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Injectable biodegradable hydrogels: progress and challenges

Ki Hyun Bae,† Li-Shan Wang† and Motoichi Kurisawa*

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Over the past decades, injectable hydrogels have emerged as promising biomaterials because of their biocompatibility, excellent permeability, minimal invasion, and easy integration into surgical procedures. These systems provide an effective and convenient way to administer a wide variety of bioactive agents such as proteins, genes, and even living cells. Additionally, they can be designed to be degradable and eventually cleared from the body after completing their missions. Given their unique characteristics, injectable biodegradable hydrogels have been actively explored as drug reservoir systems for sustained release of bioactive agents and temporary extracellular matrices for tissue engineering. This review provides an overview of state-of-the-art strategies towards constructing a rational design of injectable biodegradable hydrogels for protein drug delivery and tissue engineering. We also discuss the use of injectable hydrogels for gene delivery systems and biomedical adhesives.

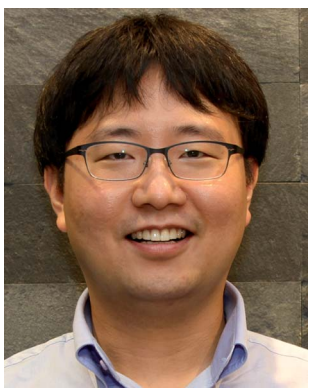
1 Introduction

Hydrogels are a class of cross-linked polymeric networks, which can absorb large quantities of water or biological fluids. These materials possess unique characteristics that make them attractive in the biomedical field. First of all, hydrogels are highly biocompatible because of their high water content and structural similarity to the natural extracellular matrices.[1] Second, the soft and pliable nature of hydrogels minimizes mechanical irritation and damage to the surrounding tissue.[2,3] Third, they have excellent permeability for transport of nutrients and metabolites, which supports the survival and growth of encapsulated cells.[4] For these reasons, numerous hydrogel systems have been widely applied for diverse biomedical applications, including drug delivery, tissue engineering, diagnostics, immunoisolation, and blood-compatible coating of medical implants.[5–8]

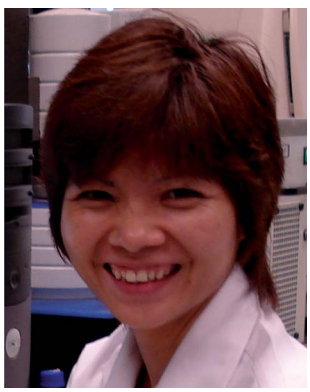
In principle, hydrogels can be made from virtually all types of natural and synthetic polymers. The formation of hydrogels is accomplished by physical or chemical cross-linking of polymer chains. Physical cross-linking employs non-covalent associations among the polymer chains *via* electrostatic, hydrophobic, or hydrogen-bonding forces.[9,10] On the other hand, chemical

Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos #04-01, Singapore 138669. E-mail: mkurisawa@ibn.a-star.edu.sg; Fax: +65 6478 9083; Tel: +65 6824 7139

† These authors contributed equally to this work.



Ki Hyun Bae received his Ph.D. degree under the guidance of Prof. Tae Gwan Park in the Department of Biological Sciences at Korea Advanced Institute of Science and Technology in 2011. He is currently a postdoctoral fellow in the group of Dr Motoichi Kurisawa at Institute of Bioengineering and Nanotechnology (IBN), Singapore. His current research focuses on development of injectable hydrogels and nanoscale drug delivery systems for biomedical applications.



Li-Shan Wang studied chemistry at the Shandong University in P. R. China and received her B.Sc degree in 1995. She obtained her M.Sc and Ph.D. degrees in chemistry from the National University of Singapore in 2001 and 2011 respectively. She is currently a Research Scientist in the Institute of Bioengineering and Nanotechnology (IBN). Her current main research interests include the synthesis of biomaterials, cell responses to mechanical properties of substrates and its microenvironment, as well as their application in drug delivery and tissue engineering.

cross-linking introduces covalent linkages between the polymer chains to form gel networks. These linkages are usually generated by photo-irradiation, disulphide formation, Schiff-base formation, and Michael-type addition reactions.[11] Although physically cross-linked hydrogels can avoid the use of harsh chemical reactions, they tend to disintegrate in the body mainly due to disruption of the non-covalent associations.[12,13] In contrast, chemically cross-linked hydrogels exhibit a high physical stability with enhanced mechanical strength, making them promising candidates for long-term *in vivo* applications.

Recently, there has been growing interest in injectable hydrogels which can be formed *in situ* after being injected into the body.[14] They provide superior benefits over conventional preformed hydrogels. For example, the use of injectable hydrogels eliminates the need for complicated surgical procedures that are required to implant preformed hydrogels. Injectable hydrogels carrying bioactive molecules and cells can be administered *via* a simple and minimally invasive procedure, which ultimately reduces patient discomfort and the cost of treatment. This feature is also beneficial in reducing the recovery time and the risk of infection in patients.[15] Moreover, these hydrogels can be readily injected into locations that are hard to access through surgery. In view of their favourable characteristics, injectable hydrogels have attracted significant interest as drug delivery vehicles, tissue engineering scaffolds, medical adhesives, and dermal fillers.[16–18] As a drug carrier, injectable hydrogels can form a stable depot in the body *in situ* and release their therapeutic payloads in a controlled manner. These hydrogels are also advantageous for the regeneration of damaged tissues because they are able to form a desired shape that is coherent with the surrounding tissues at defect sites.[19]

This review aims to highlight the recent development of injectable biodegradable hydrogels for biomedical applications. First, the key criteria in the design of injectable hydrogels for drug delivery application, such as the gel network structure, drug release mechanism, and biodegradability, are introduced. Then the current approaches for controlling protein release kinetics are summarized. In addition, we review state-of-the-art strategies to modulate biological effects and physical properties of injectable hydrogels for tissue engineering. The use of injectable hydrogels for gene delivery and biomedical adhesives is also discussed.

Motoichi Kurisawa received his Ph.D. degree from the School of Materials Science, Japan Advanced Institute of Science and Technology (JAIST) in 1998. He conducted his postdoctoral research in the area of biomaterials at Tokyo Women's Medical University and Kyoto University. He joined the Institute of Bioengineering and Nanotechnology (IBN) in 2003, and is currently a Team Leader and Principal Research Scientist. His research interest is biomaterials for drug delivery and tissue engineering.

2 Applications of injectable hydrogels for protein drug delivery

With remarkable advances in recombinant DNA technologies, a wide range of biologically active proteins have become available for the medical treatment of diverse chronic diseases.[20] In order to achieve the desired therapeutic effect, an appropriate amount of the protein drugs needs to be delivered to the target site in the body. However, the effective delivery of protein drugs is very challenging because of their poor stability, rapid proteolysis, and short circulating half-lives.[21,22] For example, the protein drugs undergo degradation readily through proteolytic enzymes in the blood stream. In addition, these drugs are rapidly cleared by the kidney because of their small molecular size.[23] It has been reported that many therapeutic proteins (*e.g.*, interleukin, superoxide dismutase, and tumour necrosis factor) have plasma half-lives less than 30 min.[24,25] Accordingly, frequent injections of high doses of the protein drugs are typically required to extend their therapeutic effect. This in turn increases the risk of toxic side effects and immune response.[26] Furthermore, such multiple administrations are not desirable because these result in patient discomfort and a relatively high cost of treatment.

For the aforementioned limitations to be overcome, it is necessary to develop a controlled protein delivery system that can release the loaded protein drugs over a prolonged period of time.[27] Hydrogels have gained considerable attention as an ideal delivery vehicle for protein drugs because of their unique characteristics. First of all, hydrogels have a high water content (>90 wt%) and thus provide an aqueous environment favourable for preserving the native structure and bioactivity of proteins. It has been shown that proteins entrapped in a gel network are more resistant to denaturation than free proteins.[28] Second, the highly porous structure of hydrogels enables them to carry large quantities of protein drugs.[29] This feature is a prerequisite for developing long-term protein delivery systems that are able to provide continuous protein release. Lastly, proteins can be incorporated within a gel matrix under mild conditions (*e.g.* physiological temperature and pH) that are not detrimental to the proteins.

With these distinctive advantages, injectable hydrogels have been widely explored as formulations for the controlled release of bioactive proteins.[30–32] These formulations can be administered in a minimally invasive manner. Ideally, the *in situ* formed gel would serve as a delivery depot releasing the loaded protein drugs in a sustained fashion to maintain plasma drug concentrations continuously within a therapeutic range. As a result, the therapeutic effect of these drugs would be prolonged for extended periods of time that consequently allows for less frequent administrations. In the following sections, we will discuss some of the key criteria for designing injectable hydrogels in formulations for protein release. The present approaches to control protein release kinetics will be also discussed.

2.1 Design criteria for injectable hydrogels in protein drug delivery

2.1.1 Gel network structure. The structure of gel networks should be considered in the design of injectable hydrogels for protein delivery applications. For this reason, it is necessary to understand how the network structure of hydrogels influences their physical properties. The cross-linking density of the hydrogels, which refers to the number of chemical or physical cross-links in a given volume, is an important parameter governing many hydrogel structural characteristics.[33] Fig. 1 shows the relationship between the cross-linking density and the hydrogel properties such as the modulus, equilibrium swelling ratio, and drug diffusivity. As the cross-linking density increases, the equilibrium swelling ratio and drug diffusivity of hydrogels decrease with a concomitant reduction in the mesh size (ξ).[34,35] Since the mesh size defines the space between macromolecular chains available for molecular transport, it affects the water content of swollen gels and diffusivity of entrapped protein drugs to a large extent.[36] On the other hand, the gel modulus typically increases upon raising the cross-linking density. The modulus of injectable hydrogels is especially crucial for their *in vivo* applications.[37] For example, if a gel is too soft, it is easily deformable in a mechanically dynamic environment in the body. This deformation is often accompanied by a change in the total surface area of the hydrogel networks. As a result, the desired rate of protein release may not be achieved. The biocompatibility of hydrogels is also influenced by their modulus. It has been shown that stiffer hydrogels can cause a more severe foreign body reaction *in vivo* possibly by stimulating the spreading and activation of macrophages at the host–hydrogel interface.[38,39] The subsequent formation of a thick fibrous capsule around the gels can hamper the release of protein drugs from them by creating an additional diffusion barrier.[40] Accordingly, for the development of successful injectable hydrogel systems, it is highly desirable to control the physical properties of the hydrogels *via* tailoring their cross-linking density.

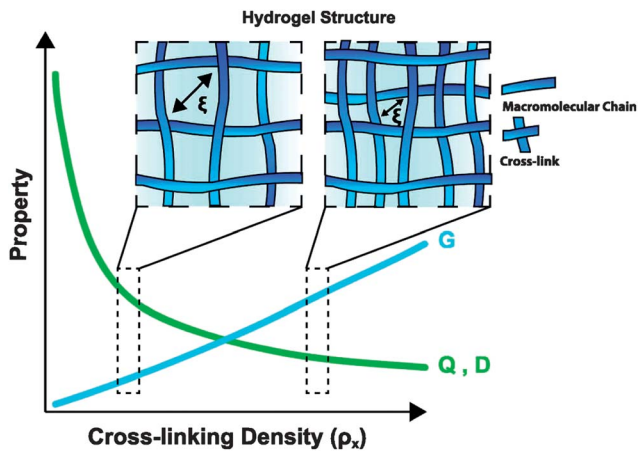


Fig. 1 The relationship between cross-linking density and physical properties of hydrogels (modulus (*G*), equilibrium swelling ratio (*Q*), and drug diffusivity (*D*)). The increase in the cross-linking density yields more dense gel networks with a concomitant reduction in mesh size (ξ). Reprinted with permission from ref. 35. Copyright (2013) Elsevier Ltd.

2.1.2 Drug release mechanism. In general, protein drugs loaded in hydrogels are released by several mechanisms, such as diffusion, swelling, erosion, or a combination of these mechanisms.[41] The mesh size of the hydrogel networks is a critical factor for determining the release mechanism of the encapsulated proteins.[42] For instance, when a hydrogel has a larger mesh size than the proteins in its swollen state, diffusion is the main mechanism governing the protein release. In contrast, when the mesh size of a swollen gel is smaller than the protein diameter, then diffusion of these protein molecules is hindered. As a result, the swelling and bulk or surface erosion of hydrogels will control the release of entrapped proteins from the gel matrices. A more detailed description of these release mechanisms can be seen in several other reviews.[43–45] Through altering the hydrogel mesh size rationally, it is possible to manipulate the release kinetics of protein drugs from the hydrogels.

In practice, it is not possible to directly measure the mesh size of swollen gel networks. Instead, the mesh size has been estimated based on equilibrium-swelling theory and rubber-elasticity theory.[46,47] In the case of highly swollen hydrogels, where the cross-links are introduced in solution, the mesh size (ξ) can be calculated according to the following equation: $\xi = Q^{1/3}(C_n N L^2)^{1/2}$, where Q is the volumetric swelling ratio of the hydrogel, C_n is the Flory characteristic ratio of the polymer, N is the number of bonds between two cross-links, and L is the length of the bond along the polymer backbone. Among the variables, C_n and L are easily calculated from the chemical structure of the polymer chains, whereas Q and N are determined experimentally by measuring the volume of the hydrogel before and after equilibrium swelling.

2.1.3 Biodegradability. Currently, many injectable hydrogels have been designed to be either hydrolytically or enzymatically degradable in the body.[48–50] Since these biodegradable hydrogels do not require a surgical procedure to recover them, patient compliance can be significantly enhanced. Degradation of these hydrogels is accomplished by bulk or surface erosion. Bulk erosion is the cleavage of the polymer chains that occurs homogeneously throughout the whole matrix.[51] The majority of biodegradable hydrogels reported to date exhibit bulk erosion because of their high water content and permeability.[40] Surface erosion occurs when the rate of water uptake or enzyme transport into the gels is much slower than the rate of polymer chain scission.[52] For protein delivery applications, surface-eroding hydrogels are advantageous because they allow for the continuous release of entrapped proteins with nearly zero-order kinetics.[53]

The degradation rate of hydrogels is closely related to their *in vivo* residential time. Slower degrading hydrogels stay in the body for a longer period of time than faster degrading ones. Ideally, the *in vivo* residential time of hydrogels should be matched with the entire duration of the drug release.[37] For example, if a gel is eliminated from the body before releasing all the loaded protein drugs, an abrupt leakage of large amounts of the drugs may occur. This decreases the therapeutic efficacy of

the protein drugs while increasing the risk of undesired side effects. Hence it is required to optimize the degradation rate of hydrogels to maximize their *in vivo* performance. The degradation rate of gel matrices can be tuned by tailoring the polymer architecture, the cross-linking density, and the amount of incorporated degradable moieties.[54–56] The biocompatibility of hydrogels can also be influenced by their degradation rate. Typically, a prolonged residence of the *in situ* formed gel encourages a lasting immune response.[57] The rapid degradation of hydrogels is beneficial because their fast clearance leads to a decreased immune response.

2.2 Approaches for controlling release of protein drugs

As discussed in the earlier section, injectable biodegradable hydrogels have received significant attention as protein delivery systems. Yet several obstacles restrict the practical use of injectable hydrogels for therapeutic protein delivery. The main challenge is the premature leakage of encapsulated protein drugs. Typically, many hydrogel systems quickly release the majority of the loaded protein drugs during the initial release stage.[58,59] This so-called "initial burst" release is not desirable because it leads to a drastic increase in the plasma drug concentrations, which often causes harmful side effects and ineffective treatment of diseases.[60] A variety of approaches have been developed to overcome this limitation through attempting to suppress the initial burst and to extend the release period of protein drugs. In the present section, we will summarize the current approaches being applied to design injectable hydrogels for controlled protein release.

2.2.1 Gel network engineering. The most common approach to control the drug release kinetics is by engineering the network structure of hydrogels. In this approach, the permeability of gel networks is adjusted on the cross-linking density through a variation of the cross-linking parameters. Such parameters include the polymer concentration, the amount and molecular weight of cross-linkers, and the gelation conditions.[61–63] van de Wetering *et al.* have reported injectable polyethylene glycol (PEG) hydrogels for sustained release of the human growth hormone (hGH).[64] These hydrogels were produced by Michael-type addition reactions of eight-arm PEG acrylates with thiol compounds.[65] The resulting gel network was degradable under physiological conditions by hydrolysis of the ester groups. The hGH was precipitated by linear PEG for encapsulation to protect the protein from chemical reactions with the gel precursors during gelation.[66] By varying the molecular weight of the eight-arm PEG acrylates, the hGH release kinetics could be finely tuned (Fig. 2). For instance, virtually 100% of hGH was released from the 10 kDa PEG acrylate gels within 12 h, while the 2 kDa PEG acrylate gels liberated approximately 35% of their protein contents over 500 h in a sustained manner. On the other hand, the gels made from a 1 : 1 (w/w) mixture of these PEG acrylates exhibited intermediate release kinetics. This revealed that decreasing the chain length (molecular weight) of the polymers reduced the mesh size and diffusivity of the network, resulting in a delayed protein release. Moreover, the good agreement between observed and predicted release curves indicates that hGH was released from the PEG hydrogels in its native form.

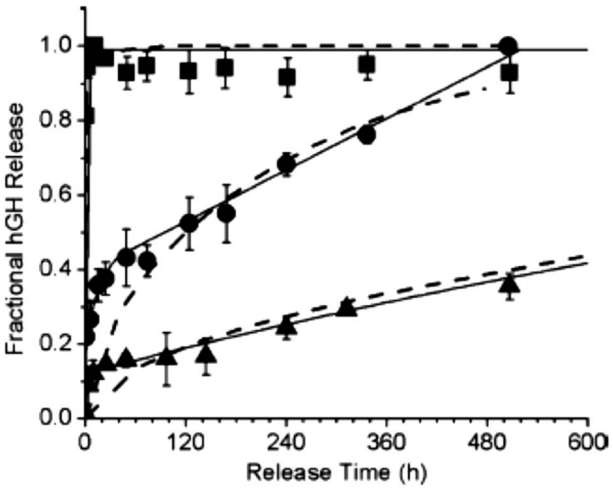


Fig. 2 Release kinetics of hGH from PEG hydrogels formed from eight-arm PEG acrylates of different molecular weights: 10 kDa PEG acrylate gels (■), 2 kDa PEG acrylate gels (▲), and the gels made from a 1 : 1 (w/w) mixture of these PEG acrylates (●). The best-fit curves of the experimental data (solid lines) showed good agreement with the release curves predicted by a mathematical model (dashed lines). Reprinted with permission from ref. 64. Copyright (2004) Elsevier Ltd.

Our group developed an injectable hyaluronic acid–tyramine (HA–Tyr) hydrogel system for protein delivery (Fig. 3).[67,68] These hydrogels were formed through the oxidative coupling of tyramine moieties, which was catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). The stiffness and gelation rate of the hydrogels could be independently tuned by H_2O_2 and HRP concentrations, respectively.[12] *In vitro* release experiments demonstrated that α-amylase and lysozyme were released from HA–Tyr hydrogels in a diffusion controlled manner.[69] The released α-amylase retained more than 95% of its bioactivity, indicating that the enzyme-catalysed cross-linking reactions did not cause denaturation of the proteins. Recently, we have incorporated interferon-α2a (IFN) into HA–Tyr hydrogels for liver cancer therapy.[70] IFN is a class of cytokines that displays antiproliferative activities against a number of human cancers such as leukemia, melanoma, and hepatocellular

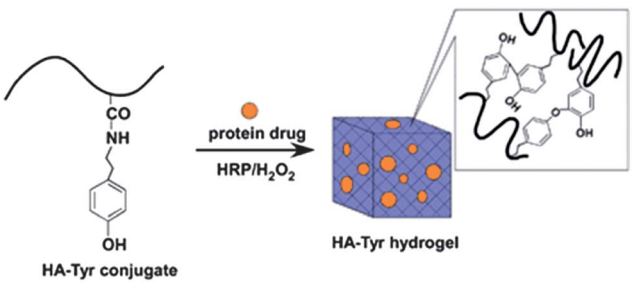


Fig. 3 *In situ* formation of HA–Tyr hydrogels by an enzyme-mediated oxidation for protein delivery. Reprinted with permission from ref. 68. Copyright (2010) The Royal Society of Chemistry.

carcinoma.[71] The release rates of IFN could be regulated by tuning the cross-linking density of the hydrogels through the control of the H_2O_2 concentration. It was found that stiffer hydrogels formed by a higher H_2O_2 concentration exhibited slower protein release, as compared with softer hydrogels formed by a lower H_2O_2 concentration (Fig. 4a). During the first 8 h, a linear plot was obtained by plotting the release of IFN as a function of the square root of time, indicating that the drug release was governed by a typical Fickian diffusion with first-order release kinetics (Fig. 4a inset). The *in vivo* anticancer effect of IFN-incorporated HA–Tyr hydrogels was examined in a HAK-1B tumour-bearing nude mouse model. Notably, the IFN-loaded hydrogels effectively suppressed the tumour growth when compared to IFN solution injected at the same dose (Fig. 4b). The pharmacokinetics study revealed that the enhanced anticancer effect was attributed to the continuous release of IFN from the HA–Tyr hydrogels for prolonged periods of time *in vivo*.

To date, a number of studies have shown that the initial burst and release rate of incorporated proteins can be decreased by raising the cross-linking density of hydrogels.[72–74] However, this approach has been found to be less effective in controlling the release of low-molecular-weight proteins. Delgado *et al.* have investigated the effect of protein molecular size on release kinetics from cross-linked poly(*N*,*N*-dimethylacrylamide) hydrogels.[75] These hydrogels were loaded with proteins of different hydrodynamic diameters (d_h), *i.e.* immunoglobulin G (IgG, $d_h = 10.7$ nm), bovine serum albumin (BSA, $d_h = 7.2$ nm), and lysozyme ($d_h = 4.1$ nm). There was only 7% of IgG liberated into the external medium in 80 h, whereas about 30% of BSA was released during the same period. On the other hand, virtually 100% of lysozyme was released in 24 h, indicating that the mesh size of gel networks was too large to restrict the diffusion of lysozyme molecules.[76] Although it is likely that the diffusion of such small proteins can be prevented by substantially increasing the cross-linking density, this often diminishes the hydrophilicity and biocompatibility of hydrogels.[38,39] Moreover, an incomplete release would occur *via* the permanent entrapment of proteins in high-density regions of the gel network that possess a smaller mesh size than the protein.[58] Hence, the degree of network cross-linking has to be carefully considered when designing hydrogels for protein delivery.

2.2.2 Pendant chain systems. One viable way to avoid the initial burst release of protein drugs is covalently grafting them to the polymer backbone of hydrogels through a cleavable linker. The hydrogels based on this approach are usually referred to as "pro-drug" or "pendant chain" systems. The tethered protein drugs can be released from the gel matrices by hydrolysis, reduction, or enzymatic degradation.[77–79] Recently, Verheyen *et al.* developed an efficient strategy towards temporary immobilization of protein drugs onto the gel matrices.[80] The lysine residues of lysozyme were first modified with protected thiol groups using the succinimidyl-*S*-acetylthioacetate (SATA) reagent (Fig. 5a). Subsequently, the thiol groups were deprotected and coupled to the linker molecule *via*

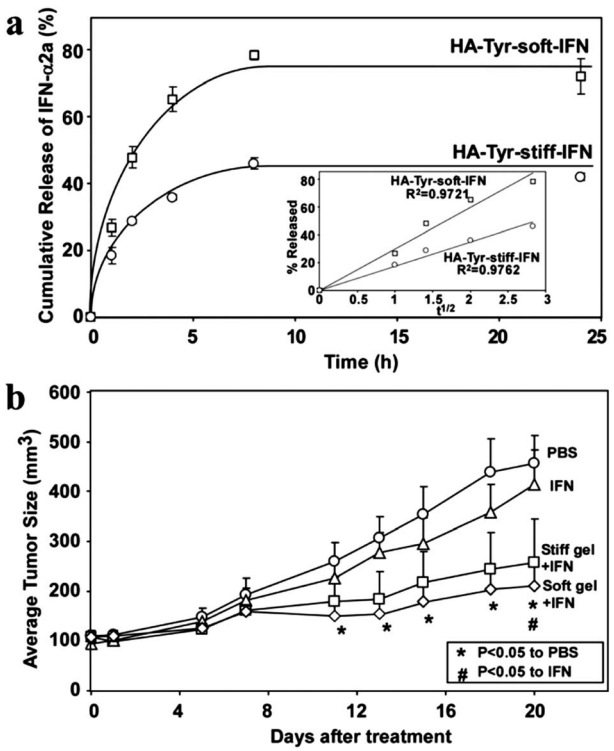


Fig. 4 (a) Cumulative release profiles of IFN from HA–Tyr hydrogels formed with (□) 437 and (○) 728 μM of H_2O_2. The inset shows the cumulative release of the protein as a function of the square root of time. (b) Tumour regression study of HAK-1B tumour-bearing nude mice treated with PBS, IFN solution, or IFN-loaded HA–Tyr hydrogels. Reprinted with permission from ref. 70. Copyright (2013) Elsevier Ltd.

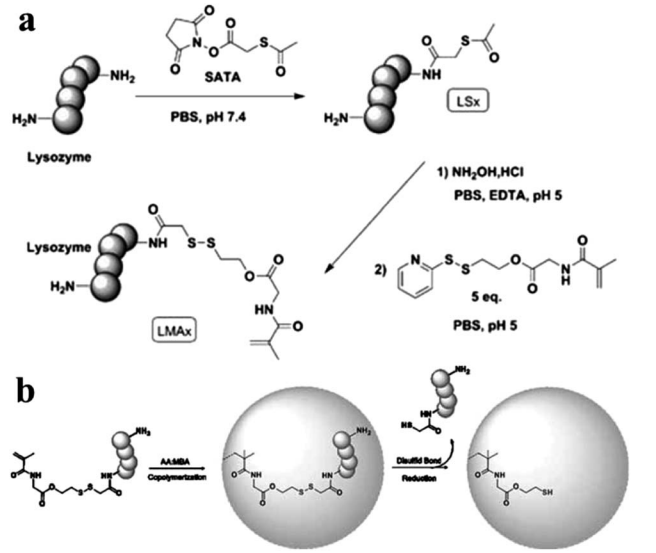


Fig. 5 (a) Synthetic scheme of methacrylamide-modified lysozyme. The lysine residues of lysozyme were modified with protected thiol groups using the SATA reagent. Subsequently, the thiol groups were deprotected and coupled to the linker molecule *via* thiol–disulfide exchange. (b) Schematic illustration of a hydrogel releasing the grafted lysozyme molecule by reduction of the spacer. Reprinted with permission from ref. 80. Copyright (2010) Wiley-VCH Verlag GmbH & Co. KGaA.

thiol–disulfide exchange reactions. The resultant methacrylamide-modified lysozyme was copolymerized with acrylamide monomers to form hydrogels (Fig. 5b). These covalently bound lysozyme molecules could be liberated from the gel network *via* hydrolysis and/or glutathione-mediated reduction because the linker molecules contained both an ester bond and a disulfide bond.[81] For instance, the hydrogels released almost 74% of their protein contents upon exposure to 2.5 mM of glutathione, whereas they exhibited a smaller degree of release (less than 20%) in the absence of glutathione. Such pendant chain systems can eliminate the burst release of proteins without compromising the hydrophilic nature of gel matrices. In addition, the release rate of protein drugs can be modulated simply by tailoring the degradation rate of the linker molecule.[82] Despite these advantages, some drawbacks still limit the practical use of pendant chain systems. First, this approach necessitates the chemical modification of proteins, which may adversely affect their biological activity.[83] Second, protein drugs can be denatured by organic solvents commonly used in the conjugation reactions. This is unfavourable for clinical applications because the denatured proteins can stimulate an immunological response.[29] Lastly, a complicated multi-step procedure is typically required to attach the cleavable linker to the protein drugs.[84]

2.2.3 Composite hydrogel systems. Another approach for controlling protein release is based on composite hydrogel systems, where protein-loaded micro or nanoparticles were embedded in a bulk hydrogel matrix. These particles serve as a drug reservoir from which the loaded protein drugs are slowly released in a sustained manner.[85] The poly(lactic-*co*-glycolic acid) (PLGA) microsphere has been extensively utilized to create composite hydrogel systems because of its biocompatibility and controllable degradation rate.[86–88] Lee and co-workers recently fabricated injectable alginate hydrogels containing PLGA microspheres for localized protein delivery.[89,90] These hydrogels were prepared by mixing protein-loaded PLGA microspheres with an alginate solution prior to cross-linking with calcium ions. The homogeneous dispersion of microspheres in the alginate gel was observed (Fig. 6a). A heat shock protein 27 (HSP27) fused to a cell-penetrating peptide TAT was loaded into the composite hydrogel system for the treatment of ischemic heart disease.[91] The release kinetics of the recombinant TAT–HSP27 fusion proteins could be tuned by varying the mixing ratios between PLGA microspheres and alginate gels (Fig. 6b). For example, almost 80% of proteins were released in the first 3 days from the alginate gels without microspheres. When the mixing ratio of PLGA/alginate was increased up to 1.5, the amount of protein liberated in the initial burst was considerably reduced to ~30%. The controlled release of TAT–HSP27 from the composite hydrogel systems was achieved for 3 weeks. Such an improvement in the protein release kinetics was likely attributed to a combination of diffusional resistances of the microspheres and the surrounding gel.[40]

Although PLGA microspheres have been extensively utilized as a sustained-release formulation of protein drugs, they have some drawbacks associated with the protein stability.[92,93] First, the use of organic solvents during their formulation often

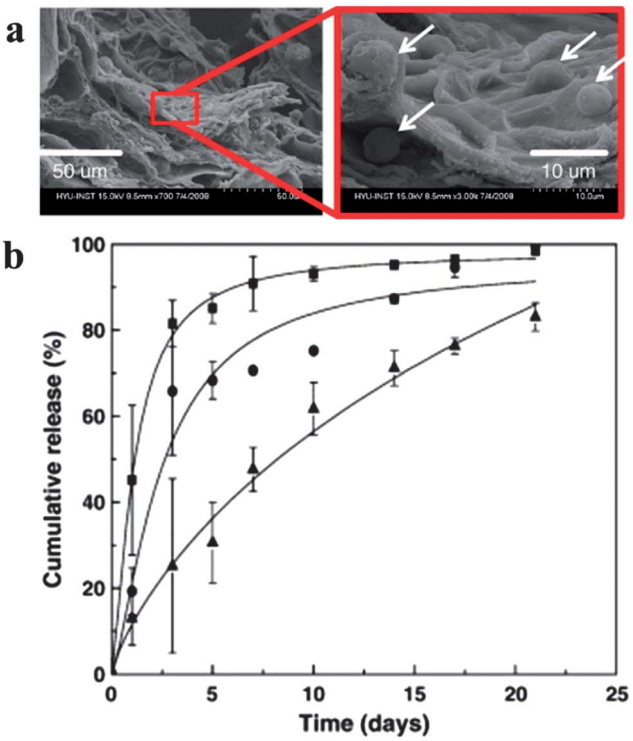


Fig. 6 (a) SEM images of PLGA microspheres incorporated in an alginate gel. The arrows indicate the embedded microspheres. (b) Release kinetics of the TAT–HSP27 fusion protein from composite hydrogel systems formed at different mixing ratios of PLGA microspheres and alginate gels (■, 0; ●, 1; ▲, 1.5; PLGA/alginate = w/w). Reprinted with permission from ref. 90. Copyright (2009) Elsevier Ltd.

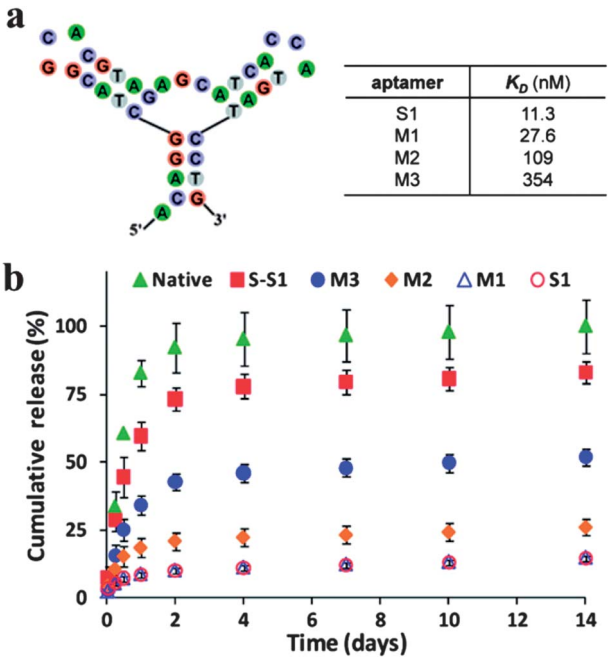
decreases the bioactivity of protein drugs. Second, proteins are susceptible to denaturation during the harsh fabrication processes (*e.g.*, emulsification, homogenization). Lastly, the degradation products (*e.g.*, lactic acid, glycolic acid) generate an acidic microenvironment in the microspheres, which may cause the denaturation of the entrapped proteins. In order to circumvent these shortcomings, hydrogel microparticles have been exploited as an alternative to PLGA microspheres. For example, gelatin microparticles that incorporate growth factors were entrapped in oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue repair.[94,95] Additionally, a sustained release of bone morphogenetic protein 2 (BMP-2) was achieved by the encapsulation of BMP-2-loaded HA microparticles in a cross-linked HA hydrogel network.[96]

2.2.4 Affinity hydrogel systems. Hydrogels can be designed to have a specific affinity toward a protein drug of interest for prolonged drug release. These so-called "affinity hydrogel" systems are typically prepared by incorporating high-affinity ligands capable of binding to the protein drug reversibly. One of the most studied ligands is heparin, a highly sulfated glycosaminoglycan found in the body. Particularly in extracellular matrices, heparin binds to growth factors primarily *via* electrostatic interactions.[97] The binding of heparin not only regulates release of these growth factors from the extracellular matrices, but also stabilizes them against proteolytic degradation.[98,99] Heparin has been used to create affinity hydrogel

systems for controlled release of growth factors because of these characteristics. Cai *et al.* have reported heparin-immobilized HA hydrogels as a localized delivery vehicle for the basic fibroblast growth factor (bFGF).[100] Heparin and HA were modified with thiol groups and then co-cross-linked with PEG diacrylate to form hydrogels. The incorporation of heparin in the gel matrices enabled the sustained release of bFGF over a 35-day period through the specific and reversible interaction between heparin and bFGF.[101] The heparin-containing hydrogels promoted the formation of new blood vessels significantly when compared to those lacking heparin. This hydrogel system was also applied for the local delivery of a variety of growth factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), and keratinocyte growth factor (KGF) to stimulate *in vivo* angiogenesis.[102,103] However, such heparin-based affinity hydrogels have some limitations. For example, only a limited range of proteins (*i.e.*, heparin-binding growth factors) are applicable for these systems. In addition, the heparin–protein interactions in gel matrices can be disrupted by the competitive binding of heparin molecules that are normally present in the plasma.[104] This in turn can cause an uncontrolled leakage of the entrapped protein drugs, resulting in a decreased therapeutic efficacy.

There have been tremendous efforts to develop advanced affinity hydrogel systems that can regulate the release of a wide range of protein drugs in a well-defined manner. Willerth and colleagues have identified peptide domains with specific affinity to the nerve growth factor (NGF) and incorporated these peptides in fibrin matrices to tailor the release kinetics of NGF.[105] Lin and Metters fabricated PEG hydrogels tethered with iminodiacetic acid that chelates a metal ion to regulate the release rate of hexahistidine-tagged recombinant proteins.[106,107]

Recently, Soontornworajit *et al.* developed injectable poloxamer block copolymer hydrogels that are functionalized with nucleic acid aptamers.[108] Aptamers are single-stranded DNA or RNA oligonucleotides that can recognize specific target proteins with high affinity.[109] A series of aptamers with varying affinities for the platelet-derived growth factor BB (PDGF-BB) were selected from DNA libraries (Fig. 7a). The equilibrium dissociation constants (K_D) were varied from 11.3 to 354 nM by tuning the sequence and functional structure of the aptamers. The aptamer S1 was able to bind to PDGF-BB with the highest affinity. The release rates of PDGF-BB were found to decrease gradually with the increasing affinity of the aptamers (Fig. 7b). While more than 80% of the loaded PDGF-BB was diffused out of native poloxamer hydrogels during the first day, less than 10% of the loaded PDGF-BB was released from aptamer S1-functionalized hydrogels. A scrambled aptamer S-S1 with a randomly mixed sequence induced only a slight reduction in the release rates of PDGF-BB that indicated that the aptamer–protein interactions were highly specific. These results demonstrated that the release rates of proteins could be adjusted by incorporating aptamers with different binding affinities. Such aptamer-based affinity hydrogels are advantageous for protein delivery applications because aptamers can be engineered to interact with any type of proteins with high affinity and specificity. Additionally, nucleic acid aptamers themselves have many merits as affinity ligands, such as their small size, stable structure, and low immunogenicity.[110]



aptamer	K_D (nM)
S1	11.3
M1	27.6
M2	109
M3	354

Fig. 7 (a) Representative secondary structure of an anti-PDGF-BB aptamer. The table shows the equilibrium dissociation constants (K_D) of anti-PDGF-BB aptamers. (b) Cumulative release profiles of PDGF-BB from aptamer-functionalized poloxamer hydrogels. Reprinted with permission from ref. 108. Copyright (2010) American Chemical Society.

3 Applications of injectable hydrogels for tissue engineering

The concept of tissue engineering was proposed by Langer and Vacanti in the early 1990s as "the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure–function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue function".[111] This strategy of tissue engineering generally involves the incorporation of the appropriate cells into a tissue-engineered scaffold, which provides a suitable microenvironment and serves as a temporary extracellular matrix (ECM) until cells produce the matrix along the time and finally neo-tissue replaces the scaffold in the case of a biodegradable scaffold. Tissue engineering approaches mainly consist of the following key components: cells, biomaterial scaffolds and growth factors or other biological or mechanical signals. The scaffold plays an important role in regulating cell migration, proliferation, and ECM production.[112] The scaffolds should provide physical and biological properties such as appropriate mechanical strength with sufficient stability, prevention of cells from floating out of the defect, facilitation of cell proliferation, cell signalling, and stimulation of matrix production by cells. Therefore, the engineering of such scaffolds is an essential requisite for successful tissue engineering.

In the most general sense, tissue engineering seeks to fabricate living replacement parts for the body. In order to create such biologically and functionally active replacements, currently numerous strategies have been used including the incorporation of cells, various growth factors and other bioactive signals into a material scaffold such as the hydrogels. In one method for the implantation of such hydrogels to be achieved, the hydrogels are preformed and processed *in vitro* prior to encapsulating the bioactive agents or cells and the subsequent *in vivo* implantation. In another approach, the cells can be incorporated and suspended in the gel precursors prior to gelation and the gel precursors can be injected into the body as a liquid that forms gel *in situ*. In comparison to its counterpart, the *in situ* polymerization provides the advantage that the gel precursors containing cells can be injected into the defect site through small incisions and their subsequent polymerization enables a homogenous encapsulation of cells within the hydrogel. Since the fluidic precursors of the cell-hydrogel system can fill any irregular defect shapes, hydrogel-based scaffolds are highly suitable for treating defects which are otherwise not easily accessible without an invasive surgical procedure. These injectable hydrogel-based scaffolds can be easily formulated with cells by simple mixing, and do not require a surgical procedure to be implanted or in the case of biodegradable ones, to be removed. Their *in situ* polymerization also results in improved contact between the native tissue and the hydrogel.

3.1 Design criteria for injectable hydrogels in tissue engineering

The injectable hydrogels serve as a synthetic ECM to organize cells into three-dimensional (3D) architecture, and to present stimuli that guide the regulation of the cellular functions during the formation of a desired tissue. Therefore, they are expected to provide a specific biological and mechanical environment to the encapsulated cells. They also assign predefined architecture to the regenerated tissue. Thus, the selection of the appropriate hydrogel scaffold materials is governed by their physical properties, mass transport properties, and the biological interaction requirements of each specific application. These properties or design variables are specified by the intended scaffold application and the environment where the scaffold will be placed.[112–114]

In a rational design for hydrogels in tissue engineering, several variables must be considered with respect to both biochemical and physical properties.[112,114] In general, all of the hydrogel scaffolds used in biomedical applications must be biocompatible and should promote cell growth. Those involving encapsulation of the cell or bioactive agents must be capable of being formed into gels without damaging the cells or compromising activities of bioactive agents. The hydrogel scaffold should degrade into non-cytotoxic segments for easy elimination. Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. The hydrogel must have adequate mass transport properties to allow diffusion of nutrients and metabolites to and from the encapsulated cells and the surrounding tissues. The hydrogel scaffolds are required not only to have sufficient mechanical integrity and strength to withstand manipulations associated with implantation and *in vivo* existence until the cells placed at the application have produced their own functional ECM, but also to provide an appropriate mechanical environment that supports cell migration, proliferation, and differentiation.[115] As each tissue provides its own mechanical microenvironment, the mechanical characteristics of hydrogels used in tissue engineering have to be adapted to each intended application in light of the physical cues that regulate cell function and tissue morphogenesis. Therefore, the physical characteristics of hydrogels used in tissue engineering applications are equally as essential as their biological effects.

3.2 Strategies to form injectable hydrogels

Advances in polymeric materials engineering have offered new opportunities for minimally invasive surgeries that are aimed at minimizing patient trauma and speeding up recovery. To this end, various *in situ* polymerization techniques/hydrogels that use either chemical or physical cross-linking methods, such as photopolymerization, stimuli responsive polymers, multi-functional polymers, self-assembling peptide-based systems, and enzyme-mediated cross-linking systems, have been widely explored for minimally invasive applications. In photopolymerization, the components of the hydrogel along with viable cells are injected in the fluid state into the defect site arthroscopically, followed by subsequent polymerization within the defect site using a light source such as ultraviolet (UV) radiation.[116] The advantage of injectable hydrogels is that they provide spatial and temporal control, as well as a fast curing rate that is obtained under physiological conditions at room temperature. The stimuli-responsive hydrogels consist of polymer networks that can undergo a discontinuous and macroscopic phase transition between the liquid and the solid state when subjected to a small change in one or more environmental stimuli, such as temperature, pH, light, radiation forces, and chemical triggers.[117–121] The *in situ* forming protein-based hydrogel is driven by self-assembly with respect to the temperature, pH, and chemical triggers in the presence of biological fluids.[122–125] This self-assembly is generally mediated by secondary forces such as ionic interactions, hydrogen bonds, hydrophobic interactions, and van der Waals interactions.[123] In addition to their *in situ* gelation capability, these protein-based hydrogels also provide the necessary biochemical cues to support cell proliferation and tissue formation.[125]

The stability and mechanical properties of the resultant hydrogel are closely linked to the method employed to form such a hydrogel. Covalent bonds are usually strong and permanent unlike physical cross-links. Hence, another approach that has been employed for *in situ* hydrogel formation involves the mixing of two precursor solutions that have functional groups which react with each other to form hydrogels. Schiff-base reactions of amine and aldehyde groups and Michael-type addition reactions between the vinylsulfone end

groups and thiol-bearing compounds have been extensively explored for this reason.[126–129] Hydrogels formed by enzyme-mediated cross-linking reactions take advantage of the high substrate specificity of the enzyme to control and predict the cross-linking reaction. Transglutaminase (TG) is one of the typical enzymes that are capable of catalyzing cross-linking reactions. It has the ability to form an amide linkage between the γ-carboxamide group of glutamine residues and primary amines such as the one in lysine.[130] Horseradish peroxidase (HRP) is a single-chain β-type hemoprotein that catalyzes the coupling of phenols or aniline derivatives in the presence of hydrogen peroxide.[131] The hydrogels were formed through the oxidative coupling of phenol moieties, which was catalyzed by HRP and H_2O_2.[132,133] More comprehensive overviews on the mechanisms of gel formation *in situ* by chemical or physical triggering processes are found in excellent recent reports.[15,37,134] Whereas the focus of this review is limited to strategies applied to design *in situ* forming hydrogels with respect to their physical characteristics and biological effects.

3.3 Strategies to enhance biological effects

A large variety of ligand-functionalized materials have been prepared for tissue engineering with the identification of small oligopeptide sequences within ECM proteins. These cell-adhesive ligands have been grafted or incorporated into either synthetic gels or natural biopolymer matrices to enhance the biological effects of the resultant hydrogels.

In recent years, there has been a surge of interest in tissue engineering approaches that use synthetic hydrogels. The usage of synthetic hydrogels has been appealing because it is possible to molecularly tailor their properties, such as their hydrophilic–hydrophobic balance, mechanical and structural properties, degradation profile, *etc.* They have small batch-to-batch variation and are easy to scale up in comparison to naturally derived polymers. However, the vast majority of synthetic materials have inadequate interactions between the substrates and the cells. Approaches to enhance their interaction include the incorporation of bioactive ligands through chemical or physical processes. Lutolf *et al.* have developed a novel class of molecularly engineered synthetic ECM analogs to address a concept of responsiveness to cellular stimuli.[135] These synthetic hydrogels are based on vinylsulfone-functionalized multiarm PEG macromers that are reacted *via* Michael-type addition under mild conditions with cysteine- and biscysteine-containing peptides (Fig. 8). The cysteines were joined by matrix metalloproteinases (MMPs) or a plasmin-sensitive polypeptide in the biscysteine. The monocysteine was functionalized with adhesion peptides such as RGD, a well-known cell adhesion ligand found in fibronectin. The adhesion peptides and the MMP or plasmin-sensitive peptide were incorporated to mimic the two essential biological functionalities of an ECM analog: cell adhesion and degradability. PEG acted as an inert structural platform due to its hydrophilicity and resistance to protein adsorption. Hence, it exhibited the desired biological signals uniquely from incorporated peptides or proteins, with minimal structural or chemical background. The mild gelation conditions allow gel

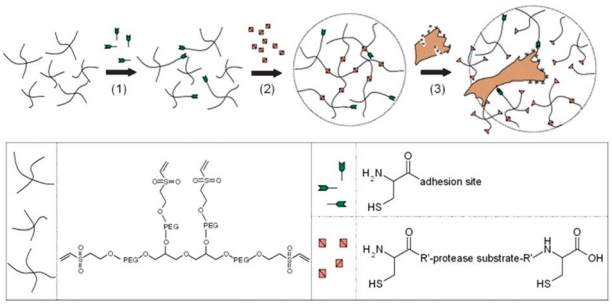


Fig. 8 Michael-type addition reaction between VS-functionalized multiarm PEGs and mono-cysteine adhesion peptides. Reprinted with permission from ref. 135. Copyright (2003) VILEY-VCH Verlag GmbH & Co. KGaA.

formation in the presence of cells.[129,135–137] A synthetic poly(ethylene glycol)-tethered integrin binding peptide (PEG-RGDs) hydrogel formed by photo-cross-linking, combined with an endothelial cell/pericyte precursor co-culture enabled microvascular network formation *in vitro*.[138]

An idea of producing synthetic materials that are able to self-assemble to form fibrillar matrices *in situ* was inspired by the intricate fibrillar architecture of natural ECM components. A class of supermolecular gels formed by self-assembling oligomeric-amphiphiles was designed by Niece *et al.*[139] These gels not only served as scaffolds that biomechanically organize cells in a 3D environment, but also allowed for the incorporation of specific biomolecular signals through electrostatic attraction. Thus, electrostatically driven co-assembly between two peptide-amphiphiles created mixed nanofibers that simultaneously presented two biological signals to cells. The peptide epitopes, such as RGD, IKVAV8 and YIGSR9, were chosen for a demonstration of the biomedical potential of these self-assembling systems (Fig. 9). IKVAV8 and YIGSR9 are laminin sequences known to interact with mammalian neurons. Neural progenitor

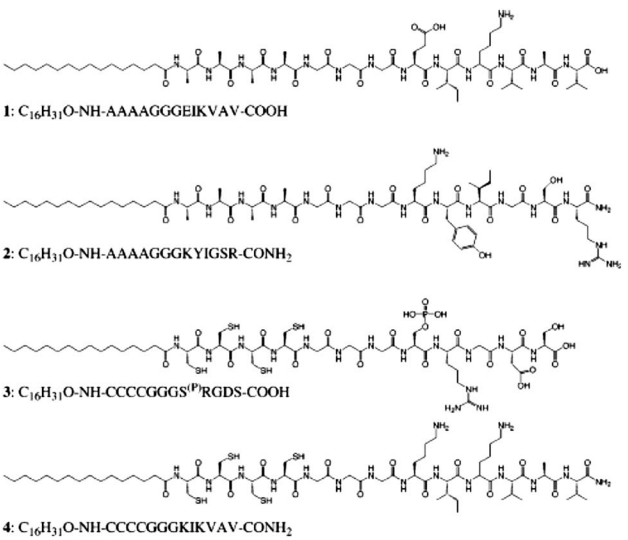


Fig. 9 Chemical structures of four peptide-amphiphiles used for self-assembly. Reprinted with permission from ref. 139. Copyright (2003) American Chemical Society.

cells that were incorporated inside the hydrogels with the laminin-derived peptide IKVAV were observed to differentiate into neurons.[140]

Besides synthetic polymers, natural biopolymers have been modified with bioactive epitopes to enhance their biological effects. A very flexible biomaterial platform was developed to incorporate various bioactive peptides into fibrin gels by Hubbell *et al.*[141–143] On this hydrogel platform, the enzymatic activity of Factor XIIIa was employed to covalently incorporate exogenous bioactive peptides within fibrin during coagulation. Specifically, bi-domain peptides were designed and synthesized that contained a bioactive sequence of interest in one domain as well as a substrate for Factor XIIIa in the second domain. The fibrin gels were formed with incorporated peptides from laminin or heparin-binding peptides. The neurite extension *in vitro* was enhanced when gels were augmented with an exogenous peptide, with the maximal improvement reaching 75%. Park *et al.* have reported an injectable cell-free gelatin–PEG–tyramine hydrogel with Ser-Val-Val-Tyr-Gly-Leu-Arg peptide conjugation through an enzyme-mediated cross-linking reaction (Fig. 10). It was found that the conjugation of the peptide influenced the activity of endothelial cells in surrounding tissues and enhanced angiogenic activity and cell migration in the hydrogel matrix.[144]

Other approaches to enhance the biological effects of injectable hydrogels include combining the inherent benefits in both the natural and synthetic polymers by grafting or in the form of a hybrid hydrogel or through an interpenetrating polymer network (IPN). Wiltsey *et al.* developed an injectable hydrogel based on poly(*N*-isopropylacrylamide)-*g*-chondroitin sulfate (PNIPAAm-*g*-CS) with adhesive properties for nucleus pulposus tissue engineering.[145] The thermal sensitivity allowed this hydrogel to be injectable. Chondroitin sulfate (CS), an ECM component of the native tissue, was grafted into the PNIPAAm matrix to offer the potential for enzymatic degradability, anti-inflammatory activity and bioadhesive characteristics. Sargeant *et al.* reported an injectable hydrogel system composed of Type I collagen and multi-armed PEG containing a reactive succinimidyl ester.[146] It exhibited suitable mechanical and biological properties for tissue engineering.

A hierarchically designed and injectable IPN hydrogel for bone or cartilage tissue engineering applications was recently reported by Geng *et al.*[147] The IPN hydrogel possessed good mechanical properties, controllable degradation and favorable biocompatibility. In this work, a two-step process was introduced to fabricate the injectable hydrogel from oxidized dextran (ODex), amino gelatin (MGel) and 4-arm poly(ethylene glycol)-acrylate (4A-PEGAcr) for cell encapsulation. A primary network was formed based on a Schiff-based reaction between ODex and MGel, and then a UV light-induced radical reaction of 4A-PEG-Acr was used to produce the independent secondary network. Both of the reactions were carried out under physiological conditions in the presence of living cells with no toxicity.

3.4 Strategies to modulate physical characteristics

The previous approaches to engineer artificial tissues have focused largely on optimization of polymer chemistry and selection of appropriate biochemical properties. It is also increasingly recognized that physical parameters are also essential design variables of the substrates used in tissue engineering applications besides biochemical ones.[113] The structure and function of the adherent cell depend in a crucial way on its microenvironment, including the stiffness of its substrate.[148] Cells adhering to a substrate are able to sense the mechanical stimuli and consequently regulate many important physiological processes including cell morphology,[149,150] adhesion,[151,152] migration,[149,153,154] phenotype,[155] differentiation,[156,157] proliferation,[158] apoptosis[159] and gene expression.[160]

Recent developments in producing biocompatible materials and understanding how cells typically react to environmental stimuli in the field of injectable hydrogels have enabled numerous demonstrations in the 3D context. The 3D demonstrations show that cells can be exquisitely sensitive to changes in the mechanical properties of their substrates even when their chemical environment remains unchanged.

From this perspective, the injectable hydrogel scaffold systems with tunable stiffness that were developed in our laboratory enable an independent study on the effect of hydrogel stiffness on various cell functions in a 3D environment.[161–164] The HA–Tyr hydrogel system has demonstrated its ability to control protein and drug delivery, and has showed efficacy in cancer therapy as mentioned in the previous Section 2.2.1. In tissue engineering applications, cells were encapsulated inside the hydrogels instead of incorporating drugs or proteins. HA–Tyr hydrogels with tunable stiffness were explored as biomimetic matrices for caprine mesenchymal stem cells (MSCs) in cartilage tissue engineering.[161] It was found that the tunable three-dimensional microenvironment of the HA–Tyr hydrogels modulated cellular condensation during chondrogenesis and had a dramatic impact on the spatial organization of cells, matrix biosynthesis, and overall cartilage tissue histogenesis. Within higher cross-linked matrices, the cells adopted a more elongated morphology, with a reduced degree of cellular

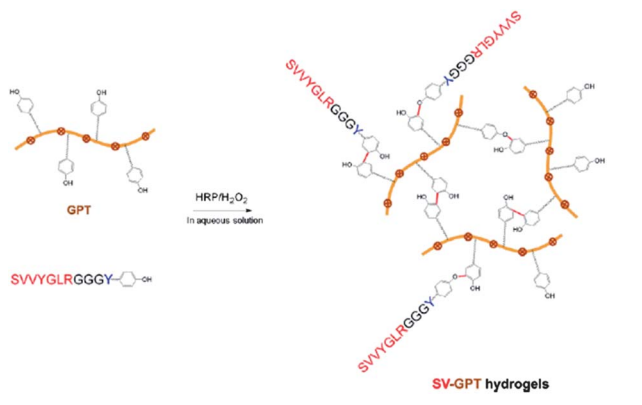


Fig. 10 Schematic representation of the *in situ* SV-Y conjugated gelatin–PEG–tyramine hydrogels (SV-GPT) formed *via* an enzyme-mediated cross-linking reaction using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) in aqueous solution. Reprinted with permission from ref. 144. Copyright (2012) American Chemical Society.

condensation. A lower cross-linked matrix enhanced chondrogenesis with an increase in the percentage of cells with chondrocytic morphology, the biosynthetic rates of glycosaminoglycan and type II collagen, and the hyaline cartilage tissue formation. With increasing cross-linking degree and matrix stiffness, a shift in MSC differentiation towards fibrous phenotypes was observed with the formation of fibrocartilage and fibrous tissues. Besides the HA–Tyr hydrogel, another hydrogel system composed of the gelatin–hydroxyphenylpropionic acid (Gtn–HPA) conjugate was also developed. It was formed by the same oxidative coupling of phenol moieties catalyzed by H_2O_2 and HRP as for the HA–Tyr hydrogel system. The H_2O_2 and HRP were also found to modulate the hydrogel stiffness and gelation rate of the injectable hydrogel, respectively. The Gtn–HPA hydrogel system has enabled an independent study on the effect of stiffness on the proliferation, migration, oxidative stress resistance and differentiation of human mesenchymal stem cells (hMSCs), adult neural stem cells (aNSCs) or human fibroblasts (HFF-1) in 3D.[162–164] It was found that the proliferation of hMSCs inside the Gtn–HPA hydrogel increased with a decrease in the hydrogel stiffness and the neuronal differentiation was also enhanced when the cells were cultured in hydrogels with lower stiffness.[162] Gtn–HPA hydrogels are capable of modulating proliferation and migration of aNSCs *via* their tunable cross-linking. Proliferation and migration of aNSCs were evidently in an inverse trend with the cross-linking degree. Gtn–HPA hydrogels exerted a selective effect where aNSCs driven towards the neuronal lineage exhibited a higher survival than those driven towards the astrocytic lineage. Furthermore, the soft Gtn–HPA hydrogel increased the proportion of aNSCs expressing the neuronal marker β-tubulin III to a greater extent than that expressing the astrocytic marker glial fibrillary acidic protein, thus indicating an enhancement in differentiation towards the neuronal lineage.[163] The cell proliferation rate of HFF-1 in Gtn–HPA hydrogels was strongly dependent on the hydrogel stiffness, with a dimensionality-specific response.[164] In the 2D studies, the HFF-1 exhibited a higher proliferation rate when the stiffness of the hydrogel was increased. In contrast, the HFF-1 cultured inside the hydrogel remained non-proliferative for 12 days before a stiffness-dependent proliferation profile was shown. The proliferation rate decreased with an increase in the hydrogel stiffness in a 3D culture environment, unlike in the 2D culture environment.

In an effort to develop a stable yet native ECM mimetic hydrogel system, a novel approach for designing injectable hydrogels with tunable swelling properties without increasing chemical cross-linking was reported by Oommen *et al.*[165] These hydrogels utilized a more stable hydrazone linkage by the delocalization of the positive charge, and consisted of modified hyaluronic acid (HA) with the hydrazone linkage. The hydrogels showed exceptional stability with controlled swelling and enzymatic degradation. In this study, the hydrazine cross-linked hydrogels with HA aldehyde and three different HA hydrazides, namely carbodihydrazide (CDH), oxalyldihydrazide (ODH) and adipoyldihydrazide (ADH), were compared (Fig. 11). It was found that grafting CDH hydrazide can deliver an exceedingly stable hydrazine linkage, which is nearly 15 fold more stable than the others in the study. *In vivo* evaluation of this material with CDH hydrazide confirmed its efficacy for bone tissue regeneration.

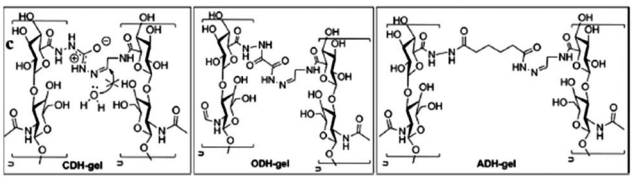


Fig. 11 Representative chemical structure of the three types of hydrazine cross-linked HA hydrogels. Reprinted with permission from ref. 165. Copyright (2013) VILEY-VCH Verlag GmbH & Co. KGaA.

To decrease and potentially eliminate hydrogel syneresis due to the hydrophilic to hydrophobic transition and the coil to globule chain collapse associated with thermo-responsive *in situ* forming hydrogels, a novel two-component hydrogel system was developed through a physical and chemical dual-gelation mechanism by Ekenseair *et al.*[166] This injectable hydrogel consisted of a PNIPAAm-based macromer with pendant epoxy rings and a hydrolytically degradable polyamidoamine-based diamine cross-linker (Fig. 12). The epoxy-amine cross-links were shown to be rapid and facile with the reaction reaching completion in less than 3 h after an initial thermogelation time of 2 to 3 s. It offers a promising and versatile family of injectable *in situ* forming hydrogels with a dual-hardening mechanism, high stability, tunable water content and degradability for tissue engineering.

The feasibility of introducing an inorganic phase into an injectable hydrogel has also been proposed as a promising choice to form an organic–inorganic composite that mimics natural tissue for bone tissue repair. Injectable bone cement composed of nanocrystalline apatite and cross-linked HA–Tyr conjugates was developed (Fig. 13).[167] The mechanical strength of the apatite/HA–Tyr cement was tuned by varying the apatite loading and H_2O_2 concentration. This rapid enzyme-mediated setting of our bone cement results in minimal heat release as compared to conventional bone cement. The crystalline phase and crystallite size (20 nm) of the apatitic phase in our bone cement matched those of trabecular bone. This biocompatible bone cement also successfully healed small bone and joint defects in mice within 8 weeks. Dessi *et al.* have reported the design of an injectable and degradable paste of polycaprolactone (PCL) reinforced with nanocrystals of hydroxyapatite for the application of local bone tissue repair in low-load areas.[168] The sol–gel method to prepare this nanohydroxyapatite allows a composite paste to be obtained with homogeneously distributed nanometric hydroxyapatite particles, which resemble the natural bone ECM.

4 Other biomedical applications

4.1 Localized gene delivery systems

Injectable hydrogels have also been utilized for local delivery of nucleic acid drugs, such as small interfering RNA (siRNA) and

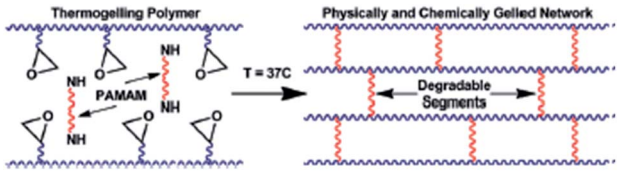


Fig. 12 Schematic representation of the hydrogel through a physical and chemical dual-gelation mechanism. Reprinted with permission from ref. 166. Copyright (2012) American Chemical Society.

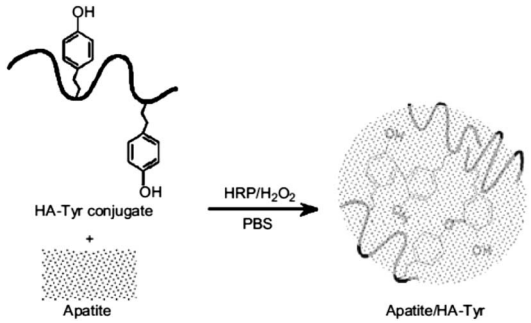


Fig. 13 Enzyme-mediated cross-linking of the apatite/HA–Tyr cement. Reprinted with permission from ref. 167. Copyright (2008) Elsevier Ltd.

plasmid DNA. Particularly, siRNA is emerging as a therapeutic tool for the treatment of numerous genetic disorders because it silences the expression of a target gene in a highly specific manner.[169] Krebs *et al.* have reported localized siRNA delivery systems based on cross-linked alginate hydrogels.[170] In these systems, the encapsulated siRNA molecules were released from the alginate gels for ~1 week in a sustained manner. These hydrogels substantially inhibited gene expression in incorporated and neighbouring cells for 3–6 days.

In general, free siRNA is not readily internalized by cells because of its strong negative charge. Additionally, it is susceptible to enzymatic degradation by nucleases when administered into the body.[171] In order to address these issues, siRNA has been incorporated into various types of nanoparticulate carriers, such as polyelectrolyte complex micelles and polymeric nanoparticles.[172–174]

Recently, Kim and colleagues have developed injectable and biodegradable poly(organophosphazene) hydrogels for the long-term delivery of siRNA.[175,176] These hydrogels were composed of poly(organophosphazene) substituted with hydrophobic L-isoleucine ethyl ester (IleOEt) and hydrophilic α-amino-ω-methoxypoly(ethylene glycol) (AMPEG). The modified poly(organophosphazenes) exhibited thermo-sensitive sol–gel transition behaviour *via* an intermolecular association of hydrophobic peptide chains.[175] The poly(organophosphazenes) were further conjugated with protamine, a cell penetrating peptide (Fig. 14), for the intracellular delivery of siRNA.[176] The resulting protamine-poly(organophosphazene) conjugates were able to form 30 nm sized polyplexes with siRNA through electrostatic interactions.

Upon injection into the body, the polyplex solution turned into a macroscopic hydrogel *via* self-assembly of hydrophobic peptide chains at the body temperature. The *in situ* formed gel slowly released the entrapped polyplexes for up to 28 days. VEGF was selected as the therapeutic target because it regulates angiogenesis processes essential for the survival and metastasis of rapidly growing tumours.[177] An intra-tumoural injection of VEGF siRNA solution induced only a marginal degree of tumour regression, which suggested that the free siRNA molecules were quickly diffused from the site of injection and cleared by the body. In contrast, the treatment of a polyplex hydrogel carrying VEGF siRNA markedly inhibited the tumour growth for 28 days with only a single injection. The reduction in the amount of VEGF in the tumours was also observed. Taken together, these results revealed that this hydrogel system enabled long-lasting and localized gene silencing by releasing the cell penetrable polyplexes in a sustained manner.

4.2 Biomedical adhesives

Over the past decades, biomedical adhesives have gained tremendous interest as useful components of the surgical toolbox. These adhesives have been widely utilized as tissue sealants and hemostatic agents to minimize bleeding in surgery, reduce operative time, and promote wound healing.[178] Representative examples of clinically available adhesives are fibrin glue and cyanoacrylates. Fibrin glue is a biological adhesive formed from concentrated fibrinogen and thrombin. Although fibrin glue offers improved tissue healing with excellent biodegradability, its use has been limited to low-pressure bleeding because of insufficient bonding strength.[179] In addition, there are concerns about the transmission of blood-borne pathogens as a result of the use of pooled human plasma.[180] Cyanoacrylate-based adhesives provide relatively strong adhesion to tissues, but they release heat *via* the exothermic reaction and decompose into toxic substances (*e.g.*, cyanoacetate, formaldehyde), often limiting their use to skin wound closure.[178,181] Furthermore, both fibrin glue and cyanoacrylates are not effective for internal organ surgeries because they adhere weakly to moist tissue surfaces.

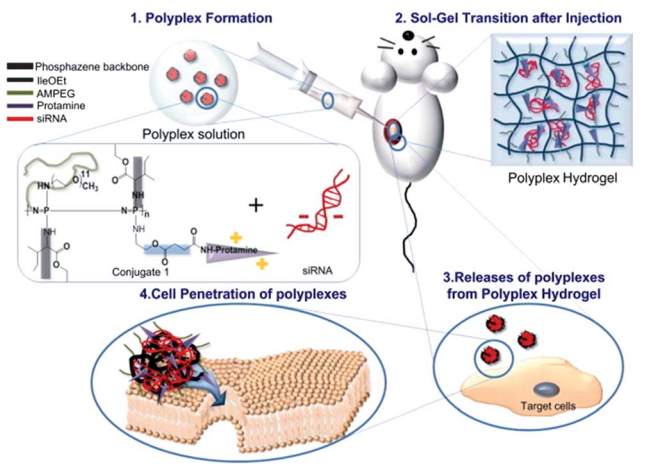


Fig. 14 Poly(organophosphazene) hydrogel self-assembled from cell penetrable polyplexes for effective localized siRNA delivery. Reprinted with permission from ref. 176. Copyright (2013) Elsevier Ltd.

There have been significant efforts to develop hydrogel-based adhesives as an alternative to the conventional adhesives. However, it has been considered difficult because most swollen hydrogels do not adhere strongly to tissues.[182] In recent years, researchers have exploited the adhesive strategy of marine mussels to enhance adhesiveness of hydrogels.[183,184] It is known that the mussels produce specialized adhesive proteins to stick to underwater substrates. These proteins are rich in 3,4-dihydroxy-L-phenylalanine (DOPA), a catechol-containing amino acid.[185] It has been shown that DOPA plays an essential role in the strong adherence of mussels to organic surfaces. Slightly alkaline conditions in the ocean trigger oxidation of DOPA to highly reactive DOPA–quinone, which can be covalently coupled with primary amines available on the organic substrates *via* Schiff-base formation or Michael-type addition reactions.[186] In addition, DOPA is able to interact non-covalently with the substrates through hydrogen bonding and π–π stacking.[187] Such versatile and moisture-resistant adhesive properties of DOPA make mussels effectively adhere to virtually all types of organic surfaces in a material-independent manner.

Messersmith and colleagues have developed mussel-inspired adhesives based on DOPA-functionalized four-arm PEG (PEG–DOPA) precursors (Fig. 15a).[188–190] Upon application, PEG–DOPA hydrogels were formed in less than 1 min *via* the intermolecular cross-linking of oxidized DOPA moieties.[188] An *ex vivo* study using punctured human fetal membranes demonstrated that these hydrogels provided superior durability and sealing properties over fibrin glue in a moist environment.[189] The adhesive hydrogel was also utilized as an injectable tissue sealant to immobilize pancreatic islets onto the surface of the epididymal fat pad in diabetic mice (Fig. 15b).[190] Histological analysis revealed that the immobilized islets were intact and well vascularised for at least 4 months, indicating that the hydrogels provided excellent tissue adhesion with little inflammatory response (Fig. 15c). Moreover, the gel-immobilized islets had hypoglycemic effects comparable to those transplanted in the liver. These results suggested that the adhesive material did not interfere with the viability and function of the engrafted islets. Despite their remarkable stability and adhesive properties, the applicability of PEG–DOPA hydrogels was restricted because they were not biodegradable. To circumvent this problem, Brubaker *et al.* incorporated an elastase-cleavable Ala–Ala dipeptide substrate into the polymer structure.[191] The hydrogels formed from the modified PEG–DOPA slowly degraded over several months after subcutaneous implantation in mice.

Hydrolytically degradable mussel-inspired adhesives were also reported by Mehdizadeh *et al.*[192] In this study, a family of injectable citrate-based mussel-inspired biodegradable adhesives (iCMBAs) was synthesized by using a condensation polymerization reaction between PEG, citric acid, and DOPA or dopamine. These adhesives were degradable under physiological conditions through a hydrolytic cleavage of ester linkages. The iCMBAs were utilized as hemostatic agents for sutureless wound closure (Fig. 16). Aqueous solutions of iCMBAs readily formed hydrogels upon mixing with sodium periodate solution. As an oxidizing agent, periodate ions initiated the cross-linking reaction of DOPA moieties present in the backbone of the iCMBA polymers. The resultant iCMBA network can adhere strongly to surrounding tissues *via* DOPA-mediated formation of covalent linkages and hydrogen bonding. An *in vitro* lap shear strength test revealed that the adhesion strength of iCMBAs was 2.5–8.0 times stronger than that of fibrin glue under wet tissue conditions presumably due to moisture-resistant adhesive properties of the DOPA moieties. *In vivo* animal studies have revealed that iCMBA effectively closed open wounds created on the back of rats within a few minutes. After 28 days, the iCMBA-treated skin was found to have much higher tensile strength than the suture-closed skin, suggesting that these adhesives enhanced healing of the wounds. Histological evaluation indicated that iCMBA was completely degraded and absorbed in rats while eliciting only minor acute inflammation. Such

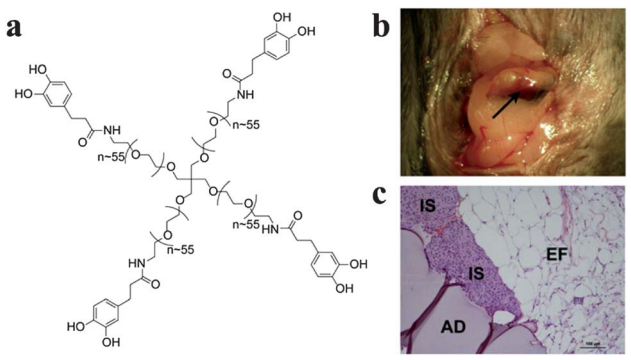


Fig. 15 (a) Chemical structure of the PEG–DOPA adhesive precursor. (b) Photographic image of adhesive-immobilized islet bolus on the epididymal fat pad surface. The black arrow indicates the location of PEG–DOPA adhesives. (c) Representative micrographic image of hematoxylin and eosin (H&E)-stained graft explants. Adhesive, AD; islet, IS; epididymal fat tissue, EF. Reprinted with permission from ref. 190. Copyright (2009) Elsevier Ltd.

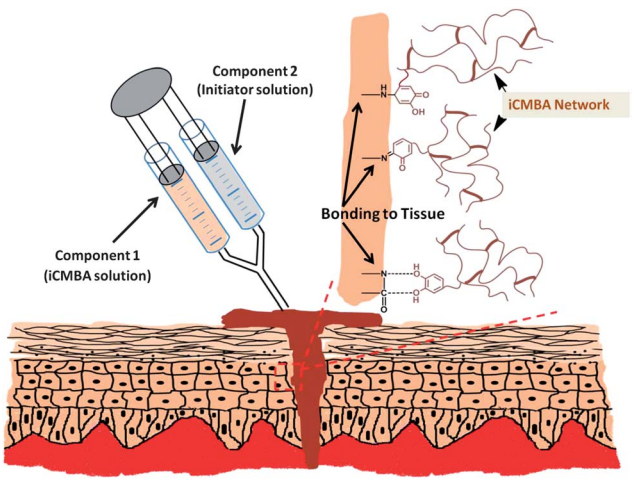


Fig. 16 Injectable citrate-based mussel-inspired biodegradable adhesives (iCMBAs) for sutureless wound closure. Solutions of iCMBA and sodium periodate can be easily applied to a wound by using a double-barreled syringe. The *in situ* formed iCMBA network is able to adhere strongly to surrounding tissues through covalent linkages and hydrogen bonding. Reprinted with permission from ref. 192. Copyright (2012) Elsevier Ltd.

hydrogel-based adhesives are expected to be broadly applied for both external and internal applications.

5 Conclusion and future perspectives

In our review, we have summarized the current design strategies and biomedical applications of injectable biodegradable hydrogels. These hydrogels allow for the administration of bioactive molecules and cells without the need for surgical procedures. Moreover, they can reduce patient discomfort, recovery time, and the risk of infection. These unique characteristics have gained injectable hydrogels considerable attention for their potential as controlled protein delivery systems. Several important criteria such as gel network structure, drug release mechanism, biodegradability, and safety issues should be carefully considered when designing protein-releasing hydrogels. A variety of approaches have also been developed in attempts to achieve the sustained release of protein drugs while minimizing the initial burst release. Although remarkable progress has been made in controlling the release kinetics of protein drugs, the current approaches need to be further improved for the clinical use of hydrogel-based delivery vehicles.

The use of injectable hydrogel systems in the field of tissue engineering is attractive in light of the drive towards clinical applications owing to their easy integration into the existing clinical procedures. Developments in materials science provide opportunities for the rational design of injectable hydrogels with appropriate physical characteristics and biological effects for intended tissue engineering. Both biochemical and mechanical signals interplay to guide cell functions and tissue regeneration. Therefore, new tissue engineering approaches aimed at regenerating lost or diseased tissues must incorporate both biochemical and physical design variables, in order to most effectively induce tissue repair and potential organ regeneration in the future. Despite tremendous efforts to design novel hydrogels for tissue repair and regeneration, challenges to reproduce the complexity of the biological microenvironment still remain. In-depth research on the basic mechanisms that involve interactions between cells and ECM, as well as other biological signals during tissue repair is helpful in proposing multiple integrated approaches to customize the physical or biological properties of the materials tailored to the tissue of interest.

Recent advances in polymer science and nanotechnology are broadening the applicability of injectable hydrogels. Nanoscale gene delivery systems with cell-penetrating abilities are rationally designed and incorporated into injectable hydrogels for the long-term delivery of nucleic acid drugs. The combination of mussel adhesive proteins and injectable hydrogels creates a new type of adhesive providing excellent biocompatibility and strong adhesiveness to moist tissues. In addition, significant efforts have been made to develop intelligent hydrogel systems through integrating stimuli-sensitive moieties into the hydrogel structures. These systems can be designed to sense biological or environmental cues in the body and self-regulate their physical characteristics in a real-time manner. Such emerging injectable hydrogels hold great potential in overcoming the limitations of conventional hydrogels.

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