Abstract: Biodegradable and biocompatible hydrogels have many different applications in the field of biomedicine. The hydrogels could be utilized as drug carriers and cell scaffolds for drug delivery and tissue engineering. The hydrogels are appealing scaffolds because they are structurally similar to the extracellular matrix (ECM) of many tissues, which can often be delivered in a minimally invasive manner. A major issue in the use of a hydrogel scaffold is that the establishment of a biomimic environment is necessary for engineered tissue survival. In tissue engineering, there is significant interest in exploiting biocompatible conjugation to construct mimics of ECM for cells culture applications. Recently, a variety of crosslinking strategies have been developed for the fabrication of hydrogels, which are based on an affinity interaction, ionic interaction, electrostatic interaction, hydrophobic interaction, hydrogen bonding, crystallinity and covalent conjugation. This review will discuss recent advances in the field of biodegradable hydrogels crosslinked via biocompatible conjugation, including both physical and covalent crosslinking, which can be potentially used as drug and cell scaffolds in regenerative medicine applications.

Subjects: Biomaterials; Drug Targeting; Biomaterials & Medical Devices

Keywords: hydrogel; tissue engineering; biopolymer; drug delivery; regenerative medicine
1. Introduction

1.1. Hydrogels in regenerative medicine

Regenerative medicine, which combines tissue engineering and drug delivery, takes the multi-disciplinary principles of materials science, medicine, and life science to generate tissues and organs of better biological structures and functions (J. Chen & Zou, 2019; Dragan & Dinu, 2019). Regenerative medicine is to implant scaffolding materials for tissue regeneration based on the recruitment of native cells into the scaffold, and subsequent deposition of extracellular matrix (ECM) (Dragan & Dinu, 2019; Ong et al., 2019). Cell scaffolds provide the initial structural support and retain cells in the pores for cell growth, metabolism and matrix production, thus playing an important role during the development of engineered tissues (Ong et al., 2019; Razavi et al., 2019). A major issue in the use of scaffolds and tissue reconstruction procedures in general, is that the establishment of a biomimic environment into the engineered tissue is necessary for tissue survival (Nuttelman et al., 2008; Razavi et al., 2019).

Biodegradable hydrogels have attracted considerable attention as drug delivery systems and cell scaffolds for soft tissue engineering applications owing to their high biocompatibility and resemblance to biological tissues (H. Liu et al., 2019; Slaughter et al., 2009; Sui et al., 2008). Injectable hydrogels are promising substrates for tissue engineering applications due to high tissue-like water content, ability to homogeneously encapsulate cells, efficient mass transfer, easily manipulated physical properties and minimally invasive delivery (X. Chen et al., 2019; Nicodemus & Bryant, 2008). The hydrogel precursor loaded with growth factors (GFs) and/or targeted cells can be injected into the wound site and experiences a solution-to-gelation transition (sol–gel) in situ due to physical or chemical stimuli. Highly hydrated hydrogels can better mimic the chemical and physical environments of ECM and therefore are ideal cellular microenvironment for cell proliferation and differentiation.

Cellular growth is dependent on both intrinsic and extrinsic cues that can be provided by ECM. In vivo interaction between cells and ECM includes cells interacting with and responding to biochemical and biophysical signals like GFs and microstructures, directing the cell fate and tissue formation (Khan et al., 2009; Kretlow et al., 2007; Leach & Schmidt, 2005; Qian et al., 2019; Xing et al., 2019). In soft tissue engineering, adipose-derived stem cells (ASCs) have great potential in the field of regenerative medicine (Chiu et al., 2009; DeForest et al., 2009; Lutolf & Hubbell, 2003; Ren et al., 2018; Wieland et al., 2007). ASCs have been used in conjunction with various scaffolds and bioactive signaling molecules, such as GFs, to engineer human tissue. There is evidence that the ability of ASCs to grow and differentiate varies among physical structures and changes with bioactivities of scaffolds. Defining these variations in ASCs function and molecular mechanisms of adipogenesis will facilitate the development of cell-based therapies.

1.2. Biopolymers for hydrogels

A variety of naturally- and synthetically derived materials have been utilized to form hydrogels for tissue engineering applications (D.-A. Wang et al., 2007; Hudalla et al., 2011; Zhao et al., 2009). Natural polymers have been widely used as hydrogels for tissue engineering approaches due to excellent biocompatibility. Naturally derived hydrogel-forming polymers have frequently been used in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM (Prabhakaran et al., 2011; Zhao et al., 2009). However, hydrogels derived from natural polymers often undergo rapid degradation upon contact with body fluids or medium. Therefore, limitations of natural hydrogels have motivated approaches to modify these polymers as well as to utilize various synthetic polymers.

Synthetic biopolymers may potentially have suitable physical and chemical properties for tissue engineering applications. Synthetic polymers are appealing for hydrogels because their chemical and physical properties are typically more controllable and reproducible than those of natural polymers. Synthetic polymers can be reproducibly produced with specific block structures, molecular weights, and degradable linkages (Elisseeff et al., 2000; Kennedy et al., 2014; Khademhosseini & Peppas, 2013;
Rice et al., 2013; H. Tan, Ramirez et al., 2009; Van Vlierberghe et al., 2011). Particularly, the ability to control the crosslinking density provides the flexibility and tailorability to synthetic hydrogels for cell encapsulation and tissue growth. However, synthetic polymers may lack an informational structure for positive cell biological response. Compared to natural hydrogels, synthetic hydrogels offer improved control of the matrix architecture and chemical composition, but tend to have lower biological activity. As a consequence, the modification of synthetically derived hydrogels is usually required. An appealing and effective strategy is to incorporate bioactive species such as GFs, peptides and proteins into the material, resulting in biomimetic composite hydrogel scaffolds with bioactive functions for the optimal cellular response.

1.3. Hydrogel systems

Biocompatible hydrogels can be classified into physical and chemical (covalent) formulations, according to their gelation mechanism (Kirschner et al., 2014). The hydrogel network crosslinked by the physical association between polymeric chains or nanoparticles is the so-called physical gel, while the formation of a chemical gel takes place via covalent bonds between polymeric chains (Holland et al., 2004; Leach et al., 2004; Moffat & Marra, 2004). To develop a suitable hydrogel as a cell carrier, the degradation rate and mechanical properties of the hydrogel must complement the tissue growth and natural ECM. In general, these properties can be fine-tuned through variations in the chemical structure and crosslinking density in hydrogels. For a given hydrogel system, activities of seeded cells can be regulated by attaching specific bioactive moieties to the polymer matrix backbone. Comprised of various ECM-like macromolecules and proteins, hydrogels control the tissue structure, regulating the function of the cells (Cai et al., 2005; Seal & Panitch, 2006; Tae et al., 2006; Tan & Marra, 2010; C. Y. Xu et al., 2005, Yin et al., 2010). Remarkably, hydrogels prepared using a variety of biocompatible crosslinking methods exhibit tunable biochemical and biophysical properties, including proliferation, migration and differentiation. Over the past decade, many methods have been employed for the preparation of biodegradable and biocompatible hydrogels including physical and chemical (covalent) crosslinking. The reported biocompatible hydrogels systems by physical and covalent crosslinking are listed in Table 1.

1.4. Smart hydrogels

While these are promising steps towards the development of self-healing hydrogels, developing smart hydrogels as glucose-responsive scaffolds for adipose tissue engineering such as cell growth factor delivery still represents a challenge. For practical adipose regeneration, the challenge is to dynamically deliver the key adipogenic factors such as insulin and insulin-like growth factor-1 (IGF-1) in hydrogels

| Table 1. Biocompatible conjugation of hydrogels |
|------------------------------------------------|
| **Hydrogel Types** | **Gelation Mechanism** | **Biopolymers** |
| Physical hydrogels | Molecular recognition | Natural/synthetic biopolymers |
|                    | Hydrogen bonding      | Polysaccharides/synthetic biopolymers |
|                    | Electrostatic interaction | Polysaccharides |
|                    | Ionic crosslinking    | Alginate |
|                    | Hydrophobic interaction | Synthetic biopolymers |
| Covalent hydrogels | Michael-type addition | Natural/synthetic biopolymers |
|                    | Schiff base reaction  | Polysaccharides/synthetic biopolymers |
|                    | Boronate ester crosslinking | Polysaccharides |
|                    | Diels-Alder addition  | Polysaccharides/synthetic biopolymers |
|                    | Click chemistry       | Natural/synthetic biopolymers |
|                    | Enzymatic reaction    | Protein/peptides |
| Multiple hydrogels | Physical/covalent crosslinking | Natural/synthetic biopolymers |
to induce adipogenesis (Alhadlaq et al., 2005; T. Yang et al., 2014). Unlike conventional drug delivery, insulin delivery systems require a glucose-sensing ability to specifically interact with glucose and sense its levels (Luo et al., 2009; Miyata et al., 2004). Steady release of insulin in a normal glucose environment is not desirable because it could cause hypoglycemia in the patient when the blood glucose level is decreased (Tanna et al., 2006). In particular, glucose-responsive hydrogels possess special properties in response to external stimuli, which renders these hydrogels advanced compared to typical systems for self-regulating delivery (S.-Y. Cheng et al., 2006; Ravaine et al., 2008). There is a need for self-regulated gel delivery systems having the capability of adopting the rate of insulin release in response to changes in glucose concentration in order to keep the blood glucose levels within the normal range. In order to achieve dynamic GFs release, smart hydrogels to sense the change in the blood glucose concentration is required when glucose concentration increases. Several approaches have been utilized for self-regulated insulin delivery designs as rate-control mechanisms including enzyme–substrate reactions, competitive binding and pH-sensitive polymers (M. Fan et al., 2017; Otto & Lane, 2005; Ravaine et al., 2008). These systems showed a differential delivery of insulin in response to glucose with in vitro diffusion experiments. Recently, benefits of dynamic covalent bonds for tissue development have stimulated efforts to design new emerging smart hydrogels exhibiting moldable and self-healing characteristics (S.-Y. Cheng et al., 2006; Miyata et al., 2004; Tanna et al., 2006). Such systems are of particular interest for clinical use as they can not only manage external damages and repair themselves as self-healing materials but also exhibit multi-responsive properties to environmental stimuli (M. Fan et al., 2017; Otto & Lane, 2005; Ravaine et al., 2008; Rubin et al., 2009).

2. Physical crosslinking strategies
Noncovalent assembly of polymeric materials via specific molecular recognition interactions such as protein–protein, peptide–peptide, and ionic crosslinking interactions has become increasingly prominent in the production of responsive, reversible and injectable hydrogels (Vermonden et al., 2012; Q. Wang et al., 2007; J. Zhang et al., 2010). In many of the natural hydrogels, e.g., collagen and fibrin glue, the physical and ionic crosslinking mechanisms are difficult to control, which limits the final network structure and properties. A variety of assembly has been developed for the fabrication of injectable hydrogels, which are based on affinity interactions, ionic interactions, electrostatic interactions, inclusion complex, hydrophobic interactions, hydrogen bonding and crystallinity (Estroff & Hamilton, 2004; Jayawarna et al., 2006; Mahler et al., 2006; Schenning & Meijer, 2005; Sekine et al., 2011; Smith et al., 2008; Z. Yang et al., 2007).

2.1. Affinity interaction
The ability of heparin and related glycosaminoglycans (GAGs) to sequester and stabilize GFs has been exploited in the production of hydrogels that can mediate cell proliferation and differentiation (Beahm et al., 2003; Capila & Linhardt, 2002; Chinen et al., 2003; Grande et al., 2001; Jin et al., 2010; Shu et al., 2002; H. Tan, Zhou et al., 2012; Wissink et al., 2001; Yamaguchi & Kiick, 2005; Yamaguchi et al., 2007). Recently, low molecular weight heparin (LMWH) and heparin interacting protein (HIP) or vascular endothelial growth factor (VEGF) have been transiently immobilized in a star poly(ethylene glycol) (PEG) matrix via affinity interaction between the HIP/VEGF and covalently incorporated heparin (Capila & Linhardt, 2002; Grande et al., 2001). The initial studies suggest that the star PEG-heparin copolymer and its incorporation into hydrogels are capable of VEGF delivery. Therefore, the synthesis of new polysaccharide hydrogels conjugate via specific heparin-peptide binding affinity and that might therefore permit manipulation of inductive properties for ASCs differentiation and adipogenesis. We developed a hydrogel producing, via the interaction of LMWH modified hyaluronic acid (HA) and peptide CRPKAKAKAKAKDQTK, a GF sequence derived from the heparin-binding domain of HIP (H. Tan, Zhou et al., 2012). This hydrogel precursor was designed with LMWH on an HA backbone, which was accessible for conjugating with HIP in an aqueous environment (Figure 1a). The LMWH-functionalized HA (HA-LMWH) was employed via Michael addition of thiol-functionalized HA to maleimide-functionalized LMWH. The HIP-functionalized PEG (PEG-HIP) was synthesized via Michael addition of vinyl sulfone terminated four-arm PEG to HIP, which contains flexible star PEG grafts with HIP located at the PEG termini. The terminal LMWH of HA-LMWH was easily accessible to HIP in the aqueous solution, thus enabling affinity
interactions at the hydrophilic PEG termini. Hydrogels were formed via the mixing of homogeneous, low viscosity solutions of HA-LMWH and PEG-HIP (Figure 1b). Cell culture studies demonstrated that the hydrogels were bioactive and preserved the proliferation of the entrapped ASCs. After 14 days incubation, as the importance evidence for ASCs differentiation, the hydrogel was found to cause a statistically significant increase in lipid accumulation (Figure 1c). Roundly shaped ASCs were residing in the hydrogel after 24 h incubation, which possessed normal spherical morphologies, indicative of the highly bioactive nature of the gel (Figure 1d). Furthermore, SEM images also demonstrated that lots of lipid accumulated on the ASCs (Figure 1e). Coupled with the cell proliferation data, this HIP-mediated hydrogel induced differentiation of ASCs, which suggested a novel potential mechanism for targeted differentiation of stem cells via the therapeutic affinity interaction cross-links.

2.2. Nucleobase pairing
Nucleobase functionalized biopolymers could assemble into biomaterials through specific nucleobase pairing (M. Fan, Zhang et al., 2015; Manna et al., 2009; Van de Manakker et al., 2009; Q. Wang et al., 2007). These successful results clearly illustrate that specific nucleobase pairing promise as scaffolding materials for cell cultures and tissue engineering. More recently, a biological hydrogel was self-assembled via the Watson-Crick base pairing of thymine- and adenine- functionalized star PEG (H. Tan, Xiao et al., 2012). This work should bring up a novel methodology to generate robust injectable scaffolds with tailorable properties for biomedical applications. The biological self-assembly PEG hydrogel system is established by the Watson-Crick base pairing between thymine (T) and adenine (A) via the hydrogen bonding (Figure 2a). Compared with linear PEG, multi-arm star-shaped PEG has the nature to induce stereo-complex formation, which shows promise in biomedically relevant hydrogel systems (M. Fan, Yan, Tan, Miao et al., 2014; H. Tan, Xiao et al., 2012). Firstly, thiol thymine (T-SH) and thiol adenine (A-SH) were synthesized, respectively. Maleimide terminated four-arm PEG (PEG-Mal) was functionalized
with either T-SH or A-SH functionalities as self-assembly precursors via the Michael-type addition.
After dissolution and a mixture of precursors in an aqueous environment, a stereo-PEG hydrogel network was self-assembled due to the formation of pairing complexes. Remarkably, incubation temperature has a significant influence on the swelling of this self-assembly PEG hydrogels (Figure 2b & c). Compared to the 20°C and 37°C, the swelling ratio of hydrogel significantly increased after 24 h incubation at 50°C. Insulin-loaded hydrogels caused a significant increase in ASCs proliferation in vitro after 7 days of incubation. The attached ASCs were present in the superficial area of the biological hydrogel and maintained their polygonal morphologies with size as ~20 μm (Figure 2d). Viable cells were observed after 7 day of culture, and more than 98% of the ASCs survived. Elliptical or round-shaped cells were uniformly distributed in the hydrogel (Figure 2e), which is an indicator of phenotype retention of ASCs and is essential for matrix formation. Spherical-shaped ASCs were distributed in the scaffold, indicative of the biological nature of the hydrogel for cell survival. These unique characteristics of this biological hydrogel make it a promising candidate as an injectable scaffold for pharmaceutical and biomedical applications.

Furthermore, this flexible way to assemble a polysaccharide hydrogel has been presented via the specific pairing of functionalized nucleobases that are capable of inducing osteogenesis (M. Fan, Yan, Tan, Ben et al., 2014; M. Fan, Yan, Tan, Miao et al., 2014). The strategy is to use opposite charged polysaccharide derivatives, e.g., quaternized cellulose (QC) and heparin, as gel scaffold precursors, which could be additionally crosslinked by extra electrostatic interactions. Comparing to unmodified cellulose, QC has positive charges and been applied in medical application due to the better hydrophilicity, biodegradability, biocompatibility and antibacterial properties. The potential application of this gel scaffold in bone tissue engineering was confirmed by the encapsulation behavior of osteoblasts (M. Fan, Yan, Tan, Ben et al., 2014). In combination with cell growth factor, e.g., bone morphogenetic protein (BMP-2), the gel scaffold exhibited beneficial effects on osteoblast activity and differentiation, which suggested a promising future for local treatment of pathologies involving bone loss.

2.3. Drug crosslinking

Drug-crosslinking hydrogels have been developed on the basis of drug–target interactions. The hydrogels are based on the specific dimerization of the protein gyrase B (GyrB) by the aminocoumarin antibiotic coumermycin and the subsequent dissociation of the proteins upon the addition of novobiocin (Betre et al., 2006; Hildner et al., 2010; Kemmis et al., 2010). This stimulus inducible dissolution
can be used to adjust the release rate of a previously embedded GFs by varying the novobiocin concentration in the medium. For example, to exploit adipose-derived stem cells (ASCs) as a new resource for cartilage repair and guide implementation of therapy, significant research endeavors have been devoted to prepare biopolymer-based platforms capable of inducing ASCs in the chondrogenetic differentiation (Diekman et al., 2010; Ino et al., 2008; Perea et al., 2006; Shimizu et al., 2007). Most biomaterials currently used to trigger chondrogenesis of ASCs share GFs such as BMP and transforming growth factor-beta (TGF-β) as a prerequisite for chondrogenetic differentiation in scaffolds (Akiyama et al., 2010; Ehrbar et al., 2008). It is a difficult challenge to create a functional platform from stem cells to in vitro build an organized cellular construct that would permanently stimulate chondrogenetic differentiation and native cartilage regeneration. Recent studies have demonstrated the cell aggregation undergoing a cell condensation step as a prerequisite for commitment to the chondrogenic lineage in vitro (Akiyama et al., 2010; Alexander, 2008; Ehrbar et al., 2008). However, ASCs may be potentially impaired during differentiation by a mechanical stimulus such as magnetic forces. Innovative approaches are thus needed to drive 3D cellular organization by using biotechnologies in vitro. Compared to magnetic forces, biological stimuli may offer a promising alternative for shaping the multi-cellular organization, having the major advantage of biomimetic inducement.

In order to produce cellular aggregates for chondrogenetic differentiation, ASCs have been confined in three dimensions by using home-designed biological stimulus. Different from the existing cell aggregation technologies, our group pioneered a biological cell-affinity stimulus, by which a hydrogel substrate was utilized to induce cell aggregation. The idea is to couple cell-specific biopolymer brush onto the hydrogel substrate synchronously providing a reservoir of GFs on ASCs chondrogenetic differentiation (Figure 3a). This approach integrates a BMP-2 delivery system and a substrate able to induce cell aggregation in the same 3D hydrogel scaffold (H. Tan et al., 2013). For the coupling aggregation agent brush, we strategically introduced covalently linked GyrB. This coupling strategy is based on the specific dimerization of the GyrB by the aminocoumarin antibiotic coumermycin and the subsequent dissociation of the proteins upon the addition of novobiocin. Through dimerized by coumermycin and dissociated by novobiocin,

Figure 3. (a) Conceptual illustration of a hydrogel-based substrate capable of not only inducing ASCs aggregation with superb efficiency, but also harvesting these cellular aggregates. In order to confer cellular aggregation induction onto hydrogel, galactosylated chitosan (GC) brushes are coupled onto hyaluronic acid (HA) hydrogel through coumermycin-mediated GyrB dimerization. Via a competitive displacement of novobiocin, GC can be released by the dissociation of dimerization, enabling highly efficient cellular aggregation harvest from the substrate. (b) Release kinetic of cellular aggregation as a function of time. (d-e) CLSM images of the ASCs on gel-substrates containing BMP-2 before and after GC dissociation at 24 h.
the aggregation agent can be introduced onto and subsequently dissociated apart from the gel-substrates, enabling a highly efficient cellular aggregation and easy harvest.

Galactosylated chitosan (GC), the cell aggregation agent, is a lactose derivative of a highly deacetylated chitosan, which can stimulate the biosynthesis of chondro-specific ECM (Donati et al., 2005; H. Tan, Wu et al., 2010). Previous experience demonstrated the efficacy of GC on inducing 3D cellular aggregates with the high-level of chondrocyte proliferation, viability and GAG secretion. To study the performance of cellular aggregation, a cell suspension containing ASCs was introduced onto the GC coupled gel-substrate. The GC coupled substrate containing BMP-2 displayed the highest cellular aggregation and the lowest single-cell retention. We hypothesize that the cellular aggregates could be harvested as a result of the dissociation of GC from the gel-substrates. To validate this hypothesis successively, a release study of cellular aggregates was carried out by the addition of novobiocin (0.5 mM). A gradual aggregate release can be observed as a function of culture time (Figure 3b). Cytoviability of the harvested cellular aggregates was significantly higher than that of the cells on the BMP-2 conjugated substrate without GC for a 24 h culture. The induced cellular aggregates by the bio-functionalized substrate can significantly improve the viability performance, with the very distinct morphologies of cellular aggregates. Normally, the cells cultured on HA hydrogels remained spherical and isolated, indicative of the highly hydrophilic nature of the HA. The morphology change was particularly evident when the cells were cultured on the GC coupled substrate containing BMP-2 (Figure 3c). After being cultured for 24 h, the adherent cells aggregated to form nodules of various sizes on the GC coupled substrate containing BMP-2. Furthermore, cellular aggregates could be easily harvested from the substrate by rinsing using PBS with novobiocin (Figure 3d). It is conceivable that the incorporation of GFs and cell aggregation induced agent improved the cell–material interactions. This approach demonstrates the potential of ASCs aggregates for building large cartilage substitutes of defined geometry and high tissue integration.

2.4. Biotin-Avidin conjugation

In soft tissue reconstruction, ASCs can secrete multiple angiogenic growth factors, including VEGF, that contribute to enhanced viabilities of encapsulated cells and engineered tissue (H.-H. Jung et al., 2010; Mao et al., 2007; Singh et al., 2009). Delivering ASCs with VEGF in a biodegradable hydrogel scaffold serves as an alternative approach and may hold greater therapeutic value in terms of engineered tissue survival. While the versatility of injectable scaffolds has been broadly exploited, conventional scaffolds are limited by the intrinsic inability to translate VEGF into soft tissue engineering and instability of the VEGF delivery for ASCs proliferation and differentiation (Aksu et al., 2008; Mao et al., 2007; Singh et al., 2009). Biological conjugation of biotin-avidin in polymeric materials has become increasingly prominent in the production of biomaterials (Aksu et al., 2008; Mao et al., 2007).

The synthesis of a multifunctional nanofibrous hydrogel integrated with the VEGF-entrapped polysaccharide nanoparticles has been reported that are capable of inducing ASCs proliferation and differentiation for adipogenesis in vitro. The novel hydrogel network was conjugated via the biological interaction of biotin terminated four-arm PEG (PEG-Biotin) and streptavidin-functionalized HA (HA-Streptavidin) (H. Tan, Shen et al., 2012). Furthermore, transfer of VEGF via a polysaccharide nanoparticle system assemble in the physiological environment can be a valuable prophylactic option (H. Chen et al., 2016; Saito & Tabata, 2012; De Souza et al., 2009; X. Xu et al., 2011; Zieris et al., 2011). Delivering the VEGF-entrapped polysaccharide nanoparticles in a hydrogel scaffold can serve as an alternative approach and may hold greater therapeutic value in terms of engineered tissue survival due to its unique delivery system. Therefore, the polysaccharide nanoparticles can be assembled via the electrostatic interaction between N,N,N-trimethylchitosan chloride (TMC) and LMWH. VEGF was entrapped in LMWH/TMC nanoparticles via the affinity interaction associated with the LMWH (Figure 4a). TMC, a cationization of a water-soluble chitosan derivative, is employed as a positive polyelectrolyte for electrostatic assembly with the negative LMWH (Figure 4c). TMC has a higher solubility than chitosan over a broader pH range, and is
capable of opening tight junctions of cells at physiological pH, which leads to an increase of paracellular permeability (Mao et al., 2007). As a kind of promising gene delivery vector, TMC has been employed to improve the carrier property on gene transfection. In this study, the quaternization degree plays an important role in assembly efficiency, and a higher quaternization results in a higher VEGF entrapment efficiency. These results are important to guide the molecular design and the optimization of chitosan family for pharmaceuticals delivery. The VEGF-loaded LMWH/TMC nanoparticles (VEGF-LMWH/TMC) were distributed homogeneously in the nanofibrous hydrogel (Figure 4e). After 14 days of incubation, provide images of the VEGF-particle/gel showed significant lipid accumulation on some ASCs as the important evidence for ASCs differentiation (Figure 4f, g). These results demonstrated that the multifunctional nano-hybrid VEGF-particle/gel was bioactive and preserved the proliferation and differentiation of the encapsulated ASCs.

3. Covalent crosslinking strategies
Covalent crosslinked hydrogels offer many advantages such as controllable crosslinking density and structure properties. Particularly, the ability to control the crosslinking density provides the flexibility to design a wide range of polymeric networks for cell encapsulation and tissue growth. Conventionally, biodegradable hydrogels have been synthesized by chemical crosslink methods such as crosslinking agents (e.g., glutaraldehyde, genipin, adipic dihydrazide and bis-sulfosuccinimidyl suberate),
3.1. Schiff-base reaction

Recent studies have identified that the Schiff-base reaction can be utilized to crosslink functional amine and aldehyde groups and present in natural biomaterials with non-cytotoxic effects compared to studies performed with commonly used chemical crosslinkers (H. Tan, Chu et al., 2009; H. Tan, Rubin et al., 2010). Our laboratory has examined the utilization of the Schiff base reaction to crosslink natural polymers to form biodegradable hydrogels that have the potential to produce novel scaffolds for soft tissue engineering applications (D-A. Wang et al., 2007; Ito et al., 2007; Maia et al., 2005). The Schiff base coupling chemistry has the advantage that it introduces no potentially cytotoxic groups into the gels formed and can create a more biomimetic microenvironment for cell survival, rendering them more suitable for potential in vivo applications. The carbon–carbon bonds of the cis-diol groups in the molecular chain of polysaccharides can be cleaved to generate reactive aldehyde functions by periodate oxidation, which can develop chemical crosslinking action with amino functions via the Schiff base linkage. Several polysaccharides such as hyaluronic acid (HA), alginate, dextran, gum arabic and chondroitin sulfate can be partially oxidized and employed for Schiff base linkage (D-A. Wang et al., 2007; Ito et al., 2007; Maia et al., 2005; Martino et al., 2005).

For example, the utilization of Schiff base reaction to crosslink natural biocompatible polymers, such as chitosan and HA, to form biodegradable hydrogels has the potential to produce cell scaffolds for tissue engineering applications (H. Tan, Chu et al., 2009; H. Tan, Rubin et al., 2010). The gelation is attributed to the Schiff base reaction between amino groups of N-succinyl-chitosan and aldehyde groups of oxidized HA (Figure 7a). Since the microstructure and high water content are very similar to that of the ECM of natural cartilage, the chitosan-HA hydrogels may preserve the phenotype of chondrocytes. The cells that were encapsulated within the composite chitosan-HA hydrogel possessed normal spherical morphology, as per that in normal cartilage, predicting a potential application of the hydrogel as an injectable scaffold in cartilage tissue engineering. Elliptical or round-shaped chondrocytes were uniformly distributed in the hydrogel. The 3D calculation result demonstrated that more than 93% of the encapsulated chondrocytes survived (Figure 7b). However, further optimization of the system is required to promote cell proliferation and ECM production in addition to the maintenance of their phenotype.

Self-healing hydrogels have the potential to broad pharmaceutical applications via the Schiff base reaction. The traditional hydrogels come across some challenges that will limit its application, such as suffering mechanical forces after injection that deform or even damage them. The key point to maintain both the structural and functional integrity is to give hydrogels the ability of self-healing (Burattini et al., 2010; Cong et al., 2013; Li et al., 2015; Y. Zhang et al., 2012). Different from traditional hydrogels, self-healing hydrogels possess a self-repair capability after damage and can be used as injectable materials (Cho & Ooya, 2018; Deng et al., 2012; Ding et al., 2015; Gaina et al., 2013; Pepels et al., 2013; Wei et al., 2016). Self-healing hydrogels could arrive at the spot and overcome fracture during the delivery during injection in vivo, then enhance the medicine delivery efficiency and improve the therapeutic effect. Herein, we put forward the Schiff base reaction to broaden the biopolymer-based self-healing hydrogel system for drug delivery and tissue engineering. The self-healing biopolymer hydrogel is presented by the crosslinking of aldehyde groups from oxidized alginate (OAlg) and amino groups from water-soluble carboxyethyl chitosan (CEC). The compressive and rheological tests exhibited that the composite hydrogels had good self-healing properties. Digital pictures were directly demonstrated the self-healing ability of hydrogels (Figure 7c). We prepared two identical hydrogels, used Rhodamine to stain one of them, put them together, and allowed them to self-heal at room temperature. After 2 h, the healed hydrogel was solid enough to overcome the stretching force from the outside and maintained the self-integrity undamaged matrix. The dynamic reversible covalent
bonding made them lightly crosslink together, especially on the surface of the hydrogels, and resulted in the excellent self-healing ability.

Despite extensive studies of pharmaceutical usage in vitro, the use of a glucose-responsive hydrogel as an injectable cell scaffold for tissue engineering has received limited attention (S.-Y. Cheng et al., 2006; Tan & Hu, 2012; Tanna et al., 2006). A biodegradable and glucose-responsive dextran hydrogel system immobilized with concanavalin A (ConA) has been designed via a Schiff base cross-linking reaction for insulin delivery (Figure 7d). The formation of glucose-sensitive gel is attributed to the Schiff base reaction between amino and aldehyde groups of dextran derivatives, respectively (Tan & Hu, 2012). The morphologies and compressive modulus of the freeze-dried hydrogels demonstrated that the incorporated ConA results in the formation of a tighter network structure in hydrogels due to a physical lectin-saccharide interactions by free glucose of the dextran from the lectin receptor sites. The immobilized ConA as an additional cross-linker progressively dissociated from the gel upon adding glucose, and results in a decrease cross-linking density. The preliminary results indicate that the insulin would be released from this hydrogel device into the local microenvironment in response to glucose by the swelling of hydrogel network.

3.2. Diels-alder addition
The Diels-Alder reaction in aqueous environments, which involves a highly selective [4 + 2] cycloaddition reaction between a diene and a dienophile, is diverse in scope and efficient in reactivity, results in very high yields, produces no byproducts, and occurs under mild reaction conditions (Dantas De Araujo et al., 2006; Jia et al., 2015; Sun et al., 2006; H. Tan et al., 2011; Tiwari & Kumar, 2006). The compatibility of aqueous Diels-Alder chemistry with biomolecules has been exploited elegantly in the bioconjugation of protein, peptides and oligonucleotides, which were site-specific without the interference of the many functional groups present in the polysaccharide backbone (Hill et al., 2001; Kim et al., 2005; Shi et al., 2009, 2007; Tona & Häner, 2005). Given the high specificity and efficiency, the success of aqueous Diels-Alder chemistry in biomolecule conjugation and immobilization may be extended to the synthesis of biodegradable hydrogels. The aqueous Diels-Alder cycloaddition reaction was used as a new methodology to conjugate a polysaccharide hydrogel, which provides a competitive alternative to conventional methodologies to prepare biodegradable hydrogels. For example, a polysaccharide derivative hydrogel with novel structures has been developed via aqueous Diels-Alder cycloaddition reaction of bio-conjugation that specifically allows for biopharmaceutical delivery (H. Tan et al., 2011). Hydrogel precursors were designed with furan groups (diene) on the outer PEG, corona that are accessible for reaction with maleimide (dienophile) functionalized HA derivatives in an aqueous environment at 37°C (Figure 6a).

For construction of maleimide-functionalized polysaccharides, sodium HA was oxidized by sodium periodate, and the carbon-carbon bonds of the cis-diol groups in molecular chains are cleaved and generate reactive aldehyde functions that conjugate with the maleimide containing molecule via Schiff-base linkage. The furan-functionalized HA derivative contains a biodegradable backbone of polysaccharide and hydrophilic grafts of PEG with furan groups located at the PEG termini. The terminal furan groups are easily accessible to maleimide-functionalized HA derivative in the aqueous solution, thus enabling cycloaddition at the PEG termini. The Diels-Alder HA gel underwent significant volume changes with temperature, with the gel swelling when the temperature was increased from 20°C to 80°C. A cytocompatibility study showed the formed hydrogels were non-cytotoxic and preserved the viability of the entrapped cells. Roundly shaped ASCs were distributed on the insulin-loaded hydrogels, and migrated into the gels, indicative of the highly hydrophilic nature of the gels (Figure 5c). The ASCs residing in the pure and insulin loaded hydrogels were further observed after 5 days culture and possessed normal spherical morphologies (Figure 5d). This Diels-Alder hydrogel derived from natural polysaccharide derivatives can be used as a potential delivery system as it resembles the extracellular matrices of tissues comprised of GAGs and can create a more biomimetic microenvironment with excellent biocompatibility.
3.3. Boronate ester crosslinking

While retaining the benefits of reversibility, the high strength of dynamic covalent bonds as compared to supramolecular interactions such as host-guest interactions leads to the increased structural stability of polymer assemblies (Cambre & Sumerlin, 2011; S.-Y. Cheng et al., 2006; Cheng & Jäkle, 2011; Dong et al., 2016; Guan & Zhang, 2013; Roberts et al., 2008; B. Wang et al., 2010). A particularly interesting type of dynamic covalent bond for the fabrication of hydrogels dedicated for biomedical applications is the boronic ester bond based on the boronate–diol interaction by the reaction of derivatives of phenylboronic acid (PBA) with 1,2- and 1,3-diols (Baldwin & Kiick, 2013; Collins et al., 2017; Jay et al., 2011). For example, Tarus et al. described an original and flexible approach to form dynamic covalent hydrogels based on biocompatible...
polysaccharides crosslinked with boronic acid-carbohydrate ester bonds at physiological pH. They demonstrated the ability to control the pH at which the sol–gel transition occurs depending on the anionic nature of the polymer backbone.

Development of dynamically released nanospheres containing IGF-1 from hydrogels with high bioactivity would greatly broaden the application for adipose tissue regeneration. The use of a glucose-responsive heparin hydrogel has been reported to dynamically deliver IGF-1 contained heparin nanospheres via the boronate–diol interaction. The method for preparing heparin gel scaffolds relies on in situ cross-linking of heparin with built-in boronate groups and maltose moieties (Ren et al., 2008). The gel scaffold was faciely prepared in physiological conditions by the formation of boronate-maltose ester crosslinks between boronate and maltose groups of heparin derivatives (Figure 6a). Heparin nanospheres exhibit residual functional maltose that can be used as reactive handles for subsequently dynamic conjugation of boronate–maltose hydrogels. Exposure of the hydrogel to glucose induces maltose functionalized heparin nanospheres dissociation off the gel network and thereby could dynamically move into the microenvironment.

The glucose-dependent viscoelastic properties of boronate–maltose hydrogels are useful for the development of smart IGF-1 carriers with physiologically responsive structural transformations. Dynamically controlled release of IGF-1 can be achieved through their anchorage at predetermined locales of the nanospheres system (Figure 6b). The in vitro nanospheres delivery diffusion experiments demonstrated the delivery of nanospheres by using the hydrogels at 37°C in response to physiologically relevant glucose levels. Compared the glucose challenge and removal, it is apparent that glucose-induced increased release of nanospheres with the flux reverting to a low level on the removal of glucose from the receptor solution, was observed on hydrogel challenged with 0.4 and 0.8 wt% glucose, respectively. The hydrogel has maintained the activity of the glucose-responsibilities in terms of controlling the diffusion of nanospheres as a function of glucose content. An increase in release intensity was observed through the action of 0.8 wt% glucose added to the system after 2 min, and the hydrogel rapidly dissociated and released the nanospheres. Exposure of the gel scaffold to 0.8 wt% glucose-induced network dissociation and
thereby released nanospheres into the microenvironment, which also was revealed by SEM images (Figure 6c). The results illustrated the hydrogels can dynamically tune the release of IGF-1 loaded nanospheres to the edge of the hydrogel with the response to external glucose. Competitive displacement results in temporarily lowering of the crosslinking of the hydrogel, which would trigger fluidity in the hydrogel and consequently facilitating the movement of nanospheres by diffusion mediated process.

3.4. Copper-free click chemistry
The copper-catalyzed click chemistry has received much attention in hydrogel fabrication due to its high chemoselectivity in mild reaction conditions with a variety of functionalizations (Crescenzi et al., 2007; Malkoch et al., 2006; Ossipov & Hilborn, 2006; Van Dijk et al., 2009). Typically, 1,3-dipolar cyclo-addition catalyzed by Cu (I) can be accomplished with high efficiency, reliability, and no by-products under the physiological condition (Polizzotti et al., 2008; X-D. Xu et al., 2009). Some macromolecular derivatives, such as poly(vinyl alcohol) (PVA), PEG and hyaluronan, were functionalized with pendant acetylene and azide groups to form hydrogels by the 1,3-dipolar cyclo-addition reaction (Altin et al., 2010; Hu et al., 2004; Pahimanolis et al., 2006; Polizzotti et al., 2008; S. Q. Liu et al., 2009; R. T. Chen et al., 2012; Van Dijk et al., 2010; X-D. Xu et al., 2009; Yilmaz et al., 2011). Generally, these hydrogels prepared via this cycloaddition reaction have controlled architectures and improved mechanical properties. Although the versatility of copper-catalyzed click chemistry has been broadly exploited for hydrogels, it is important to note that a major limitation is the intrinsic toxicity of copper and the inability to translate these approaches into tissue engineering. In that case, the risks of metal toxicity will prevent its use in clinical applications. Therefore, many attempts have been devoted toward exploiting efficient metal-free click conjugation for molecule ligation. In the past, an example of metal-free click reaction called “the Strain-Promoted Azide–Alkyne Cycloaddition (SPPC)” involving the strain-promoted [3 + 2] cycloaddition using cyclooctyne derivatives was reported by Agard et al. that enabled the click reaction without copper catalysis under physiological condition (Agard et al., 2004; S. Jung & Yi, 2012; Steinhilber et al., 2013). Presently, the SPPC has been employed to graft bioactive peptides to PEG hydrogels for molecular patterning (DeForest & Anseth, 2012; DeForest et al., 2008; S. Jung & Yi, 2012; Steinhilber et al., 2013). Also, Takahashi et al. chemically modified hyaluronan with azide and cyclooctyne groups to prepare an in situ crosslinked hyaluronan hydrogel (Takahashi et al., 2013). Although the formed hydrogel was biocompatible, it showed fast hydrolytic degradation that may prevent its use in tissue engineering.

More recently, oxanorbornadiene (OB) moieties have been considered to be effective functional molecules to bring in a reaction with an azido group resulting in triazole linkages (M. Fan, Ma et al., 2015; Van Berkel et al., 2007, 2008). Krause and Jirawutthiwongchai have successfully modified alginate and chitosan via OB for a metal-free click ligation, respectively (Jirawutthiwongchai et al., 2013; Krause et al., 2012). Their results indicated that OB with a certain chain length is necessary as it might help minimize the steric hindrance in the reaction. The most attractive point of OB is that the reaction can progress at room temperature without any additives or catalysts. For this reason, this metal-free click conjugation would be a good pathway to design bio-structured hydrogels, on the basis of biopolymers. In order to mimic the natural ECM of adipose, which is composed of GAGs, chitosan and hyaluronan were chosen to prepare a composite hydrogel. For the metal-free click reaction, chitosan and hyaluronan were modified with OB and 11-azido-3,6,9-trioxaundecan-1-amine (AA), respectively (M. Fan, Ma et al., 2015). Hydrogel precursors were designed with OB groups on the backbone of chitosan (CS–OB), which were accessible for cycloaddition with azido groups on the backbone of HA (HA–AA) in the physiological environment. Figure 8a shows the chemical structure of polysaccharide derivatives. The CS–OB was synthesized by the introduction of trifluoromethylated oxanorbornadienes to chitosan’s N-terminal of the glucosamine units, which displays excellent water solubility at neutral pH. To achieve the metal-free click cycloaddition for hydrogel formation, 11-azido-3,6,9-trioxaundecan-1-amine containing azido groups was grafted onto the HA catalyzed by EDC, to form an azido-functionalized HA–AA. The OB moieties are considered to be effective functional molecules to bring in a cycloaddition with azido groups, resulting in a triazole linkage between
chitosan and hyaluronan. Ultimately, a biodegradable polysaccharide-based hydrogel network was generated by conjugating CS–OB with HA–AA at 37°C, which afforded high water content and elasticity similar to soft tissues.

The most important issue for the metal-free click hydrogel is the cytocompatibility for the target cells by comparing with the conventionally copper-catalyzed hydrogels. Since the strategy of metal-free click chemistry avoids the copper contamination, the metal-free biopolymer hydrogel can provide many biochemical cues to generate an artificial niche for cell proliferation and differentiation. In the metal-free hydrogel, most of the encapsulated ASCs were shown to survive after 21 days culture (Figure 8b). Elliptical or roundly shaped ASCs were uniformly distributed in the metal-free hydrogel. The residing cells within metal-free hydrogels at day 7 possessed normal spherical morphologies, and one can estimate the cell size as ~10 μm (Figure 8c). Since the microstructure and biomolecule content were very similar to those of the ECM of natural soft tissue, the metal-free hydrogels may preserve the phenotype of ASCs, predicting a maintainable bioactivity for cell survival support. After 21 days, the cells that were encapsulated within the hydrogel still possessed normal spherical morphologies, similar to those in normal tissue, predicting a potential application as an injectable cell scaffold in soft tissue engineering. The hydrogels were injected bilaterally into the dorsal subcutaneous region of Balb-C mice to investigate initial in vivo biocompatibility. Figure 8d displays the representative macroscopic images of hydrogels immediately after injection for 1 h. The original circular shape was maintained after 7 days of implantation. The contour of the pockets of hydrogels was clearly demarcated, as seen through the skin, and in subcutaneous tissue upon dissection. The gross appearance of hydrogel was transparent, was somewhat cohesive, and adhered to tissue planes (Figure 8e). Histological cross sections of the implanted hydrogel and nearby tissue are presented in Figure 8f. A significant
amount of the hydrogel remained and no infections or inflammatory reactions were observed in the skin and subcutaneous tissues at day 7. The tissues at the gel–tissue interface also showed an absence of infiltration by neutrophils and macrophages.

4. Multiple crosslinking strategies
One approach to creating an ideal cell scaffold for tissue engineering applications is to incorporate bioactive elements into hydrogels for increased cellular bioactivity. For application in the field of therapeutic cells, multiple hydrogels are designed to be highly cell-compatible to mimic the ECM in combination with physical and chemical crosslinking. Multiple crosslinking of biopolymers or other bioactive drugs can create suitable biomimetic microenvironments for cell implantation. Besides biopolymers, assembly of microspheres has become increasingly prevalent in the production of biofunctional gel scaffolds for drug or cell delivery applications (Goldberg et al., 2007; Mironov et al., 2009; Nichol & Khademhosseini, 2009; H. Tan et al., 2014; Thornton et al., 2007). Development of microsphere-based gel scaffolds with high bioactivity and suitable mechanical performance would greatly broaden the application for soft tissue regeneration. However, it is difficult to form bulk hydrogels using biopolymer microspheres, and when formed, the microsphere gels do not exhibit suitable mechanical strength or bioactive property. Many efforts have been focused on inter-particle interactions for assembly of microspheres, including electrostatic forces, magnetic forces, hydrophobic interactions and steric hindrance, but the robustness and bioactivity of formed gels still remain unsatisfactory (Goldberg et al., 2007; Huang et al., 2007; Mironov et al., 2009; Nichol & Khademhosseini, 2009; H. Wang et al., 2011; Yu et al., 2007). Although some microspheres based on synthetic polymers are quite successful for bulk scaffolds, the biomedical applications of these scaffolds are strongly limited by the intrinsic inability to translate cell GFs into tissue engineering applications (Mironov et al., 2009; H. Wang et al., 2011; Yu et al., 2007).

A double-network (DN) principle provides a general approach to developing robust hydrogels for biomedical applications. For example, Gong and Khademhosseini et al. have reported a new method of obtaining strong and tough hydrogels by making DN materials with a high molar ratio of the second network to the first network (Nakajima et al., 2012; Shin et al., 2012; Q. Wang et al., 2008). Further research into these materials as tissue engineering scaffolds determined that these DN hydrogels weakened when they were prepared in cell-compatible solutions, and the encapsulated cells did not function as well as in single network hydrogels. Following this approach, the microgel-reinforced strategy was used by Khademhosseini et al. to make hydrogels by embedding stiff microgels into a soft and ductile matrix (Sivakumaran et al., 2013; X. Xu et al., 2011). These hydrogels exhibited significantly higher strength than single network hydrogels with no microgels, and comparable strength to DN hydrogels. Motivated by the need to develop better hydrogel systems in terms of both mechanical strength and biological properties, we hypothesize that the micro-phase segregated DN concept could be applicable to developing injectable microsphere-based gel scaffolds with biological functionalities. It consists of two networks: a densely cross-linked biopolymer microspheres network (skeleton) and a sparsely crosslinked ductile biopolymer network. One can expect two advantages of this microsphere-based DN gel. First, microspheres incorporated at a higher composition ratio can potentially increase the strength of the DN gel due to their higher stiffness. Second, because GFs were encapsulated not in the ductile network but in microspheres, the GFs were expected to function better in this microsphere-based DN gel than in normal DN hydrogels. Importantly, unlike most microspheres that are chemically inert, the biopolymer microspheres would have residual functionalities that allow for further bioconjugation of a ductile network.

One strategy is to use heparin as the microspheres substrate and chitosan as the ductile network, respectively. In this system, heparin microspheres with a higher composition ratio can potentially increase the strength of the DN gel, while chitosan solution as a liquid phase can provide the injection (H. Tan et al., 2014). Heparin and chitosan are proteoglycans or GAGs, as highly charged polyelectrolytes, which could be additionally crosslinked by electrostatic interactions (Baldwin & Kiick, 2013; Ehrbar et al., 2007). Heparin microspheres exhibited residual functional peptides that could be used as reactive handles for subsequently enzymatic conjugation of
A chitosan derivative, as proposed in Figure 9. The method for preparing heparin microspheres and gel scaffolds relies on in situ crosslinking of heparin with a built-in fibrin-mimicking peptide of CTIGEGQHHLGGAKQAGDV containing Lys and Gln functionalities. This peptide derived from the transglutaminase factor XIIIa (FXIIIa) crosslinking site of fibrin, with an additional N-terminal cysteine residue. The peptides would couple and form covalent isopeptide bridges between Gln and Lys by enzymatic catalysis of the FXIIIa.

For the formation of the micro-phase segregated DN structure, the peptide functionalized chitosan solution was introduced as the second network and mixed with the peptide functionalized heparin microspheres to form the contrasting topological structure. The macroscopic gel scaffold was assembled with interlocked hierarchical organizations form through simultaneously enzymatic reactions and extra electrostatic interactions between the peptides on microspheres and those in chitosan solution. There are two levels of crosslinking in this gel scaffold: the primary network consisting of crosslinked heparin chains inside each microsphere, and the secondary network interconnecting the microspheres. Generally, fibrin is susceptible to degradation (fibrinolysis) by various proteolytic enzymes, including plasmin and matrix metalloproteases (MMPs). Due to intrinsic protease substrate in this peptide, the peptide functionalized heparin microspheres, which under in vitro conditions are stable for many weeks, are readily degraded when exposed to active MMP-1. Exposure of the heparin microspheres to MMPs induces microsphere biodegradation and thereby could release GFs into the microenvironment.

The nature of oppositely charged proteoglycans also allowed for simultaneous crosslinking of the microspheres via extra electrostatic interactions, giving rise to doubly crosslinked gel scaffold with tunable viscoelasticity. Unlike conventional microsphere-based hydrogels, this gel scaffold is much more stiff and strong than biological soft tissues. The viscoelasticity behavior makes the gel scaffolds suitable for extrusion and injection by syringes. Homogeneous gel-threads were formed
upon injection through a conventional medical syringe. As revealed the microstructure of lyophilized gel scaffold, inter-sphere distances were close and showed a clear interconnection between heparin microspheres. By the additional liquid phase of peptide functionalized chitosan, the gel scaffold showed a compact packing of microspheres via the enzyme-catalyzed reaction. The lyophilized peptide functionalized chitosan was also found between microspheres, which might contribute to increase crosslinking of the gel scaffold via extra electrostatic interactions. One can speculate that the peptide functionalized chitosan in the liquid phase could couple microspheres as bridges. Also, in vitro degradation studies indicate that the gel scaffold exhibited proteolytically sensitive, and BMP-2 could release controlled upon cell-derived proteolytic degradation of the gel scaffold. Hence, this in vitro delivery of BMP-2 from the gel scaffold is supposed to become controlled by cellular stimuli, when cells infiltrate the scaffold matrix, they secrete proteases that liberate the GFs from the scaffold, localizing the GF response.

5. Conclusions and outlook
We discussed the biocompatible conjugation of biodegradable hydrogels to be potentially used in drug delivery and soft tissue engineering applications. Many efforts have been developed to improve injectable hydrogels and thus, support the development of more natural and functional tissues. We anticipate that these multifunctional gel scaffolds can render the formulation of a therapeutically effective platform for both in vitro and in vivo soft tissue regeneration. The biocompatible coupling chemistry has the advantage that it introduces no potentially cytotoxic groups into injectable biopolymer scaffold formed and can create a more biomimetic microenvironment for drug and cell delivery, rendering them more suitable for potential in vivo biomedical applications. Each hydrogel system developed physically or covalently has its own properties for biomedical applications, especially cell therapy and tissue regeneration. Physical crosslinking strategies could be readily utilized to prepare cytocompatible hydrogels without extra reagents, but the hydrogels demonstrate weak stability and physical properties. Although the versatility of methods has been broadly exploited, chemical crosslink methods are limited by potential toxicities of chemically reactive moieties, aggregation of entrapped proteins, and irreversible modification. The success of injectable tissue constructs is highly dependent on the design of the hydrogel scaffolds including physical, chemical and biological properties. An ideal injectable hydrogel would potentially mimic many roles of ECM found in tissues, resulting in the coexistence of both physical and chemical gels. A single gel network is unable to meet all the design parameters simultaneously (e.g., degradation, biocompatibility or mechanical properties). Novel networks with multiple functionalities should be developed, both to enhance the material biocompatibility as well as control the mechanical properties. In addition, cell induction ligands such as growth factors and genes can be incorporated into the multiple hydrogels such that specific signals could be delivered in an appropriate spatial and temporal manner.

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