided into ionically crosslinked hydrogels, hydrogen-bonded crosslinked hydrogels, and temperature-induced crosslinked hydrogels, which are crosslinked by means of ions, hydrogen bonding, and hydrophobic/van der Waals interactions, respectively. Compared to chemically crosslinked hydrogels, physically crosslinked hydrogels are not created from harsh conditions such as irradiation, organic solvents or crosslinking agents and do not release heat during polymerization at the gelation site. However, physically crosslinked hydrogels are less stable than chemically crosslinked hydrogels. Besides hydrogels, microspheres have recently received increased attraction as a new type of injectable scaffolds. The following section discusses the formation, structure, and properties of physically and chemically crosslinked hydrogels and microspheres.

3.2.1 Physically crosslinked hydrogels

3.2.1.1 Ionic crosslinked hydrogels: Ionic crosslinking is one of the physical crosslinking methods that couples ionizable polymers with di- and/or tri-valent cations (See Fig. 3). Ionically crosslinked polymers include alginate and pectin, and divalent metal ions such as Ca^{2+} are used to crosslink anionic chains of polycarboxylates. Other cations such as Sr^{2+}, Ba^{2+}, and Zn^{2+} can also be used as crosslinkers. The selection of ions influences not only the permeability and degree of crosslinking [90, 91], but also the release of the content inside the polymer [92]. A dual syringe applicator in which a polymeric solution and a cation solution are held separately is often used to prepare ionically crosslinked hydrogels. When the polymeric solution contacts the cation solution, the gelation takes place upon injection.

Alginate is an ionizable polysaccharide composed of M blocks and G blocks in varying proportions and sequences. The ratio of M/G blocks, as well as the ionic strength in solution and the temperature influence the gelation rate and mechanical strength of the alginate hydrogel [66]. The most commonly used crosslinking agent for alginate is calcium chloride, which crosslinks carboxylic groups in the alginate and forms an “egg box”- like conformation [93]. The mechanical properties of alginate hydrogels can be controlled by the length of the G-block, the molecular weight of the alginate chain, and the crosslinking density [93]. Increasing the crosslinking density by adding sufficient amounts of divalent cations may interfere with various cellular processes and subsequently affect tissue

formation [94]. The slow crosslinking reaction of alginate enhances its mechanical integrity and produces a more uniform structure. The ionic crosslinking process can be slowed down using a buffer containing phosphate (e.g., sodium hexametaphosphate) because the phosphate groups in the buffer compete with the carboxylate groups of alginate during the reaction with calcium ions. A low temperature can also slow crosslinking reactions due to the decreased reactivity of ionic crosslinkers [93]. Ionically crosslinked alginate is dissolved as divalent ions are released into the surrounding media via exchange reactions with monovalent cations [93, 95]. Short-term stability in physiological environments is one limitation of using ionically crosslinked alginate. It has been shown that calcium-crosslinked alginate exhibited large variations in the degradation rate, and an uncontrollable *in vivo* degradation rate is a major disadvantage of using alginate hydrogel [96]. In addition, alginate lacks specific cell binding motifs; thus, the addition of cell-recognizable motifs (e.g., RGD) is necessary for the desired cellular responses.

Besides alginate, pectin is another natural polysaccharide and possesses a carboxylic group that can be crosslinked by cations [97]. Pectin hydrogels exhibit a poor bioresorption ability similar to alginates and can degrade under physiological conditions. Other ionically crosslinked hydrogels include poly[di(carboxylatophenoxy)phosphazene] [98]. Ionic crosslinked hydrogels also include hydrogels formed by the ionic interaction between cationic and anionic polymers. For example, polyelectrolyte complexes can be formed by cationic amino groups of chitosan or chitosan derivatives and polyanionic polymers like alginate, collagen or even DNA [99].

3.2.1.2 Hydrogen bond crosslinked hydrogels: A hydrogen bond is the electrostatic attraction between polar molecules when a hydrogen atom binds to a highly electronegative atom or function group. Hydrogen crosslinking has been observed in many biological macromolecules; for example, the double strands in DNA are bound together by hydrogen bonding [79]. In a hydrogel, hydrogen bonding occurs when electron-deficient hydrogen interacts with a region of high electron density [100]. For example, in PEG/poly(acrylic acid) (PAA) interpenetrating polymer networks, PEG acts as a proton acceptor while PAA acts as a proton donor, and the protons on the carboxylic acid groups form hydrogen bonds with the oxygen atom on the PEG chains [100].

Various factors influence the gelation process, such as the temperature, ratio and concentration of each polymer, type of solvent, and degree of association between the polymer functionalities [101]. Since hydrogen bonds are made from the electrostatic attraction between polar molecules, the strength of hydrogen bonds is rather weak and strongly dependent on the pH value and temperature of the environment. A hydrogen bond-crosslinked hydrogel itself cannot achieve tough mechanical strength, and the combination of a chemical crosslinking method is often needed. Recently, a double hydrogen bonding hydrogel was reported to exhibit elevated tensile and compressive strengths over a broad pH range [102]. Furthermore, a novel fabrication method was developed to synthesize a tough hydrogen bond, crosslinked hydrogel without any chemical initiators or covalent bonding crosslinking agents by copolymerizing poly(*N*-vinylpyrrolidone) and acrylamide [103].

3.2.1.3 Temperature-induced hydrogels: Temperature-induced crosslinking hydrogels are biomaterials that possess the property of self-gelation upon a change in temperature. According to their reaction to the change of critical temperature, temperature-induced crosslinked hydrogels can be divided into two groups. When the temperature is above a critical level, one group of polymers forms gels, while the other group changes from gels to solution. The critical temperatures of these two groups are called “lower critical solution temperatures” and “upper critical solution temperatures (UCST)”, respectively. Biomaterials with UCST property include gelatin and certain polysaccharides such as agarose, amylose, amylopectin, and carrageenan. The nucleation and growth of the helical aggregates are driven by the formation of double (e.g., polysaccharides) or triple helices (e.g., gelatin) [4,40]. Typical biomaterials with the LCST characteristic include copolymers of PNIPAM, Pluronics®, PEO/PLGA, and PEG-based amphiphilic block copolymers [39][78]. PNIPAM and Pluronics® are the most widely studied, temperature-induced crosslinking hydrogels. PNIPAM has a LCST around 32°C, and factors including polymer concentration, molecular weight, chemical structure of the copolymer, and the copolymerization of other polymers all influence the transition temperature of PNIPAM. The LCST of Pluronics® can be altered by factors like the PEO/PPO ratio, total molecular weight, relative block size and block sequence. Obviously, only a polymer with a LCST is useful as an injectable hydrogel for tissue engineering.

The polymer with a LCST is useful for *in situ* gelation. Ideally, polymer solution exists in liquid state at room temperature and forms a gel at biological temperature. Thermally-induced crosslinked hydrogels have several favorable properties including no need for organic crosslinkers, and initiators, and have no thermal effect on surrounding tissues. Similar to other physically crosslinked hydrogels, temperature-induced crosslinking hydrogels often need to be chemically crosslinked to enhance the stability of the injectable scaffolds.

3.2.2 Chemically crosslinked hydrogels—Chemically crosslinked hydrogels are crosslinked with covalent bonds from different polymer chains. In most cases, the crosslinking process forms a hydrogel with irreversible linkages and has a stronger mechanical strength than a physically crosslinked hydrogel. However, in some hydrogel systems, their covalent bonds can be broken and re-formed in a reversible manner. Those hydrogels are called reversible chemically crosslinked hydrogels which possess long-term bulk stability and local adaptability.

3.2.2.1 Irreversible chemically crosslinked hydrogels: Generally, irreversible chemically crosslinked hydrogels are prepared from coupling reaction (e.g., -OH and -COOH, or -NH_2 and -COOH), photo-polymerization crosslinking, or click chemistry [46, 104–106]. For a chemical coupling reaction, carbodiimides provide a versatile method for coupling a -NH2 group to a -COOH group. To crosslink in aqueous solution, the most commonly used carbodiimide is 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). EDC can readily crosslink primary amines to carboxylic acid groups; therefore, is a powerful tool for crosslinking natural and synthetic biomaterials and forming a hydrogel. Aldehyde-induced crosslinking can take place by crosslinking water-soluble polymers with

glutaraldehyde, so that the aldehyde groups on each molecule of the glutaraldehyde react with a hydroxyl group or carboxyl group on hydrogels. In addition to glutaraldehyde, another commonly used chemical crosslinker is formaldehyde [107].

The photopolymerization of hydrogels are typically conducted with a photoinitiator and UV/visible light irradiation, whereby free radicals dissociate from the photoinitiator upon irradiation and attack the vinyl groups on macromolecular precursors. The light irradiation creates covalent bonds that crosslink the hydrogel network within seconds to minutes [108]. 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure@2959) is commonly used for UV-curing polymerization due to its relatively good cytocompatibility [109, 110]. However, the chromosomal and genetic instability of exposed cells remains a concern when UV light is utilized [111, 112]. Visible light induced photopolymerization has been proposed for more biocompatible photopolymerization systems, which include camphorquinone [113], eosin-Y [114], lithium phenyl-2,4,6-trimethylbenzoylphosphinate [115], riboflavin [116], ruthenium/sodium persulfate [117] and Rose Bengal [118]. The photo-polymerization method exhibits multiple advantages, such as fast solidification, both spatial and temporal control over the solidification to form uniform hydrogels, and no requirement of high temperatures or extreme pH values. However, photopolymerization tends to be inefficient in cases of deep implantation where insufficient light penetration can cause insufficient crosslinking [119]. Also, care must be taken when photopolymerizing cell-encapsulated hydrogels to avoid the use of a toxic photoinitiator or intense UV light irradiation that can lead to cell damage.

Since chemical crosslinking often requires the use of the additional catalyst, crosslinkers or initiators, their potential toxicity should be taken into consideration. Novel methods including chemo-selective crosslinking strategies (e.g. click chemistry) are designed to produce controlled crosslinking of hydrogels in situ under physiological conditions without toxic additives [120, 121].

One shortcoming of irreversible chemically crosslinked hydrogels is that cell spreading, migration, and even the diffusion of the metabolites, are restricted. Moreover, although desirable degradation rates and mechanisms could be specifically engineered, the consequent loss of mechanical properties is still inevitable [122, 123].

3.2.2.2 Reversible chemically crosslinked hydrogels: Compared to irreversible chemically crosslinked hydrogels, reversible chemically crosslinked hydrogels exhibit potential to provide a dynamic environment which matches the dynamic cellular behavior and tissue remodeling process [124, 125]

The Diels-Alder reaction is one of the most prominent reactions to prepare self-healing materials. The Diels-Alder reaction is thermos-reversible, meaning that the constructed hydrogel network uncouples at higher temperature and recouples during a cooling process. Previous studies indicated that the major challenge of the thermos-reversible system for biomedical application was the high self-healing temperature [126, 127]. However, a recent study showed a hydrogel system formed from furan-modified HA derivatives and maleimide-terminated PEG, achieved reversible crosslinking at physiologically compatible

condition and had a high cell survival ratio [128]. Moreover, controlled drug release was also detected in this furan-modified Diels-Alder equilibrium system [129].

Schiff base reaction between amine groups and aldehyde or ketone groups was also used to generate reversible chemically crosslinked hydrogels. The dynamic uncoupling and recoupling of the linkages can occur fast in that reaction. One example is the chitosan/PEG hydrogel that was formed via Schiff base crosslinking between the primary amine groups of chitosan and benzaldehyde groups of the modified PEG [130]. This chitosan/PEG hydrogel retained good viability of encapsulated cells after culturing for 72h. In other reports, the hydrogels formed by aliphatic aldehyde and amine showed higher cell viability, which benefited the application in wound healing [131] and tissue engineering [132].

Hydrazone and oxime bonds are formed by the reactions of aldehyde with hydrazine and hydroxylamine. Similar to Schiff base, hydrazine and oxime are classified as imines, but are intrinsically more hydrolytic-stable than other imines. Therefore, the reaction is commonly slow at neutral pH, sometimes requires hours to days for gelation, which limits its application in *vivo*. The addition of catalysts, such as anthranilic acid, phosphonates and aniline, can accelerate the reaction [133]. However, the toxicity of the additives should be taken into consideration. Specific design of the chemical structure is an effective strategy to speed up the hydrazon/oxime formation in a catalyst-free hydrogel [134]. Studies showed that the rate of formation and hydrolysis of aliphatic aldehyde-derived hydrazon/oxime is much faster than arylaldehyde-derived hydrazon/oxime at neutral pH [135, 136]. With this design, a biocompatible hydrogel was developed to mimic the biophysical property of native tissues. The cell encapsulation study showed the development of physiologically relevant morphologies in the reversible hydrogel, while the traditional chemically crosslinked hydrogel prevented cytoskeletal rearrangement and extension [137].

Thiol-disulfide is another dynamic reaction to create reversible hydrogel. The disulfide linkages are cleaved by the reduction of thiols. The couple of glutathione and glutathione disulfide is the major redox couple in animal cells, and a higher concentration of glutathione is retained in intracellular environment (0.5–10 mM) than extracellular (2–20μM) via the nicotinamide adenine dinucleotide phosphate and glutathione reductase, maintaining a highly reducing intracellular environment [138]. The reducing environment was used for intracellular delivery of bioactive molecules such as DNA, siRNA, bioactive proteins and low molecular weight drugs [139].

3.2.3 Microspheres—Microspheres were developed mainly for controlled drug delivery. Due to their inherently small size and large specific surface area, microspheres can also act as injectable cell carriers for tissue engineering. In contrast to injectable hydrogels, microspheres allow cells to adhere and proliferate on the microspheres *in vitro* for some period of time prior to injecting the construct to the defective area, which is an advantage for certain applications.

Microspheres can be formed by natural and synthetic polymers and inorganic biomaterials. Similar to hydrogels, physicochemical properties (e.g., the hydrophilicity, strength, and modulus) and the bioactivities (e.g., the biocompatibility, biosafety, and degradability) of

microspheres are governed by multiple factors such as composition, fabrication process, particle size, surface morphology, and modification methods of microspheres.

There are several techniques utilized for the preparation of microspheres, such as solvent evaporation (single or double emulsion solvent evaporation), spray drying technique, hot melt, solvent removal, and phase inversion microencapsulation [140–143]. Solvent evaporation is the most common method utilized for the preparation of microspheres. In the oil-in-water (o/w) emulsion solvent extraction technique, the polymer is first dissolved in a solvent, such as dichloromethane and chloroform. This polymer solution is then poured into an aqueous solution including surfactants while stirring to form an o/w emulsion. As the organic solvent inside the polymer droplets evaporates, the liquid droplets solidify and the microspheres are formed. In cases of encapsulating drugs or growth factors into microspheres, a double emulsion method is utilized. Several factors affect the particle size, which include the polymer content, molecular weight, sonication time, and concentration of surfactant content [144].

Besides using them directly as injectable cell carriers, microspheres can be applied in tissue engineering through incorporating them into injectable hydrogels. The incorporation of microspheres into hydrogels increases the mechanical strength and porosity of the hydrogels [145, 146]. Also, the microspheres encapsulated in a hydrogel can act as drug or growth factor delivery vehicles to modulate cell growth and tissue regeneration within the injectable system.

3.3 Development of advanced injectable scaffolding system

As discussed in previous sections, the addition of crosslinkers is required for chemically crosslinked injectable scaffolds, which elicits toxicity concerns. In addition, a variety of injectable biomaterials, especially synthetic materials, are bio-inert or bio-conductive rather than bio-inductive, and cannot effectively recruit endogenous cells to migrate and adhere onto the scaffold. Therefore, the integration of bio-inductive factors into the scaffold is necessary. While a number of approaches have been developed to encapsulate bio-inductive factors (e.g., growth factors) into injectable scaffolds, the release of those bio-inductive factors from the scaffolds is usually a passive process and has a high burst release profile. In regard to all those obstacles, the development of advanced injectable scaffolding systems is needed for better tissue regeneration.

3.3.1 Incorporating bioactive molecules into injectable scaffolds—Bioactive molecules used in tissue engineering can be classified into two groups: cell-binding peptides and bio-functional factors such as growth factors. Cell-binding peptides include long chains from ECM proteins and short peptide sequences derived from intact ECM proteins [116].

As for hydrogels and cell adhesion peptides, typically the RGD sequences and enzymatically degradable sequences (e.g., Ala-Pro-Gly-Leu l-Arg-Asn sequences) are dispersed in polymers via chemical or physical binding methods. For example, alginate was covalently modified with an RGD peptide to improve osteoblast adhesion and spreading [147, 148]. PEG hydrogels were modified with a variety of bioactive molecules to mimic the natural ECM and modulate specific cellular responses, such as cell adhesion, enzymatic

degradation, and signal molecule-binding [149–152]. The incorporation of bio-functional factors such as growth factors is often used to enhance tissue regeneration. Compared to pre-formed scaffolds, injectable biomaterials possess a more uniform distribution of growth factors. As the injectable scaffold degrades, growth factors are released into the surrounding environment and perform their functions [115]. Design factors, including the density and spatial distribution of bioactive factors, the receptor-ligand affinity, structure of the polymer connecting peptides and materials (e.g., heparin), and physicochemical properties of hydrogel polymers (e.g., pH value, porosity, and surface charges) all govern the bioactivity of the injectable biomaterials [119].

We recently developed an approach to synthesize injectable gelatin-derived hydrogels that are capable of controlling growth factor delivery to enhance tissue regeneration [153], as shown in Fig. 4. In that approach, tyramine was first introduced into gelatin chains to provide enzymatical crosslinking points for hydrogel formation after injection. Next, heparin (a polysaccharide with binding domains to many growth factors) was covalently linked to the tyramine-modified gelatin. Finally, growth factors (e.g., vascular endothelial growth factor (VEGF)) were incorporated into the tyramine-modified gelatin by binding with the heparin in the gelatin derivative. An injectable gel with controlled growth factor release was formed through an enzymatic catalytic reaction with hydrogen peroxide (H_2O_2) and horseradish peroxidase.

The properties of the injectable hydrogel, including gelation time, mechanical strength and degradation rate, were controlled by the concentration of the reactants. The *in vitro* and *in vivo* results indicated that the gelatin-derived hydrogel provided a temporospatially controlled release of growth factor, and the released growth factor retained high bioactivity for a long time. This gelatin-derived hydrogel, therefore, shows great potential as an injectable scaffold for soft tissue regeneration and a carrier for controlled drug delivery.

3.3.2 Cell-instructive scaffolds—Cells possess the capacity to sense signals from the surrounding microenvironment and consequently develop corresponding responses to that microenvironment. Therefore, scaffolds in tissue engineering should not only serve as cell carriers, but also actively interact with cells and provide step-by-step guidance for neo tissue formation [120]. The biomaterials that selectively guide cell migration spatially and temporally control cell proliferation, differentiation, and tissue formation are cell-instructive biomaterials. A good example of a cell-instructive biomaterial is the synthetic metalloproteinase-sensitive hydrogel developed by Hubbell's group [154]. In that bioactive hydrogel network, a multi-armed, end-functionalized PEG macromer was selected as the substrate, a bifunctional peptide containing a cleavable site for MMPs was used as the crosslinker, an integrin-binding RGDSP ligand for cell adhesion was coupled with the PEG substrate, and recombinant human bone morphogenetic protein 2 (rhBMP2) was entrapped in the hydrogel (Fig. 5). The bioactive network underwent cell-mediated proteolytic degradation and bone regeneration in that system was dependent on the proteolytic sensitivity of the matrices, the matrix architecture, and delivery of the rhBMP2. The biomimetic matrices, therefore, combine the advantages of synthetic biomaterials and native protein-based biomaterials.

By controlling the substrate components, the ratio of cell adhesive factors (e.g., Tyr–Ile–Gly–Ser–Arg, Pro-His-Ser-Arg-Asn and RGD), the degradation factor MMPs in the PEG hydrogel, selective migration and invasion of the target cells into the scaffold was achieved, and subsequently, the cellular fate was modulated [155, 156]. In another study, photo-responsive domains were incorporated in a cell-containing 3D hydrogel, and the enzyme-mediated bioconjugation was spatiotemporally controlled by light exposure [157]. Therefore, the biophysical and biochemical properties of the hydrogel were modulated by light beams at the micrometer scale and at a designated time. An engineered 3D extracellular matrix was recently developed to enhance the reprogramming of induced pluripotent stem cells [158].

3.3.3 Self-assembled nanofibers—Natural ECM is composed of nanofibrous 3D networks; therefore, a number of scaffolds have been designed to mimic the nanofibrous architecture of the natural ECM. For example, Zhang and colleagues developed a class of KLD12 peptides that were nanofibrillar hydrogels with high water content and crosslinked by self-assembling amphiphilic peptides in a physiological medium [159]. Those gels showed cell organization in a 3D fashion without the addition of bioactive moieties. Self-assembled peptides were also developed to form 3D nanofibrous scaffolds using L-amino and D-amino acids [160]. Those peptides are characterized by a stable β-sheet molecular structure and are self-assembled from soluble molecules into insoluble nanofibers. RADA16-I is another class of ionic self-complementary peptides [161]. Those 16-amino acid sequence peptides have a β-sheet structure that can undergo self-assembly to form nanofibrous matrices. RADA16-I was investigated as a 3D scaffold for bone regeneration in combination with mesenchymal stem cells and platelet-rich plasma [161]. However, the relatively low pH range (approximately 3–4) of the RADA peptides impairs the activity of the adjacent cells and leads to instability of the peptides in a neutral environment. SPG-178, another self-assembled peptide with a higher isoelectric point, was more stable and biocompatible for tissue regeneration [162] (Fig. 6).

A class of amphiphilic peptides, called “multidomain peptides” (MDP) were designed to have modular ABA block motif and self-assemble into nanofibers [163]. The process of supramolecular assembly is driven by a core motif (B block) of alternating hydrophilic and hydrophobic amino acid residues where dimers form because of the predisposition of hydrophobic residues to avoid water. The self-assembled MDP nanofibers had an average diameter of a few nanometers and a length of several micrometers. Concerns about the degradability of the β-sheet-forming MDPs led to the incorporation of MMPs’ specific cleavage motif to improve the degradation rate of the MDP hydrogels [163]. The incorporation of cell adhesion motif RGD along with this cleavage site also increased cell viability and enhanced cell migration into the hydrogel matrix [163]. The MDP hydrogels were tested as an injectable scaffold for pulp tissue regeneration [164].

3.3.4 Nanofibrous microspheres—It is widely known that ECM-mimicking nanofibrous architecture enhances cell-material interactions. However, traditional microspheres have a solid-interior structure and lack nanofibrous architecture. Therefore, they are not ideal injectable carriers for cell adhesion and bioactive factor delivery. In

addition, solid-interior microspheres generate a large amount of degradation by-products when they are degraded, which may exert an adverse effect on the surrounding tissues of the defect area. To integrate the ECM-mimicking architecture into microspheres, our group recently developed the technology to prepare nanofibrous hollow microspheres [165]. First, we synthesized star-shaped poly(L-lactic acid) using poly(amidoamine) dendrimers as initiators. Next, an oil-in-oil emulsification process and a phase inversion and separation step were performed to induce the polymer solution to self-assemble into nanofibrous hollow microspheres. The nanofibrous hollow microspheres were composed entirely of nanofibers with the diameter at the same size as the collagen fibers. The open, hollow structure of the microsphere contributed to high porosity (>95%), therefore providing sufficient space for cell growth and ECM deposition. The nanofibrous hollow microspheres had an overall density of less than 1/30 of the density than that of the solid-interior counterparts. Therefore, when the nanofibrous hollow microspheres were degraded, they generated significantly fewer by-products than did the solid-interior microspheres.

When the nanofibrous hollow microspheres were tested as injectable cell carriers, a high cell attachment efficiency to the microspheres was observed, which was attributed to the nanofibrous architecture. Moreover, the chondrocytes seeded on the nanofibrous hollow microspheres had a significantly higher proliferation rate and produced higher amounts of glycosaminoglycan (GAG) than did those on the solid-interior microspheres. When the nanofibrous hollow microspheres were mixed with chondrocytes and injected into a rat femoral condyle mold to incubate *in vitro* for a few weeks, new cartilage tissue with a shape identical to the rat femoral condyle was regenerated, demonstrating the capability of the microspheres to serve as an injectable scaffold to fill complex defects and be molded into a predesigned 3D tissue shape. A critical-sized rabbit osteochondral defect-repair model further showed that the nanofibrous hollow microspheres/chondrocytes group achieved substantially better cartilage repair than the autologous chondrocytes implantation group, indicating that the nanofibrous hollow microspheres are an excellent injectable cell carrier for cartilage regeneration.

To further incorporate bioactive molecules into the nanofibrous microsphere and temporally and spatially control its release, we developed a unique hierarchical nanosphere-encapsulated-in-microsphere scaffolding system [166]. In that system, the growth factor BMP2 binds with heparin and is encapsulated into heparin-conjugated gelatin nanospheres, which are further immobilized in the injectable nanofibrous microspheres, as shown in Fig. 7. BMP2 has binding domains with heparin, and the binding of BMP2 to heparin protects BMP2 from denaturation and degradation, subsequently prolonging its sustained release. Because the heparin-binding BMP2 was encapsulated in gelatin nanospheres and entrapped by the nanofibers of PLLA microspheres, the BMP2 was released in a multiple-controlled manner (by the binding with heparin, the encapsulation of the nanosphere, and the entrapment of the microsphere nanofibers) and retained its high bioactivity. A calvarial defect model confirmed that this unique BMP2-loaded hierarchical microsphere system was an excellent osteo-inductive carrier for bone regeneration. The hierarchical microsphere system can be easily applied to other types of tissue regeneration through the use of different growth factors. Therefore, this approach expands the ability to develop new injectable biomaterials for advanced regenerative therapies.

4. Applications of injectable scaffolds in dental and craniofacial tissue regeneration

4.1 Injectable scaffolds for dental tissue regeneration

4.1.1 Dentin and pulp regeneration—Dental pulp is a highly specialized connective tissue that maintains the biological and physiological vitality of a tooth. Under certain conditions, the pulp forms tertiary or reparative dentin in response to external agents [167, 168]. Direct and indirect pulp capping with calcium hydroxide cement or mineral trioxide aggregate (MTA) forms a dentinal bridge at the exposure site. For complete necrosis of pulp tissues, a root canal treatment is often performed in the clinic. However, the tooth, losing its vitality after root canal treatment, becomes susceptible to postoperative fractures and may suffer a re-infection due to coronal or apical micro-leakage [169, 170]. Loss of pulp vitality in young permanent teeth also interferes with tooth maturation and apexogenesis. Thus, there is a great need for the development of a tissue engineering approach for the regeneration of the pulp and dentin complex.

The stem cells used for dentin/pulp regeneration include postnatal dental pulp stem cells (DPSCs) [171, 172], stem cells from exfoliated deciduous teeth (SHED) [173], periodontal ligament stem cells (PDLSCs) [174, 175], stem cells from apical papilla (SCAP) [176–178] and dental follicle progenitor cells [179, 180]. Among them, the DPSCs, SHED and SCAP are potentially more suitable cell sources for pulp/dentin regeneration because they are derived from pulp tissue or the precursor of pulp [181].

Due to the small and irregular shape of the tooth root canal, injectable biomaterials are considered excellent candidates for pulp and dentin regeneration. Collagen was the first natural biomaterial used as an injectable gel for pulp regeneration. However, the collagen gel inside the root canal contracted as it was transplanted inside the back of nude mice [181]. Fibrin was modified with PEG to form a PEGylated fibrin hydrogel with a decreased degradation rate and was used as a dental stem cell carrier [182]. The *in vivo* results indicated a pulp-like tissue formation in the canal space after five weeks [182]. Puramatrix™, a synthetic self-assembled nanofibrous peptide hydrogel, has been used for the regeneration of a variety of tissues and was also tested recently for the regeneration of a pulp-dentin complex [183]. In one study, Puramatrix^{tm} was used as a carrier to investigate the role of DPSCs in triggering angiogenesis of the root canal [184]. Human umbilical vein endothelial cells and DPSCs were cultured together in Puramatrix^{tm}, and they exhibited more ECM, vascularization, and mineralization than did the DPSC-mono-cultures *in vivo*. Self-assembling peptide amphiphiles are other synthetic biomaterials and RGD-modified PAs were evaluated as injectable scaffolds for pulp and dentin regeneration [185]. Besides, a PEG-maleate-citrate hydrogel was crosslinked using visible light and proposed for vital pulp therapy and endodontic regeneration [186].

Due to its unique anatomical structure, revascularization of the full-length root canal has been a challenge for many years. Fast revascularization is considered the key to the success of pulp regeneration in a full-length root. Current approaches to increasing fast revascularization include the incorporation of angiogenic growth factors and the co-culture

of DPSCs and endothelial cells to enhance angiogenesis. However, those approaches only regenerated a small ratio of pulp-like tissues in the lower region and no tissues in the upper region of the root canal [184].

We recently developed an injectable microsphere system for full-length pulp regeneration (Fig. 8). In that system, heparin was incorporated into gelatin and formed heparin-conjugated gelatin nanospheres, which were further immobilized in nanofibrous biodegradable PLLA microspheres. A hierarchical microsphere acts as both a cell carrier and controlled growth factor delivery vehicle. As a cell carrier, the hierarchical microsphere is self-assembled with synthetic nanofibers that mimic the structure of natural collagen fibers. The ECM-mimicking nanofibrous architecture enhanced DPSC differentiation and pulp tissue regeneration. As a growth factor delivery vehicle, the release of the VEGF from the microsphere is controlled in a multiple-layer manner (through the binding with the heparin, the degradation of the nanosphere, and the physical adsorption of nanofibers). A full-length root canal model, in which the coronal end of the canal was sealed with MTA, was adopted in the study to truly simulate pulp regeneration in a diseased human tooth. The *in vivo* results showed the regeneration of pulp tissues that fulfilled the entire lower two-thirds and reached the coronal third of the full-length root canal. In addition, a large number of blood vessels were regenerated throughout the canal [187]. Therefore, our work demonstrates the success of pulp tissue regeneration in a full-length root with one end sealed using a single installation process, making it a significant step toward regenerative endodontics.

4.1.2 Periodontal and alveolar defects—Periodontal regeneration restores the functional anchorage of a tooth by forming new alveolar bone, PDL, and cementum on the root surface. A number of injectable biomaterials have been tested for periodontal tissue regeneration. In one example, quaternized chitosan (HTCC) was mixed with α-β glycerophosphate as an injectable carrier to deliver drugs and PDLSCs [188]. The HTCC hydrogel remained in aqueous solution below 25°C and became a gel at human body temperature. The addition of HTCC slowed the drug release and provided a strong antibacterial effect on periodontal pathogens. In addition, the HTCC hydrogel was non-toxic and promoted the ALP activity of the PDLSCs *in vitro*. When the HTCC hydrogel was further conjugated with basic fibroblast growth factors, it effectively enhanced new periodontal support tissues in dogs [188, 189] (Fig. 9).

CPC is another biomaterial evaluated as an injectable carrier for periodontal regeneration. In a preclinical study reported by Hayashi et al., experimental periodontitis was induced by placing stainless-steel mesh on the mesial side of the maxillary canines in adult beagle dogs [190]. Consequently, intrabony defects were created and the CPC was injected in the defects. The results showed new cementum and periodontal ligament-like tissue were observed between the CPC and the root surface 12 weeks after surgery, indicating that the CPC provided stable wound healing and enhanced periodontal regeneration in these dogs with experimental periodontitis. However, when the CPC was used for a randomized clinical trial of human periodontal intrabony defect regeneration, it failed to demonstrate any superior clinical outcomes for the CPC group compared to the open flap debridement group [191].

The authors speculated that the filling volume and the CPC stiffness may compromise the clinical outcomes for periodontal intrabony defects.

Periodontal regeneration often uses rhBMP2 or rhBMP7 to enhance bone formation. However, the surgical implantation of a high dose of rhBMP2 (or rhBMP7) was associated with root resorption/ankylosis when evaluated in large animal models, suggesting a potential limitation of their periodontal applications. Recombinant human growth/differentiation factor 5 (rhGDF5) induces bone formation less aggressively compared with rhBMP2 and rhBMP7, and may allow the regeneration of periodontal tissues without root resorption/ankylosis. Therefore, rhGDF5 was incorporated in an injectable PLGA for periodontal regeneration. The delivery of the rhGDF5/PLGA construct using a minimally invasive procedure for periodontal regeneration was evaluated in surgically created periodontal pockets in dogs [192]. The rhGDF5/PLGA construct showed significant increases in PDL, cementum, and bone regeneration over time, suggesting that rhGDF5/PLGA was a candidate construct for periodontal regeneration. However, more work should be included to optimize the PLGA carrier and the rhGDF5 release profile, as well as to evaluate the efficacy of this approach in clinical settings using a minimally invasive approach.

Platelet rich plasma (PRP) is blood plasma that is enriched with platelets and contains a number of growth factors that play significant roles in chemotaxis, cell proliferation, angiogenesis, and bone formation. In a clinical study, PRP was used as an autologous injectable scaffold for bone marrow-derived mesenchymal stromal cells (BMMSCs) to conduct maxillary sinus-floor augmentation [193, 194]. In that study, the height of the regenerated hard tissues after two years showed mean increases of 8.8 ±1.6 mm compared to preoperative values, and no adverse effects or apparent bone absorption were detected in the follow-up years. Furthermore, the dental implants prepared with the materials in the study were clinically stable after second-stage surgery. This study indicated that an injectable construct was an alternative option as a graft material for maxillary sinus floor augmentation, making it possible to decrease healing time in the future.

Apart from growth factors, many other proteins and drugs have also been explored for periodontal regeneration. For example, a delivery system composed of PLGA-lovastatin-chitosan-tetracycline nanoparticles with an average size of approximately 100 nm was developed for periodontal tissue regeneration [195]. The lovastatin and tetracycline from the nanoparticles showed prolonged releases even after 21 and 14 days, respectively. In addition, the delivery of the loaded drugs could be further modified by the parameters such as the crystallinity and molecular weight of PLGA, and the deacetylation degree of chitosan. The nanoparticles were non-toxic, and had bactericidal activity and osteogenic potential. In an *in vivo* study, the nanoparticles promoted significant amount of new bone formation after being injected into three-walled intrabony defects of beagle dogs for 8 weeks [195].

Injectable scaffolds were also combined with gene delivery for periodontal regeneration. In one study, Pluronic F127 (PF127) was used to deliver BMMSCs modified by adenovirus BMP2 gene transformation [196]. PF127 is a polyethylene oxide and polypropylene oxide copolymer that presents in a liquid state at cold temperatures and solidifies into a gel at human body temperature. PF127 has been suggested to improve wound healing and

osteogenesis and has been considered as a carrier for osseous graft materials and growth factors [197]. Bilateral mandibular alveolar and periodontal defects were created over the premolar areas in mature male beagles and assigned to the adenovirus BMP2 group and in the control group [198]. Histology showed that the regenerated periodontal apparatus in the adenovirus BMP2 group was significantly better compared to the control. New cementum and periodontal fibers were also formed in the adenovirus BMP2 group. Therefore, the adenovirus BMP2-infected autologous BMMSC/PF127 construct offers an alternative approach to the delivery of growth factors for periodontal apparatus regeneration.

4.2 Craniofacial tissue regeneration

4.2.1 Bone regeneration—Craniofacial defects due to trauma, tumor resection and congenital abnormalities result in pain and the loss of esthetics and functionality. The reconstruction of these defects can be achieved by using tissue engineering approaches. Ideally, the scaffold used for craniofacial regeneration should mechanically support the regenerating tissue, be able to deliver bioactive factors, and fit well in complex 3D anatomic defects to restore functional anatomy [199]. A variety of polymeric hydrogels have been evaluated as injectable scaffolds for the delivery of cells and bioactive molecules for craniofacial bone defects. For example, a PEG matrix was tested for local BMP2 gene transfer in a pig calvarial critical size defect model [200]. The addition of PEG matrix facilitated cell survival and protein synthesis, and the local delivery of BMP2 gene by PEG matrix-embedded cells increased bone formation. Gelatin microspheres were also used as injectable vehicles for the dual delivery of VEGF and BMP2 for bone regeneration in a rat cranial defect model and showed a synergistic effects of these two growth factors in bone formation and bridging [201].

One study adapted a rabbit model for mandibular defects reconstruction [202]. Autogenous BMMSCs were loaded onto β-TCP micro-granules and implanted into mandibular critical-sized defects (10×15 mm) of rabbits. The results indicated that the BMMSCs/β-TCP construct regenerated a more mature and denser bone than the β-TCP-alone group. This study demonstrated that the BMMSCs/β-TCP construct promoted bone regeneration in the repair of mandibular bone defects.

Another study focused on bone regeneration around implants and used a porous rhBMP2-embedded PLGA microparticle system, which was mixed with alginate hydrogel prior to injecting into a tooth socket defect surrounding an implant [203]. Porous PLGA microspheres were first prepared via an electrospray method and had an average size of approximately 4.4 μm. After encapsulation of rhBMP2 into the PLGA microsphere, a solvent treatment step was needed to close the pores on the outer surface of the microsphere. Due to the sustained release profile of the rhBMP2 from the injectable system, the rhBMP2/hydrogel group showed a better bone regeneration and osseointegration compared to the rhBMP2 solution group and the microparticle/hydrogel group, as shown in Fig. 10.

Composites of calcium phosphates with natural and synthetic polymers are another class of biomaterials for craniofacial regeneration. The degradability and brittleness of the calcium phosphate cements were improved by the addition of polymers in the composite. An injectable construct of CPC paste and alginate microbeads was used to encapsulate human

umbilical cord mesenchymal stem cells for the regeneration of bony defects [204]. The incorporation of calcium phosphate cements improved the mechanical properties and increased osteo-differentiation and bone mineralization compared to the injectable hydrogel alone. In another study, CPC and chitosan injectable composite showed potential as a moderate load-bearing matrix and carrier of injectable osteo-inductive growth factors for bone repair [205]. Those CPC-chitosan composites were also used as carriers for stem cells [206]. In an *in vivo* study on the bone regeneration of a calvarial critical-sized defect model of rats, thermos-gelling chitosan-inorganic solution was used as a carrier for BMMSC and BMP2 [207].

Several studies used injectable biomaterials to test the potential of other cell types as a potential source of cells for craniofacial bone regeneration. For example, induced pluripotent stem cell-derived mesenchymal stem cells (iPSMSCs) were encapsulated in alginate microbeads, which were mixed with CPC paste to form an injectable cell carrier [208]. The iPSMSCs maintained relatively high viability inside the microbeads after injection. After culturing in differentiation medium, the iPSMSCs exhibited up-regulated osteogenic markers and deposited minerals. After receiving injections into craniofacial defects in nude rats for 12 weeks, more new bone tissues were regenerated in the CPC-BMP2-iPSMSCs group than other groups. DPSCs were encapsulated in a hyaluronic-based hydrogel and injected into calvarial defects of immunocompetent rats to test new bone formation [209]. While no complete healing was detected after 8 weeks, more bone was formed in the DPSC group than in the two other control groups. In another experiment, DPSCs were mixed with HA/TCP paste and injected into full-thickness cranial defects of immuno-deficient rats [210]. The *in vivo* results showed that the calcification rate and bone mineral density values in the DPSC/HA/TCP group were significantly higher than in the other groups, suggesting that DPSCs may be a suitable cell source for bone tissue engineering.

4.2.2 Cartilage regeneration—Cartilage is a flexible connective tissue. Nasal septal cartilage, the cartilages of the ear and condylar cartilage of TMJ are the main cartilages of the craniofacial region. Due to the avascular nature of cartilage, the regeneration of new cartilage is difficult. Injectable hydrogels mimic the cartilaginous matrices and have been examined for articular cartilage regeneration. There are relatively few reports on nasal and auricular cartilage tissue regeneration.

Chondrocytes are an ideal cell source for cartilage regeneration. However, they are available in limited quantities and need *in vitro* culture to obtain sufficient cell number for implantation. Meanwhile, chondrocytes easily undergo de-differentiation and lose the expression of cartilage markers once they are isolated from their native ECM and cultured in traditional cell culture dishes. Some studies suggested chondrocytes may maintain chondrogenic properties when cultured in certain environment, such as adding some special growth factors or reducing oxygen tension [211]. Recently, a heparin-based RAD16-I scaffold was developed as a 3D culture system to promote reestablishment of chondrogenic phenotype of de-differentiated human articular chondrocytes under general culture conditions [212]. In the 3D injectable scaffolding system, differentiated chondrocytes recovered elongated spherical morphology, expressed elevated chondrogenic genes, and regained proteoglycan (PG) secretion property. In another study, isolated swine articular

chondrocytes were photo-encapsulated in PEG-LA-DM/PEGDM copolymer hydrogels [213]. The ratio of the PEG-LA-DM and PEGDM affected the structural integrity between the native cartilage and the engineered cartilage construct. By modulation of the composition ratio of the copolymer, the construct showed promising results for cartilage regeneration. Not only did the chondrocyte/hydrogel construct exhibited higher GAG and hydroxyproline content as culture time prolonged, the encapsulated chondrocytes were also able to maintain their chondrocyte phenotype when implanted in subcutaneous pockets of nude mice, and eventually were able to integrate with native cartilage in a cartilage ring model when cultured in rotating bioreactors (See Fig. 11).

TMJ is a fibrocartilage between the mandible and the temporal bone. The regeneration of the mandibular condyle that is anatomically, structurally and functionally similar to the natural TMJ condyle must integrate bone and cartilage layers in an osteochondral construct. Such a construct can be prepared from multi- or bi-layer hydrogels [211, 214]. For instance, rat bone marrow mesenchymal stem cells were induced to differentiate into chondrogenic and osteogenic cells *in vitro*, and then encapsulated into a PEG-based two-stratified layer hydrogel before being implanted *in vivo* [215]. After eight weeks, a condyle-like tissue formation including both the osteogenic and chondrogenic cells formed. A similar study showed the formation of articular condyles in the shape and dimensions of the human mandibular condyle after implantation in the dorsum of immuno-deficient mice for twelve weeks, and histological images showed the formation of two stratified layers of cartilaginous and osseous tissues [216]. Furthermore, the integration of an additional matrix into soft and stiff mono-layer hydrogels would significantly increase the mechanical properties of the scaffolds. The multi- or bi-layered PEG scaffolds reinforced by fibrous collagen scaffold were proved to possess better mechanical properties to withstand compressive loads [217]. A hydroxyapatite enhanced chitosan scaffold prepared by combining a sintering and freeze-drying technique also exhibited enhanced bioactivity and mechanical strength when adopted for osteochondral regeneration [218]. The *in vitro* experiment showed that the hydroxyapatite and chitosan layers provided adequate 3D support for the adhesion, proliferation and differentiation of bone marrow stromal cells into osteoblasts and chondrocytes, respectively. Similarly, a nano-hydroxyapatite reinforced collagen-alginate hydrogel was also used for osteochondral regeneration [219]. This triple-phased composite showed enhanced tensile and compressive modulus, better cell viability and proliferation and upregulated hyaline cartilage makers.

Apart from hydrogels, PLGA microspheres with a gradient transition between cartilage-promoting factors (transforming growth factor beta 1(TGF-β_1)) and bone-promoting growth factors (BMP-2) were also constructed to mimic the osteochondral interface regeneration of rabbit mandibular condyles [220]. The BMP2- and TGF-β_1-loaded microspheres were stacked within a cylindrical glass mold to build continuously gradient scaffolds. The results suggested that the gradient design of the scaffold with bioactive factors provided consistent regeneration of a smooth condylar surface, with more uniform and thicker cartilage tissue formation than did the conventional scaffolds.

It should be noted that TMJ has a different environment from other articulating joints. In one study, PEG was used as a scaffold to examine condylar chondrocyte viability over the

culture period and under loading conditions compared to the metacarpalphalangeal joint (MCJ) chondrocytes [221]. Results showed the TMJ chondrocytes responded differently compared to MCJ chondrocytes under the same environment and loading condition. The addition of mechanical loading stimulated MCJ chondrocyte proliferation and PG synthesis, while it inhibited the TMJ chondrocyte proliferation and PG synthesis. In addition, the gene expression levels for Type I collagen was significantly higher in the TMJ chondrocytes compared to the MCJ chondrocytes. Those results confirmed a distinguishing microenvironment for TMJ and suggested a separate consideration in TMJ regeneration.

5. Future directions

Due to their capability to deliver in a minimally invasive manner and other advantageous properties, injectable scaffolds are appealing for dental and craniofacial tissue engineering. A number of synthetic and natural biomaterials have been tested for those applications. However, most conventional injectable biomaterials are not bio-inductive and cannot elicit desirable cell-material interactions. A few approaches such as the development of cell-instructive biomaterials, preparation of novel self-assembly peptides, and fabrication of bio-inspired nanofibrous microspheres, were developed recently to address this issue. When used for dental and craniofacial tissue regeneration, those bioactive materials showed more promising results than conventional biomaterials. These novel biomaterials represent the direction for the next generation of injectable biomaterials and warrant further exploration.

To fabricate an injectable scaffold from a biomaterial, a variety of parameters, such as gelation time, injectability, toxicity, biocompatibility, degradation, and mechanical properties, should be considered. It is important to understand that each parameter affects cell adhesion, proliferation, differentiation, tissue deposition, and the host response. For example, BMMSCs differentiate into neurogenesis in a soft hydrogel, myogenesis in a medium modulus hydrogel, and osteogenesis in a rigid hydrogel. The crosslinking density controls the pore size within the hydrogel network, which affects the viability and proliferation of the encapsulated cells and the release profile of the drug inside the hydrogel. To avoid damaging incorporated bioactive molecules and cells, the gelation process should occur under mild conditions. The introduction of functional groups to the polymer of a hydrogel facilitates the crosslinking reaction; however, the functional groups may show cross-reactivity with incorporated bioactive growth factors or cells during the gelation process. All those results indicated the importance of a comprehensive consideration when designing or choosing an injectable scaffolding system.

One of the major barriers to successful tissue regeneration is to regenerate a tissue with the proper structure. For example, in a natural tooth, the dentin is a mineralized hard tissue composed of multiple closely packed dentinal tubules with a diameter of several micrometers. The unique tubular architecture plays a pivotal role in maintaining normal mechanical and biological functions of a tooth. Due to the insufficient knowledge of the developmental biology of dental tissues as well as the limitation of current bioengineering technology, the regeneration of a complete layer of tubular dentin along the root canal chamber has not been achieved. With the advances in developmental biology, future work

should focus on identifying crucial chemical/biological factors and optimizing the scaffold architecture to facilitate the regeneration of a complete tubular pulpodentin complex.

A big challenge in periodontal regeneration is the simultaneous regeneration of different types of tissues (PDL, cementum, and alveolar bone) with the proper morphology. During tooth root development, the PDL, cementum and alveolar bone form simultaneously, and the PDL Sharpey's fibers are deeply inserted in the cementum and alveolar bone, which make the periodontal complex more resilient to masticatory forces. Consequently, the regenerated PDL layer should have properly organized Sharpey's fibers that connect with the cementum and alveolar bone. Otherwise, the regenerated, disorganized connective tissue cannot perform normal biomechanical function to withstand masticatory loads subjected to the teeth, resulting in either destruction of the teeth or breakage of ligaments. Similarly, a seamless stratified osteochondral construct should be used to regenerate both the cartilage and bone layers for the TMJ regeneration. It would also be crucial to observe the effect of mechanical stimulation on the regenerated tissue complex.

Revascularization has long been a challenge for dental and craniofacial regeneration, especially for full-length pulp regeneration and TMJ regeneration. As introduced in the above sections, a few approaches have sought to develop injectable hydrogels with the controlled release of angiogenic growth factors for angiogenesis and ultimately maturity of neovasculature. The spatial and temporal delivery of multiple angiogenic growth factors will enhance therapeutic angiogenesis. However, the temporospatial control of growth factor release within an injectable scaffold is still a goal to be achieved in the future.

In terms of preclinical studies, considerable success with injectable scaffolds has been achieved with craniofacial defects in small animal models. However, these scaffolding systems must be tested in larger animals to further assess their utility and applicability to craniofacial regeneration. Future research will also have to address the long-term success rates, the stability of engineered tissue, and the application of the therapy to less vascularized environments.

Despite all these challenges, injectable biomaterial-based regenerative medicine therapy for dental and craniofacial tissues is a rapidly developing field, and the advances of this field have the potential to improve the health of dental and craniofacial patients in the near future.

Acknowledgments

This work was supported by NIH/NIDCR grants R01DE024979, R03 DE22838 (X. L.) and we would like to thank Jeanne Santa Cruz for her assistance with the editing of this article.

Abbreviation

BCP	biphasic calcium phosphate
BMMSC	bone marrow-derived mesenchymal cells
CPC	calcium phaosphate cements
DPSC	dental pulp stem cell

ECM	extracellular matrix
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
GAG	glycosaminoglycan
GelMA	methacrylamide-functionalized gelatin
H2O2	hydrogen peroxide
HA	hydroxyapatite
HTCC	N-(2-hydroxy)propyl-3-trimethyl ammonium chitosan chloride
iPSMSC	induced pluripotent stem cell-derived mesenchymal stem cell
LCST	lower critical solution temperature
MCJ	metacarpalphalangeal joint
MDP	multidomain peptides
MMP	matrix metalloproteinase
MTA	mineral trioxide aggregate
o/w	oil-in-water
PA	peptide-amphiphile
PAA	poly(acrylic acid)
PCL	poly(ε-caprolactone)
PDL	periodontal ligament
PDLSC	periodontal ligament stem cell
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PG	proteoglycan
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PNIPAM	Poly(*N*-isopropyl acrylamide)
PPF	poly(propylene fumarate)
PPO	poly(propylene oxide)
PRP	platelet rich plasma

RGD	Arg-Gly-Asp
rhBMP	recombinant human bone morphogenetic protein
rhGDF	recombinant human growth/differentiation factor
SCAP	stem cells from apical papilla
SHED	stem cells from exfoliated deciduous teeth
TGF	transforming growth factor
TMD	temporomandibular joint disorder
TMJ	temporomandibular joint
UCST	upper critical solution temperatures
UV	ultraviolet
VEGF	vascular endothelial growth factor
β-GP	glycerol-2-phosphate
β-TCP	β-tricalciumphosphate

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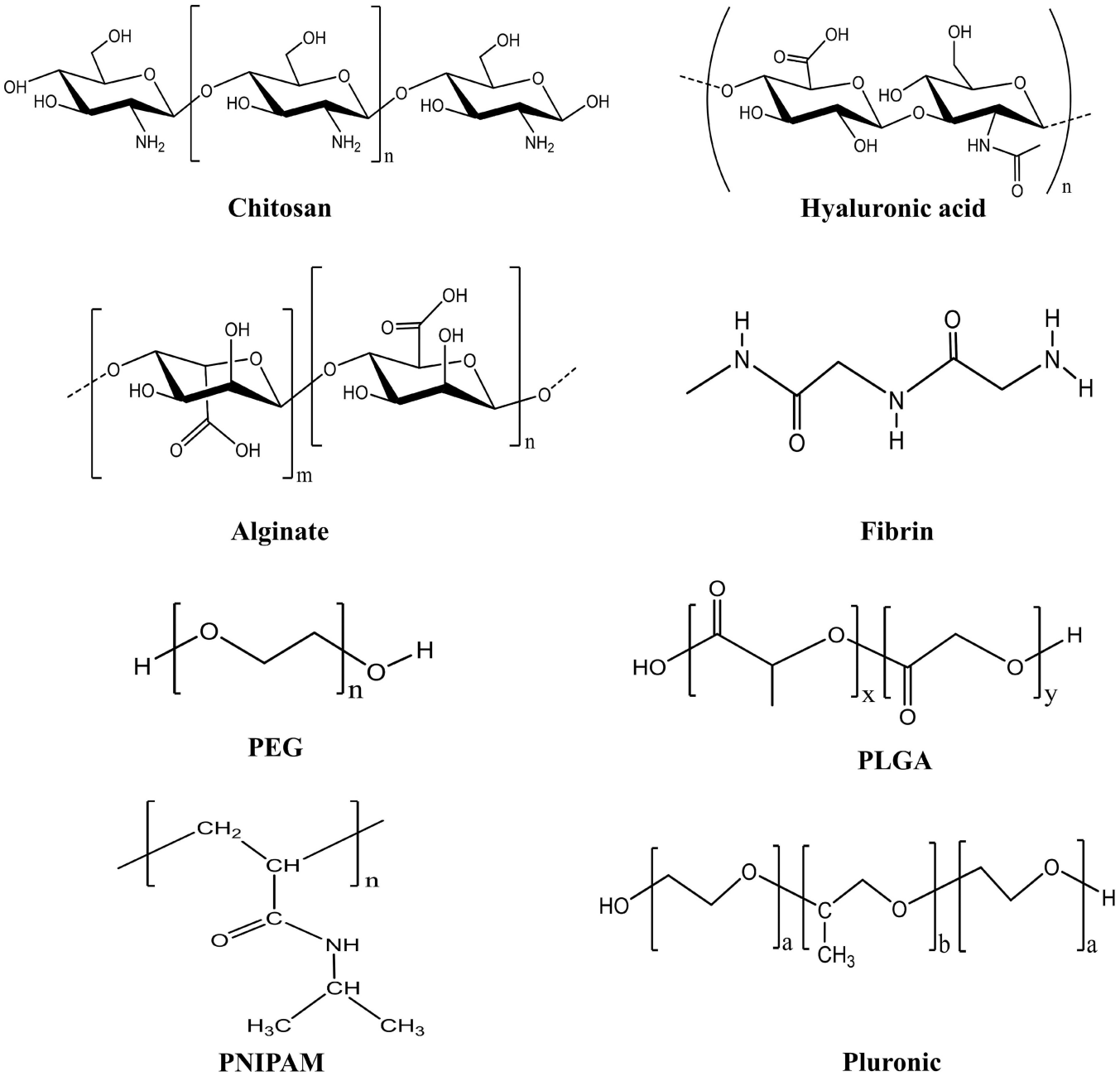

Fig. 1.
Chemical structures of representative injectable natural and synthetic biomaterials

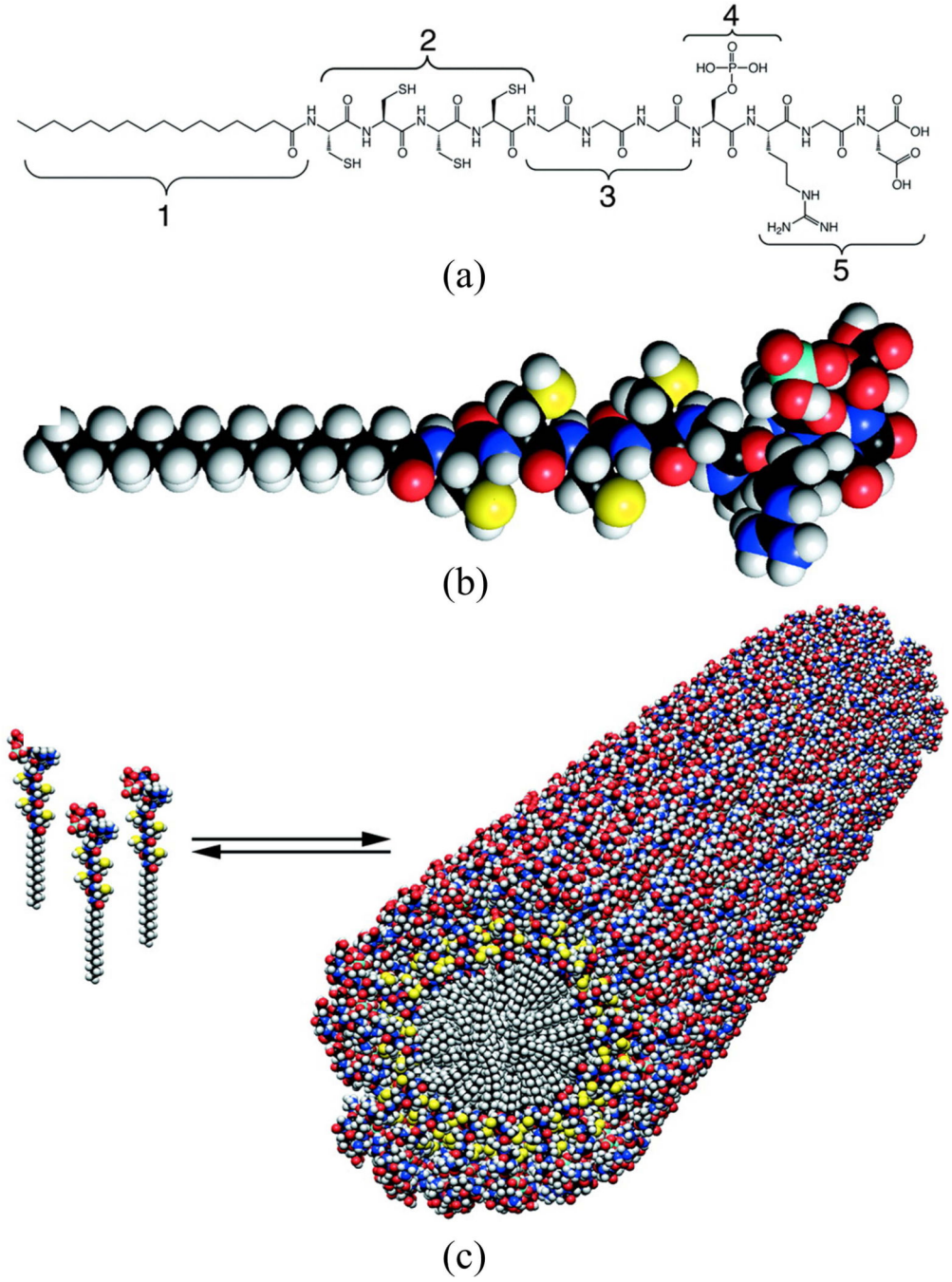

Fig. 2.

A Self-assembled peptide amphiphile (PA). (a) Chemical structure of the peptide amphiphile. (b) Molecular model of the PA. (c) Schematic showing the self-assembly of PA molecules into a cylindrical micelle. Adapted with permission from [63], Copyright 2001 American Association for the Advancement of Science.

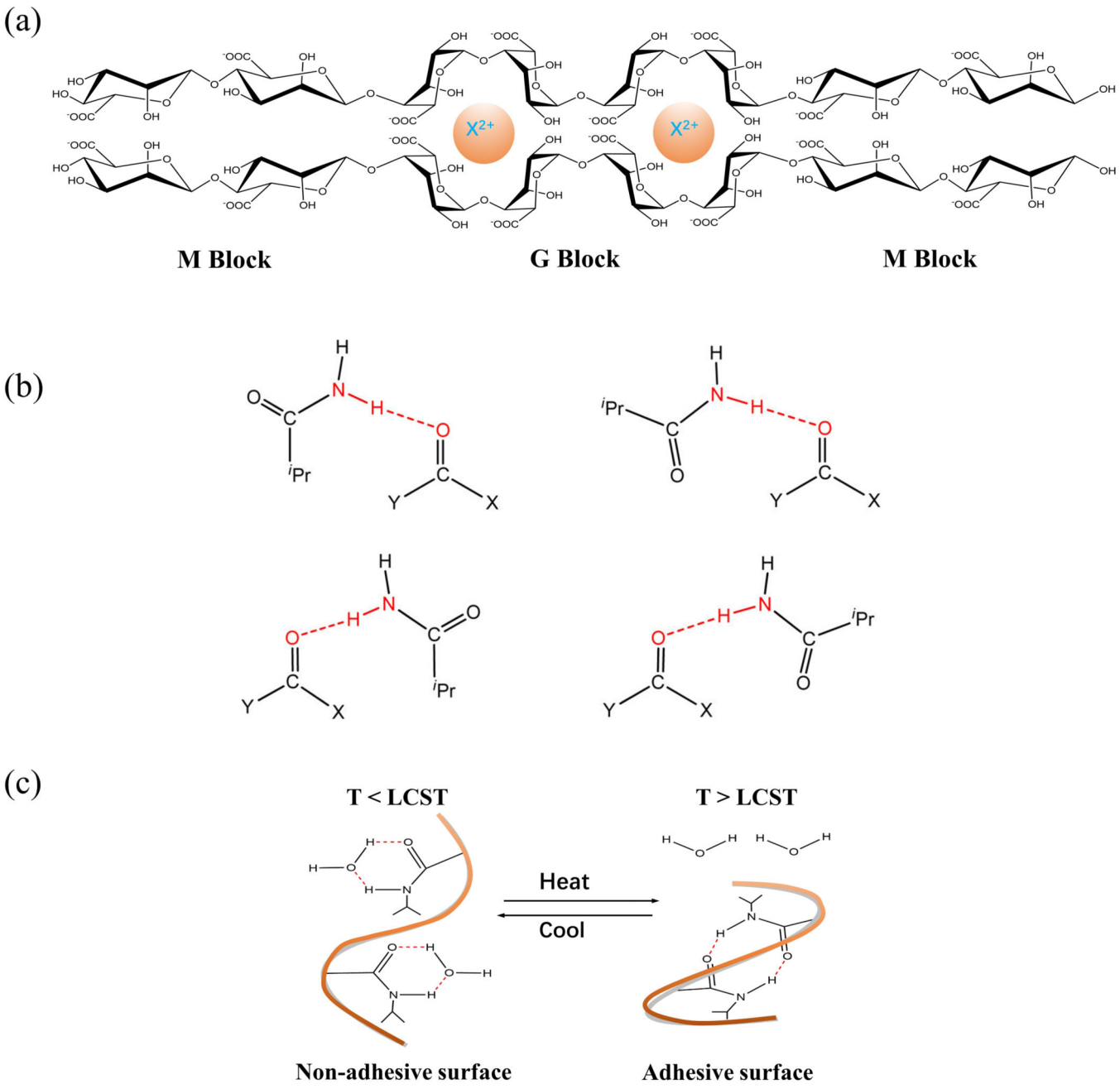

Fig. 3.

Physically crosslinked hydrogels. (a) Ionic crosslinked hydrogels. (b) Hydrogel bond crosslinked hydrogels. (c) Temperature-induced crosslinking hydrogels.

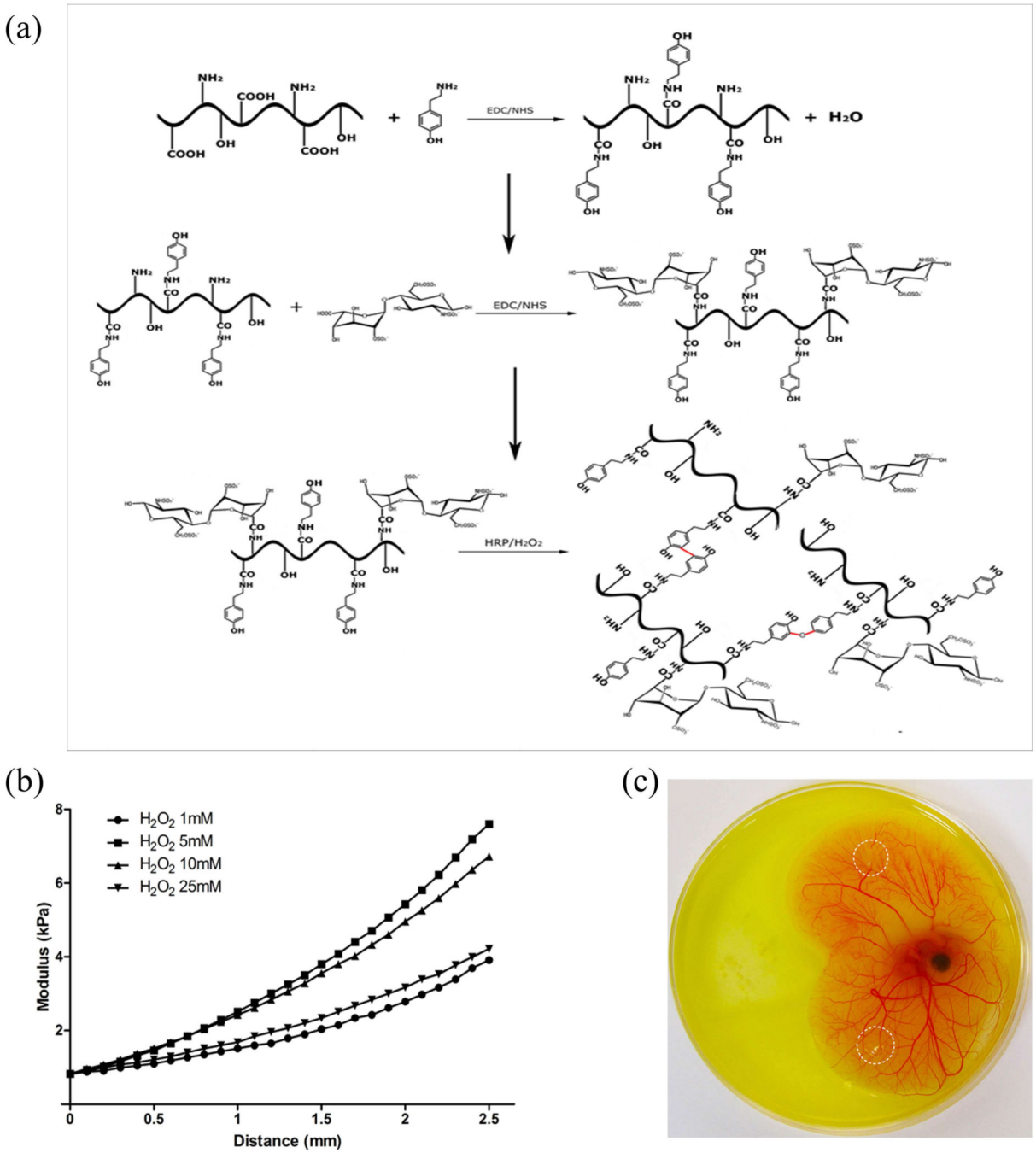

Fig. 4.

A injectable gelatin derivative hydrogel with sustained VEGF release for induced angiogenesis. (a) Synthesis of injectable gelatin-derived hydrogels. (b) Control of the mechanical strength of the gelatin-derived hydrogel using different H2O2 concentrations. (c) The released VEGF from the gelatin-derived hydrogel showed high bioactivity using a chick chorioallantoic membrane (CAM) assay. Adapted with permission from [153]. Copyright 2015 Elsevier Ltd.

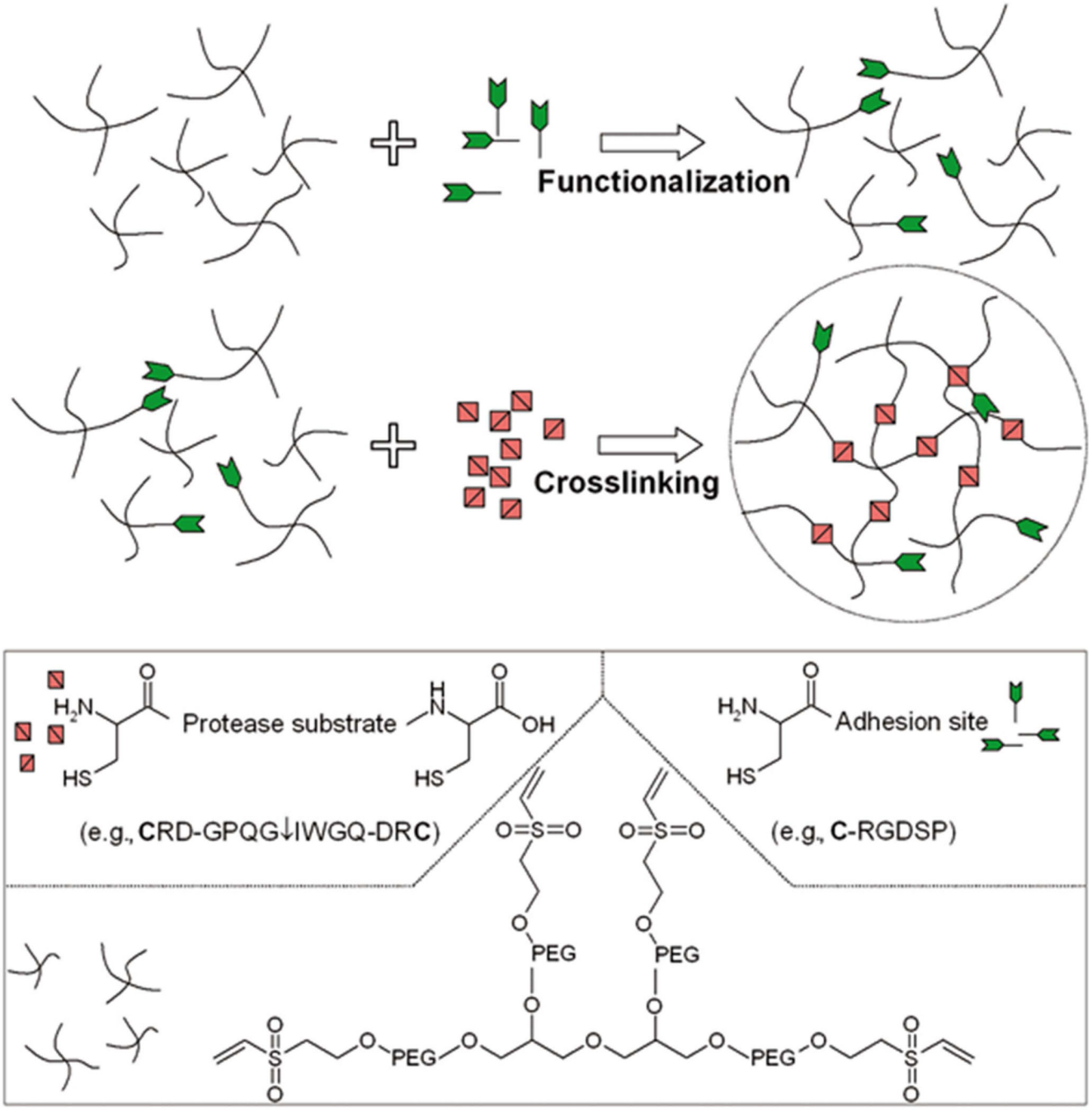

Fig. 5.

Scheme of cell-instructive hydrogel preparation using a selective conjugate addition approach. Adapted with permission from [126].Copyright 2003 Macmillan Publishers Ltd.

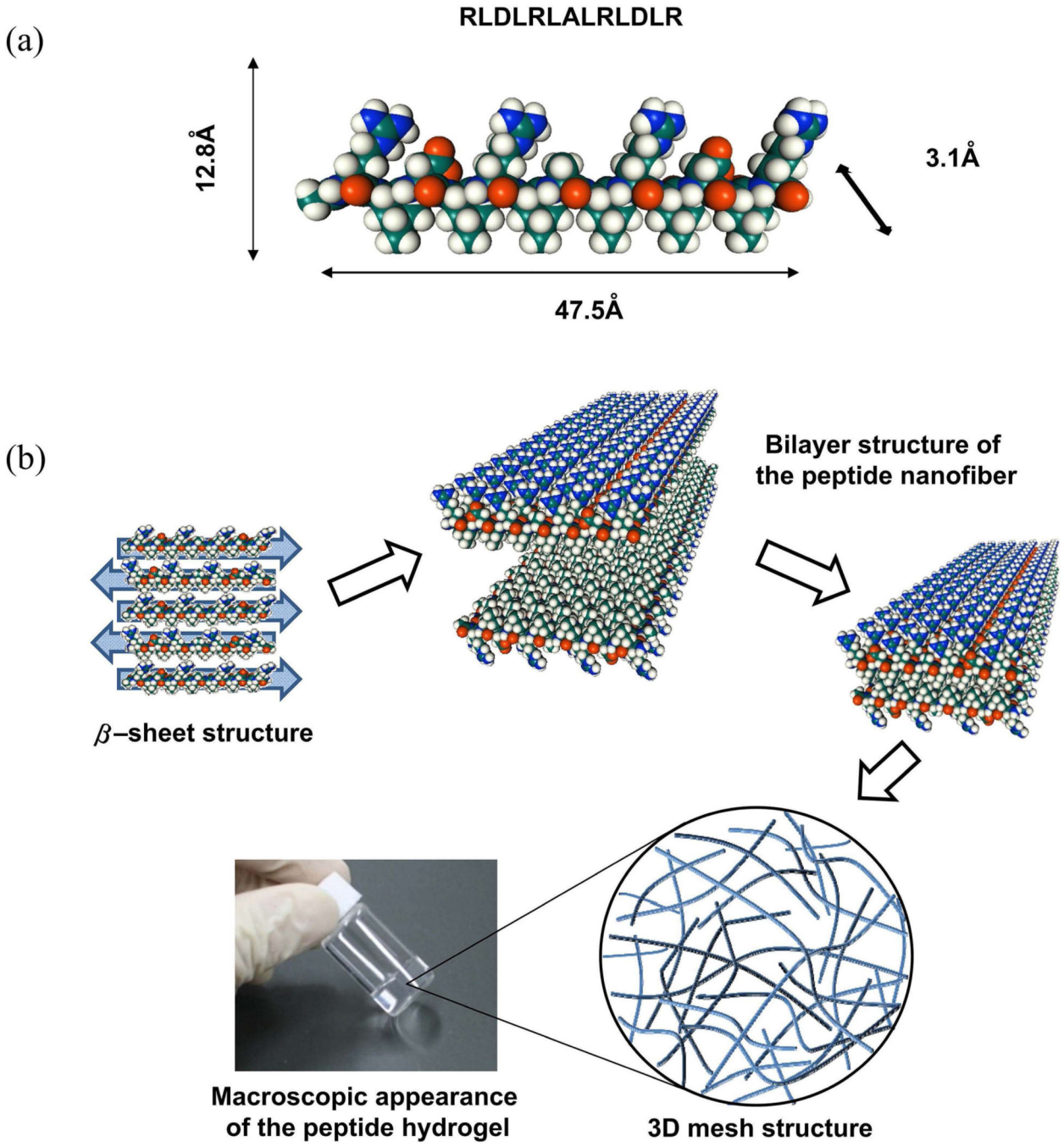

Fig. 6.

An example of self-assembling peptide hydrogels. (a) The molecular model of SPG-178 peptide, Facio was used: cyan, carbon; red, oxygen; blue, nitrogen; white, hydrogen. (b) A schematic diagram of the formation of the hydrogel from the peptide monomer. Adapted with permission from [153]. Copyright 2011 Elsevier Ltd.

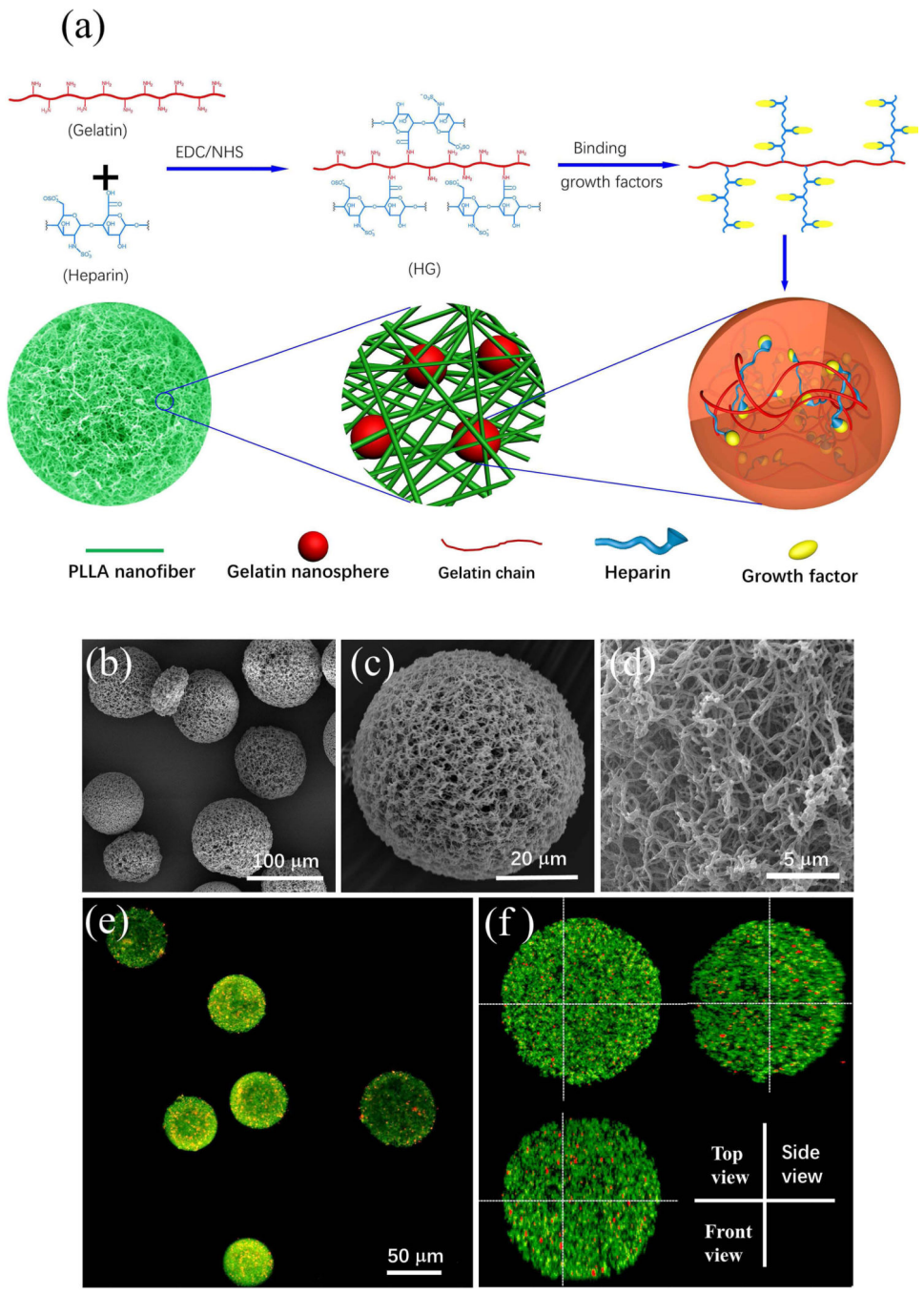

Fig. 7.

Development of hierarchical nanofibrous microspheres with controlled growth factor delivery for tissue regeneration. (a) Schematic illustration of the hierarchical microsphere structure. (b–f) Characterizations of the hierarchical growth factor-loading microspheres. To visualize the nanospheres and microspheres, TRITC-conjugated gelatin blended with heparin-modified gelatin was used and FITC-conjugated bovine serum albumin (BSA) was adsorbed on the nanofibers of the microspheres. Adapted with permission from [166]. Copyright 2015 WILEY-VCH Verlag GmbH & Co.

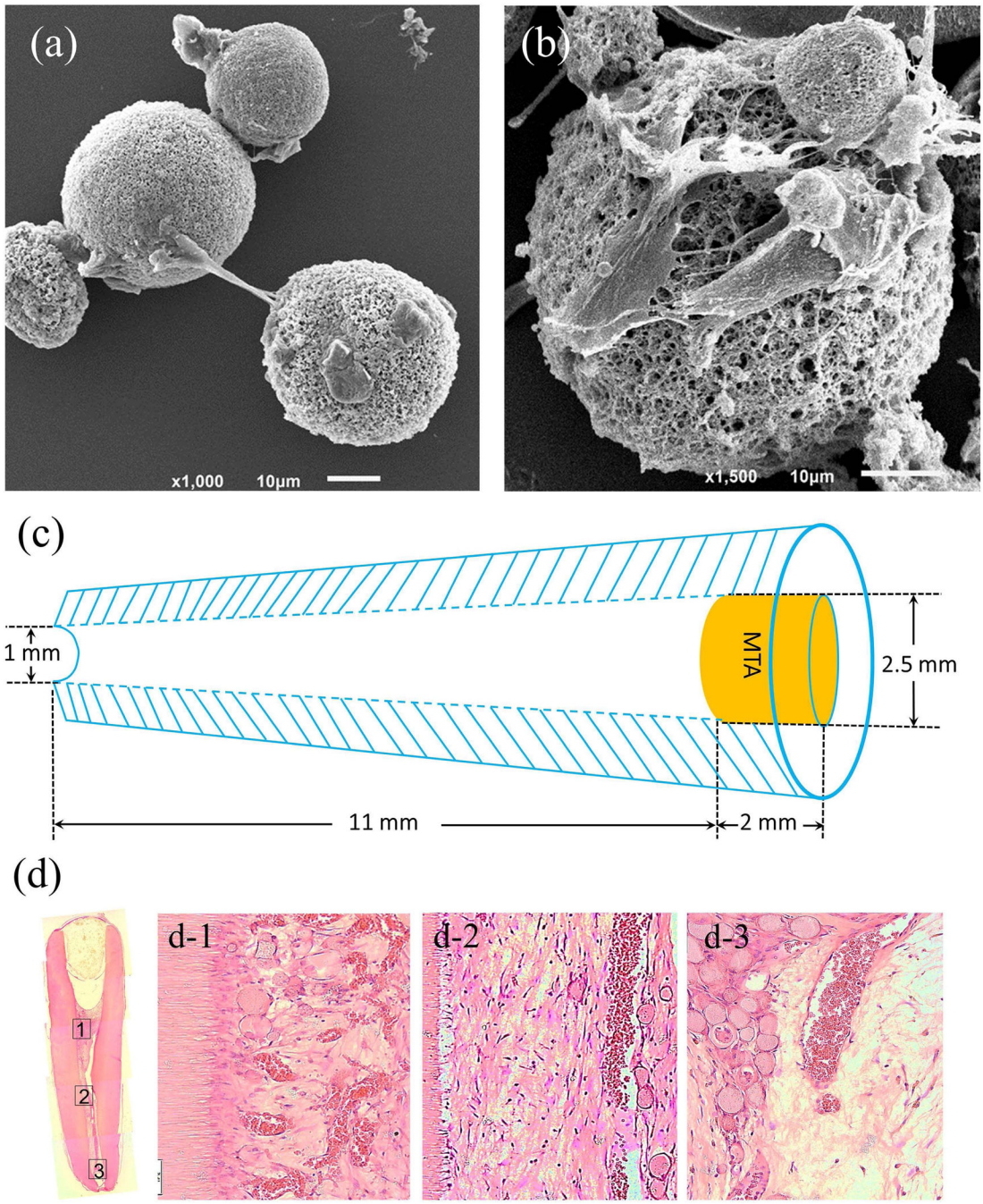

Fig. 8.

Regeneration of full-length pulp using hierarchical injectable microspheres as cell carriers. (a) Overview of an SEM image that shows the dental pulp stem cells (DPSCs) adhering to the surfaces of the hierarchical microspheres, expanded their processes and grasped the microspheres to form cell/microsphere aggregates. (b) A typical SEM image of the DPSCs adhered to a microsphere surface, showing the close interaction between the cell and the microsphere. (c) The full-length root canal model. (d) Regenerated pulp-like tissue fulfilled both the apical and middle third regions and reached the coronal third of the canal. A large

number of blood vessels were observed throughout the canals. In some regions, DPSCs differentiated into odontoblast-like cells and aligned with the existing tubular dentin of the root. Adapted with permission from [187]. Copyright 2016 Elsevier Ltd.

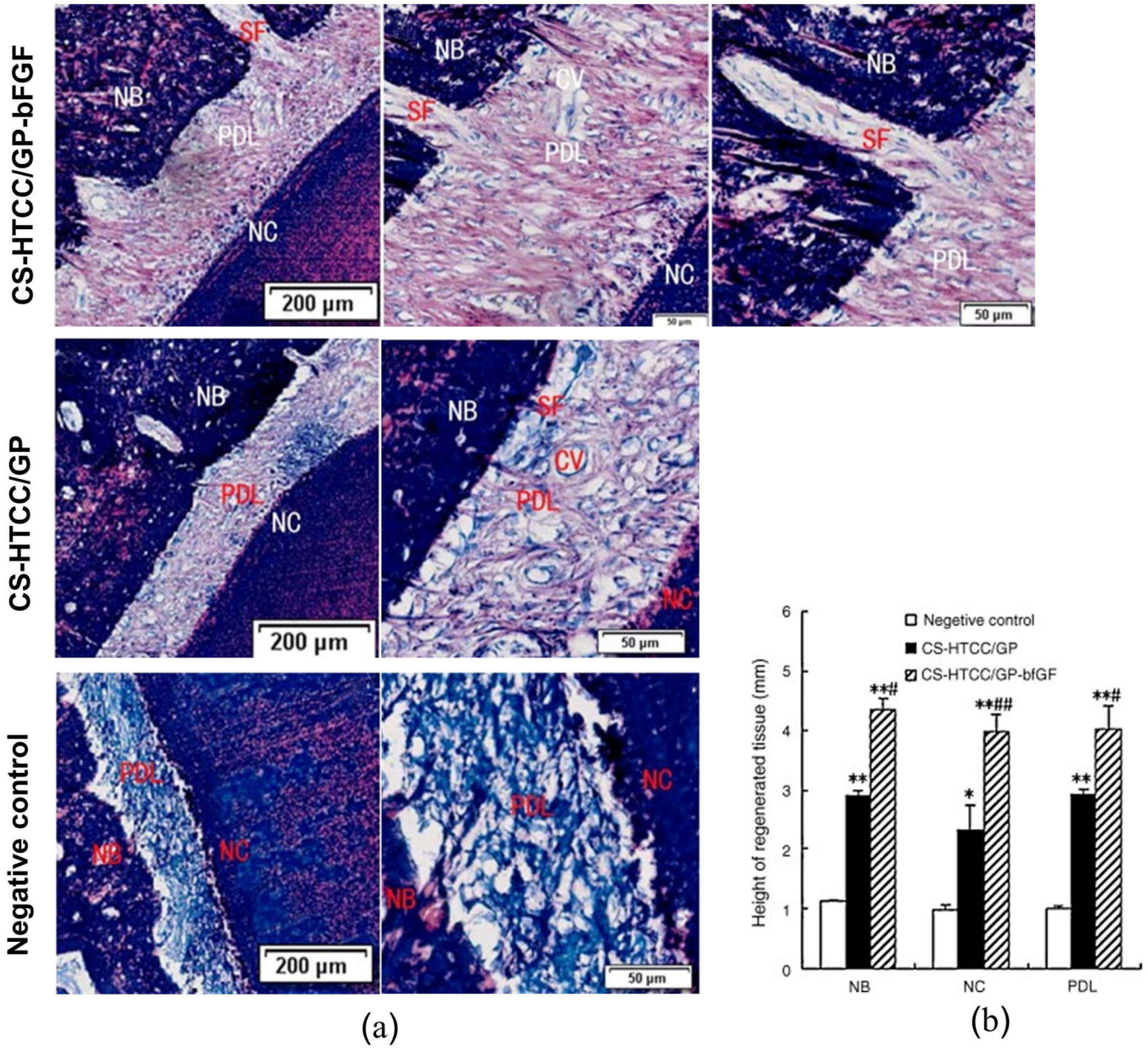

Fig. 9.

Periodontal tissue regeneration using chitosan-based injectable thermosensitive hydrogel (CS-HTCC/GP) in a dog periodontal defect model. (a) Histological examination of periodontal tissue regeneration in different groups (Mallory's trichrome staining methods). NB, new bone; NC, new cementum; PDL, new periodontal ligaments; SF, Sharpey's fibers; CV, capillary vessel. (b) Height comparision of regenerated periodontal tissues in three groups. Adapted with permission from [160]. Copyright 2010 Elsevier Ltd.

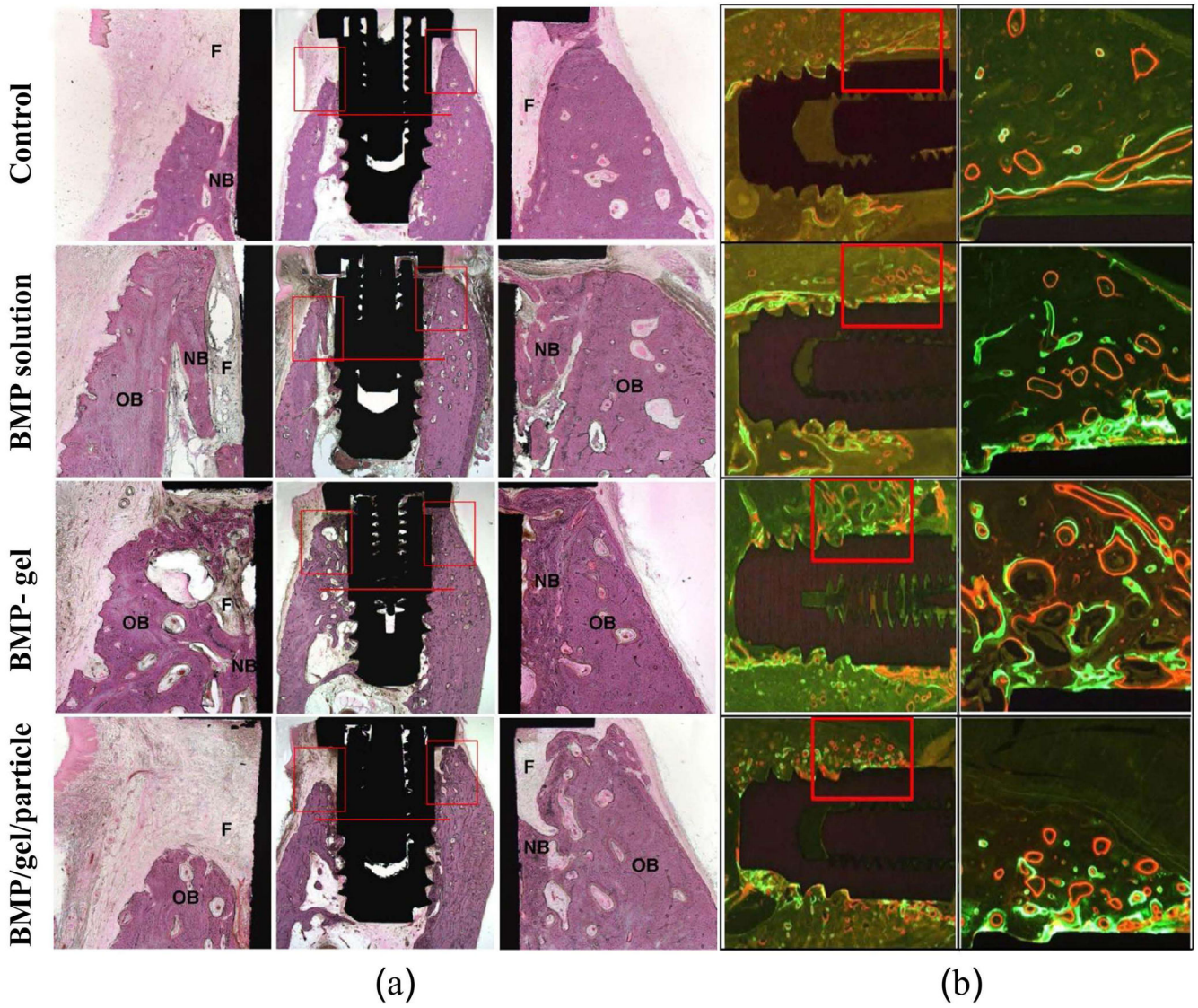

Fig. 10.

Bone regeneration in defect areas around dental implants. (a) Bone regeneration and integration evaluation from histologic images in each group. The red lines indicate the lower boundary of the defect area. F, fibrous tissue; NB, newly formed bone; OB, old bone. (b) Bone remodeling evaluation using fluorescent labelling. Green: calcein at week 2, and red:alizarin reds at week 6.Adapted with permission from [202]. Copyright 2015 IOP Publishing Ltd.

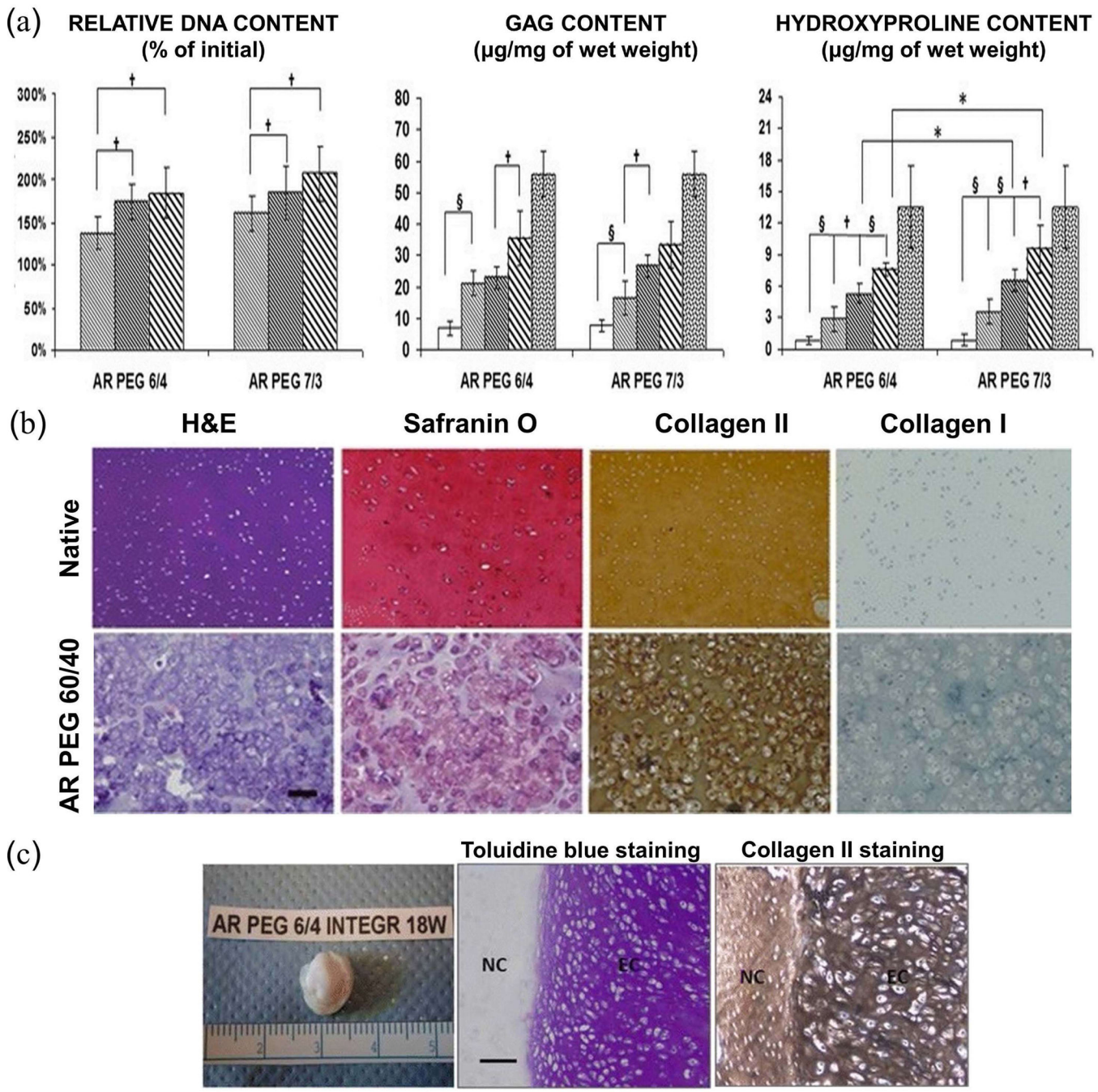

Fig. 11.

Articular cartilage generation applying PEG-LA-DM/PEGDM copolymer hydrogels. (a) Biochemical evaluation of PEG-LA-DM/PEGDM copolymer/chondrocyte constructs, including DNA content, GAG content and hydroxyproline content. (b) Histological and an immunohistochemical results from a 60/40 ratio of degradable/nondegradable PEG construct compared to native swine articular cartilage. (c) Macroscopic view, toluidine blue staining and collagen type II staining of the integration interface between the engineered cartilage

(EC) and native articular cartilage (NC) of 18 week 60/40 construct of ring model for integration study.Adapted from [215]. Open access. Copyright 2016 BioMed Central.