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Injectable hydrogels based on poly(ethylene glycol) and derivatives as functional biomaterials

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Hydrogels based on poly(ethylene glycol) (PEG) and derivatives have attracted significant interest in recent years given their capacity to be well-tolerated *in vivo* in the context of drug delivery and tissue engineering applications. Injectable, *in situ*-gelling analogues of such hydrogels offer the additional advantages of being easy and non-invasive to administer *via* the injection of low-viscosity precursor polymer solutions, expanding their scope of potential applications. In this highlight, we first review the design criteria associated with the rational design of *in situ*-gelling hydrogels for *in vivo* applications. We then discuss recent progress in the design of injectable PEG hydrogels, specifically highlighting our ongoing work on PEG-analogue hydrogels based on poly(oligoethylene glycol methacrylate) for targeted biomedical applications.

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1 Introduction

Hydrogels are water-swollen polymeric networks capable of absorbing large quantities of water and biological fluids while maintaining a distinct three-dimensional structure.¹ The high water content of hydrogels, combined with the structural similarity of hydrogels to natural extracellular matrix (ECM),² makes hydrogels attractive synthetic materials for biomedical devices including drug release depots,^{3–5} cell delivery scaffolds,^{6–8} functional tissues,^{9–11} or wound dressings.¹² The cross-linked, three-dimensional structure, while key to the ultimate applications of hydrogels, also severely limits the ability of

hydrogels to be delivered inside the body by minimally invasive routes (*i.e.* non-surgical procedures such as injections). As a result, significant focus has shifted to the design of “injectable” or “*in situ* gelling” hydrogels that form after injection *in vivo*.^{13,14} The hydrogel precursor solution can be introduced in a minimally invasive manner directly to the area of interest, significantly reducing patient discomfort, risk of infection, recovery time, and treatment cost. Once injected, hydrogel formation occurs through either physical cross-linking, typically exploiting the physiological conditions in the target tissue, or by chemical cross-linking, exploiting click and/or dynamic covalent bond chemistry.^{15–18} For many applications, the use of chemical cross-linking is preferred to improve the mechanical properties of the soft material, preserve the integrity of the hydrogel over an externally-programmed period of time (based on the type and

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density of cross-links present), and enhance the chemical flexibility of hydrogel in terms of its ability to be easily functionalized with physical or biological cues that improve its function and/or compatibility with the surrounding tissue.¹⁹

In this highlight, the relevant design criteria for injectable hydrogels will be briefly outlined and illustrated by reviewing a number of examples from the current state-of-the-art in injectable PEG hydrogels. The highlight will be concluded with a summary of our recent work on injectable PEG-analogue poly(oligoethylene glycol methacrylate) (POEGMA) hydrogels to illustrate the potential of these biomaterials as a potential substitute for conventional PEG-based biomaterials.

2 Design criteria for covalently cross-linked injectable hydrogels

The clinical lifetime of an injectable hydrogel can be divided into three separate stages: (i) pre-gelation (precursor polymers in solution), (ii) therapeutic window (after injection and gelation) and (iii) degradation (hydrogel degradation products). Rational design of injectable hydrogels thus requires careful consideration of the synthetic and biological aspects of the precursor polymers, resulting hydrogel, as well as the hydrogel degradation products. The key design criteria will be discussed accordingly, as schematically depicted in Scheme 1.^{14,16,18}

Precursor polymers

Hydrogel precursor polymers can be based on naturally occurring polymers (e.g. collagen,²⁰ fibrin,^{21,22} hyaluronic acid,²³ alginate,²⁴ agarose,²⁵ or dextran²⁶) or synthetic polymers (e.g. poly(*N*-isopropylacrylamide) (PNIPAAm),^{27,28} polyacrylamide (PAAm),²⁹ poly(hydroxyethyl methacrylate) (PHEMA), poly(vinylpyrrolidone) (PVP), poly(ethylene glycol) (PEG) or other water-soluble polymers;^{4,11,30}). Although most natural polymers can be directly cross-linked by small bifunctional molecules such as epichlorohydrin or glyoxal,³¹ a large variety of chemistries has been developed to functionalize these polymers with cross-linkable functional groups and thus avoid the need

for often toxic small molecule cross-linkers.³² Synthetic polymers can be functionalized directly through the use of functional comonomers and/or *via* post-polymerization modification. Regardless of the chemical nature of the precursor, the precursor polymer needs to be stable, non-cytotoxic, and not elicit a significant inflammatory response from the patient's immune system upon injection. This latter point is especially challenging in the context of covalently cross-linked injectable hydrogels since chemically-driven gelation requires that the hydrogel precursors are functionalized with reactive functional groups that are not necessarily bio-orthogonal³³ (e.g. amine, aldehyde, or thiol groups that are also present in, or cross-reactive with, polypeptides including enzymes, membrane proteins, *etc.*) However, it must be emphasized that the toxicity of functional polymeric precursors is typically low compared to low molecular weight chemicals with the same functionalities. For example, low molecular weight vinylsulfones (used in Michael-type addition cross-linking with thiols) demonstrate significant toxicity, but polymer-bound vinyl sulfones cannot enter cells and thus do not undergo toxic reactions with glutathione and DNA.^{34,35} Similarly, the use of low molecular weight glutaraldehyde for gelation has been reported to induce toxicity,^{36,37} but Hoffmann and co-workers noted no apparent cytotoxic effects associated with chitosan–glutaraldehyde hydrogels cultivated with ATDC5 and chondrocytes up to 14 days.³⁸ In our experience, both natural (oxidized dextran or carboxymethyl cellulose)^{39,40} as well as synthetic aldehyde-functionalized precursors do not show any significant toxicity either *in vitro* (*via* an MTT assay on 3T3 mouse fibroblasts at concentrations up to 2000 $\mu\text{g mL}^{-1}$)^{40,41} or *in vivo* (*via* subcutaneous injection in BALB/c mice).^{41,42}

The precursor polymers are likely to be stored for a considerable amount of time prior to clinical use and thus need to demonstrate high stability in aqueous solution. Furthermore, as administration occurs through a needle, the precursor solutions need to be of sufficiently low viscosity, or at least possess sufficient shear-thinning properties, to allow for easy injection. For routine applications, the maximum needle size for injections is 25G;⁴³ however, many ophthalmic or cosmetic applications require as small as a 33G needle sizes. This requirement makes both molecular weight control and polymer architecture very important in the design of a scaffold.

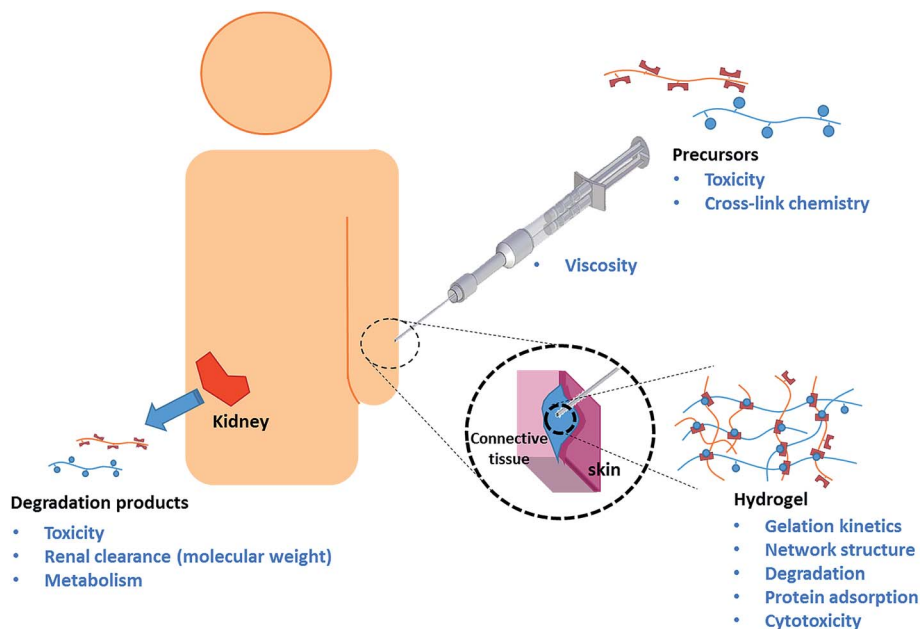
Hydrogels

For successful *in vivo* application, gelation (*i.e.* cross-linking of the precursor polymers) must occur at physiologically relevant conditions (*i.e.* $5.5 \leq \text{pH} \leq 7.5$ and $T = 37 \pm 2$ °C) relatively quickly (*i.e.* seconds to minutes) following injection. The emergence of click chemistries has introduced a variety of cross-linking approaches that are advantageous to the design of injectable hydrogels,⁴⁴ most notably copper-catalyzed alkyne azide reactions (CuAAC),⁴⁵ strain promoted azide-alkyne click cycloadditions (SPACC),⁴⁶ thiol-Michael additions,⁴⁷ Diels–Alder cycloaddition,⁴⁸ disulfide formation,^{49,50} oxime chemistry,⁵¹ and Schiff base⁵² formation. While not all of these cross-link chemistries are strictly bioorthogonal, *in vitro* and *in vivo*



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pending patent applications, and won the John Charles Polanyi Prize in Chemistry from the Government of Ontario recognizing his early career accomplishments.



Scheme 1 Schematic representation of the various design criteria for injectable hydrogels.

toxicity of such chemistries can be minimized by controlling gelation kinetics, as fast gelation kinetics reduce the amount of time that the non-bioorthogonal functional groups are exposed to native tissue prior to consumption *via* cross-linking. Gelation kinetics can be controlled by varying precursor polymer concentration, the number of reactive groups per chain, and/or the molecular weight of the precursor polymers.^{53–57} Relatively fast gelation kinetics are typically beneficial in an injectable system to minimize the diffusion of precursors from the site of injection, which may cause adverse effects in neighboring tissues and/or prevent hydrogel formation entirely if the dilution is too significant. However, it is important to minimize the formation of structural and mechanical defects in the hydrogel network, which directly influences the diffusivity of therapeutics, nutrients, oxygen and (toxic) waste products. Controlling the cross-link density of hydrogels allows for the design of injectable hydrogels that mimic a broad range of tissue mechanics ranging from soft brain tissue (0.2–1 kPa) to relatively stiff cartilage or precalcified bone tissue (20–60 kPa).⁵⁸ However, in most *in situ* gelling systems, cross-link density and gelation kinetics are inextricably linked (as both are governed by the degree of precursor polymer functionalization), representing a challenge in rational hydrogel design of highly cross-linked gels that can still be delivered by injection or weaker gels that need to be gelled quickly.

Aside from certain applications (*e.g.* dermal fillers),⁵⁹ injectable hydrogels must be either biodegradable^{60,61} or bio-erodible⁶² to avoid surgical removal after its therapeutic lifetime has expired. In tissue engineering applications, controlled degradation is even more important as a functional aspect of the hydrogel that provides temporal cues to regulate cell proliferation and migration within the hydrogel matrix.⁶³ Whereas injectable hydrogels based on natural polymers are intrinsically degradable as a result of enzymatic activity *in vivo*, the

degradation of synthetic precursor hydrogels must be programmed into the hydrogel design by using reversible chemistries that both cross-link the hydrogel and provide a hydrolyzable or oxidative link that facilitates degradation over time. The majority of biorthogonal cross-link chemistries listed earlier are reversible and therefore perfectly suited for the synthesis of injectable hydrogels.

Similar to the precursor polymers, the hydrogel itself must not elicit an inflammatory response once formed *in vivo*.⁶⁴ The ability of the hydrogel to “mask” itself from the host’s immune system by repelling protein adsorption is crucial to its success both short and long term. Protein adsorption is generally recognized as the first step of an inflammatory cascade that recruits inflammatory macrophages to the site of injury, initiates the formation of granulation tissue and (eventually, at least for slow or non-degrading materials) creates a fibrotic capsule around the material.^{65,66} This “walling-off” is particularly problematic for hydrogels designed for drug release or tissue regeneration applications as it inhibits the effective diffusion of drugs, nutrients, and/or waste products between the hydrogel and surrounding tissue.⁶⁷ Although hydrogels are less prone to protein adsorption relative to most biomaterials due to their hydrophilic and highly hydrated nature, the choice of polymeric components in the hydrogel is crucial to further reduce protein adsorption. PEG is still considered the gold-standard polymer to minimize protein adsorption, with PEG derivatives *e.g.* poly(oligoethylene glycol) (meth(acrylate)),^{68,69} natural polymers,⁷⁰ polyoxazolines,⁷¹ and zwitterionic polymers⁷² also demonstrating favorable and, in some cases, superior protein repellency properties.

Degradation products

Hydrogel degradation can occur *via* direct degradation of the polymer backbone and/or degradation of the cross-links

between the individual polymer chains. Degradation of the polymer backbone may proceed *via* an oxidative,⁵⁰ hydrolytic,^{28,73} and/or enzymatic mechanism.⁷⁴ The rate of degradation depends on the chemical structure of the polymer and the cross-link density, which govern both the thermodynamics of the bond stability as well as the kinetics of diffusion of the bond-breaking stimulus (*e.g.* water, enzymes, *etc.*) to the degradation site. Most biopolymers degrade *via* one or more of these mechanisms, resulting in non-toxic degradation products that can be metabolized by the body. Examples of synthetic polymers that contain either hydrolytically or enzymatically cleavable fragments in the polymer backbone have been reported⁷⁵ but require elaborate synthesis. Degradation of the cross-links^{76,77} is therefore the commonly used clearance mechanism for synthetic hydrogels. Consequently, the degradation products of the hydrogel are structurally similar or in some cases identical to the original precursor polymers. Since the carbon-carbon backbone synthetic precursors cannot be metabolized by the body, the molecular weight of such precursor polymers needs to be carefully controlled to prevent bioaccumulation and ensure renal clearance following degradation. Typically, a molecular weight cut-off of $20 \times 10^3 - 60 \times 10^3 \text{ g mol}^{-1}$ has been reported as the kidney clearance limit.^{78,164}

Recent progress in the design of self-immolative polymers which degrade systematically following an external stimulus to remove a capping agent should also be noted as a potentially promising strategy for driving hydrogel degradation.^{79,80} By controlling the stability of the capping material, programmed or even on-demand timed degradation may be achievable using this type of strategy. While current chemistries for the design of self-immolative polymers are not directly amenable to *in situ* gelation and some concerns persist regarding the ultimate toxicity of the degradation products resulting from many of these proposed mechanisms,² this strategy may be increasingly

important moving forward as the chemistry of self-immolative polymers continues to develop.

3 Current state-of-the-art of injectable poly(ethylene glycol)-based hydrogels

Polyethylene glycol (PEG) is a synthetic polymer that has found wide-spread application in the design of biomaterials due to its demonstrated non-cytotoxic, non-immunogenic and protein repellent properties that effectively mask it from the host's immune system.⁸¹ Hydroxyl-terminated di- and multifunctional PEG polymers can readily be functionalized with a variety of desired functional groups (*e.g.* aminoxy,⁵¹ azide,^{82,83} thiol,⁸⁴ vinyl sulfone,⁸⁵ or maleimide,⁴⁶) and cross-linked into PEG hydrogels using a step-growth polymerization approach,⁴ resulting in highly organized PEG hydrogel networks.⁸⁶ These PEG hydrogels have been demonstrated to provide excellent synthetic matrices for the controlled release of therapeutics^{4,30} as well as tissue engineering applications, particularly when coupled with functional biomacromolecules such as the cell adhesive peptide RGD to enable PEG to effectively support cell growth.⁸⁷⁻⁸⁹ The following discussion will highlight specific cross-linking chemistries that have been reported to generate injectable PEG-based hydrogels.

Azide-alkyne cycloadditions

The cycloaddition of an azide with a terminal alkyne has received considerable interest for the design of hydrogels due to the high selectivity and bioorthogonality of this cross-linking chemistry. The copper(I)-catalyzed alkyne-azide (CuAAC) click reaction⁹⁰ is of particular interest as it proceeds under physiological conditions. Ossipov and co-workers⁹¹ reported the first

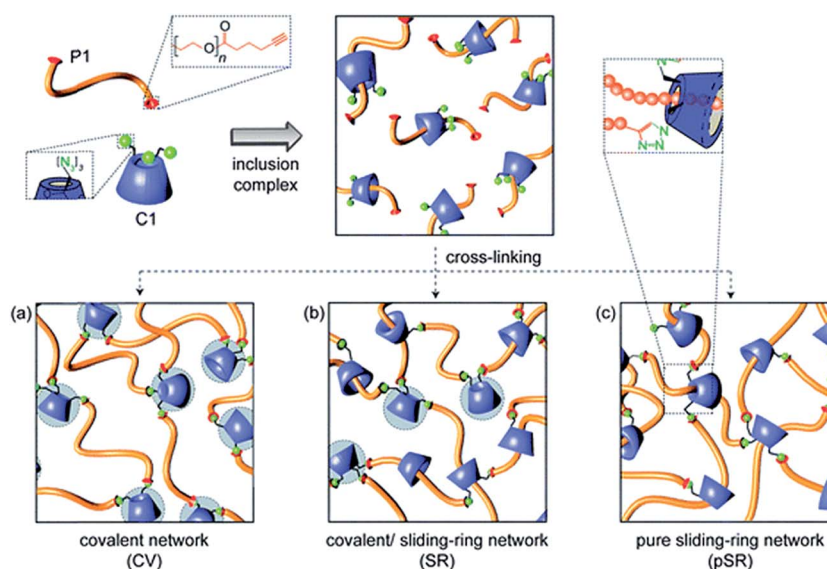


Fig. 1 Schematic representation of the formation of α -cyclodextrin (α CD)-polyethylene glycol (PEG) hydrogels through the formation of alkyne-azide chemistry (CV), inclusion complexes (pSR) and a combination of both (SR). Reproduced from ref. 95 with permission from RSC Publishing.

azide-alkyne cross-linked hydrogel for a poly(vinyl alcohol) (PVA) system. A number of PEG-based azide-alkyne cross-linked hydrogels have since been reported. For example, Adzima and co-workers⁹² reported a PEG hydrogel based on 4-arm PEG azide and a bifunctional PEG alkyne. This group demonstrated temporal and spatial control over the CuAAC click reaction by using standard photolithographic techniques to photochemically reduce Cu(II) to Cu(I). This technique offers the ability to selectively pattern hydrogels to *e.g.* promote cell adhesion and could drastically reduce the amount of Cu(I) required for the synthesis of functional and well-defined PEG hydrogels. The reduction of the Cu(I) concentration is particularly relevant as the associated cytotoxicity of copper ions and reactive oxygen species generated by copper ions *in vivo* may pose a limitation towards clinical application.¹⁹ Alternately, PEG-based hydrogels have been prepared based on mixtures of PEG with other functional entities.^{92–94} For example, Tan and co-workers⁹⁴ developed a PEG hydrogel based on PEG-alkyne and an azido-functionalized α -cyclodextrin (α CD) that can cross-link through azide-alkyne chemistry as well as the formation of PEG- α CD inclusion complexes, providing cooperative control over both cross-link density and degradation mechanism/time (Fig. 1).⁹⁵

Strain-promoted alkyne-azide cycloaddition (SPAAC)⁹⁶ has been reported as a promising alternative to CuAAC, as cross-linking can occur under physiological conditions in the absence of Cu(I). Examples of SPAAC cross-linked PEG hydrogels have been reported by Anseth's group,^{83,97} which demonstrated cross-linking of a 4-arm PEG tetraazide and a bis(cyclooctyne)-peptide. Zheng and co-workers⁹⁸ reported an injectable PEG hydrogel based on 4-dibenzocyclooctynol (DIBO) functionalized difunctional PEG and a 3-arm glycerol exytholate triazide. These hydrogels formed within 5 minutes with a

modulus of ~ 0.8 kPa and were capable of sustaining hMSC cell viability for 24 hours *in vitro*. Furthermore, the polymer precursors did not exhibit significant cytotoxicity at relevant concentrations for hydrogel preparation.⁹⁸ The high specificity, fast cross-linking kinetics, and excellent bioorthogonality will drive continuing interest in SPAAC, although the currently cumbersome synthesis of the cyclooctynes does at least now limit the broad applicability of this chemistry.

Michael additions

The 1,4-addition of a nucleophile to the β position of an α,β -unsaturated carbonyl compound such as an aldehyde or ketone, commonly referred to as a Michael-type addition, is a widely investigated approach for injectable hydrogel preparation. This cross-link chemistry occurs spontaneously under physiological conditions, especially when thiols are used as the nucleophiles,^{99,100} and the formed thioether bonds are relatively weak and can be reversible.^{101–105} Different α,β -unsaturated electrophiles have been used in conjunction with thiols for the preparation of injectable hydrogels.^{106–110} Although acrylates are the most commonly used electrophiles for Michael additions, maleimides^{48,105,111} and vinyl sulfones^{85,112–116} offer higher rates of cross-linking and have attracted more recent attention for this purpose. García and co-workers¹¹⁷ demonstrated the advantages of these alternative electrophiles in preparing functional PEG hydrogels by cross-linking a thiol-containing adhesive peptide (GRGDSPC) with 4-arm PEG containing acrylate (PEG-4-AC), vinyl sulfone (PEG-4-VS), or maleimide (PEG-4-MAL) functional groups. In the presence of 4 mM triethanolamine (TEA), gelation kinetics depended considerably on the choice of Michael acceptor, ranging from 1–5 min for PEG-4-MAL, 30–60 min for PEG-4-VS, and 60 min for PEG-4-AC.

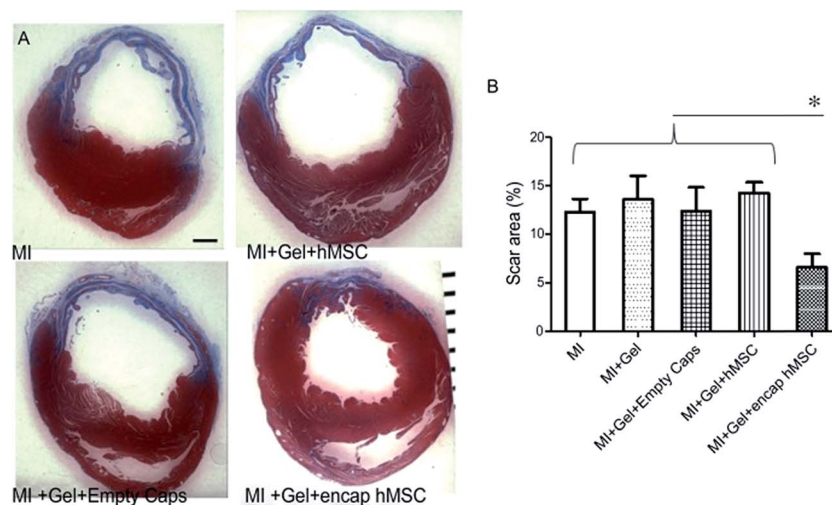


Fig. 2 Treatment of hearts using human mesenchymal stem cells (hMSC) encapsulated in a 4-arm PEG maleimide-GRGDSPC dithiol injectable hydrogel showed reduced myocardial scarring after 28 days. (A) Representative sections of infarcted hearts stained with Masson's Trichrome and treated with encapsulated hMSCs or control gels. Blue indicates fibrotic scar. $\times 15$, scale bar = 1 mm. (B) Animals treated with encapsulated hMSCs showed reduced scar area ($7 \pm 1\%$; $n = 6$) at 28 days compared to control treated hearts (MI: $12 \pm 1\%$, $n = 8$; MI + gel: $14 \pm 2\%$, $n = 7$; MI + gel + hMSC: $14 \pm 1\%$, $n = 7$; MI + gel + empty caps: $12 \pm 2\%$, $n = 5$). Data represent mean \pm SEM. * $P < 0.05$. MI indicates myocardial infarction. Reproduced from ref. 119 with permission from the American Heart Association.

Furthermore, the maleimide-based hydrogels required two orders of magnitude less TEA to gel, a significant attribute considering the known cytotoxic effects of TEA on endothelial cells, cells in the ovarian follicles, and pancreatic islet cells.¹¹⁸ These hydrogels can also readily be functionalized using a dithiol protease-cleavable RGD peptide to create an *in vivo* degradable network that promoted the spreading of encapsulated C2C12 murine myoblasts.¹¹⁹ Successful use of this hydrogel formulation was demonstrated *in vivo via* injection onto the pericardium of an excised rat heart (Fig. 2).

Michael addition cross-linked hydrogels have also been reported in the context of drug delivery applications, with Yu *et al.* cross-linking tetramaleimide and tetrathiol PEG to form transparent PEG hydrogels that quickly gelled under physiological conditions for the ocular delivery of Avastin®.¹²⁰ *In vitro* cytotoxicity indicated that the developed PEG hydrogel had no apparent cytotoxicity on L-929 fibroblast cells after 7 days of incubation; the cytotoxicity of the precursors was not determined despite concerns regarding the potential cross-reactivity and oxidation of thiols *in vivo* which persists as a potential limitation of thiol Michael additions. Prolonged Avastin® release was shown *in vitro* from these hydrogels for a period up to 14 days.¹²⁰

Disulfide formation

Disulfide bonds, formed *via* the oxidation of two thiol groups, play an essential structural and chemical role in protein folding and assembly. Thiols are protonated and unreactive in their reduced states (such as in the cytoplasm), but form S–S bonds in the presence of an oxidizing environment such as in the endoplasmic reticulum and extracellular space. Disulfide cross-linking is of significant interest for the design of injectable hydrogels as thiol-functionalized precursors readily cross-link in the presence of oxidizing agents such as oxygen¹²¹ but then degrade in the presence of reducing agents such as glutathione. Alternately, disulfide rearrangements (commonly found in nature for the formation of disulfide bonds in proteins) can be exploited for the formation of hydrogels and/or to promote hydrogel degradation. PEG-based disulfide cross-linked injectable hydrogels have been reported by Choh and co-workers,¹²² prepared by coextruding pyridyl disulfide functionalized

hyaluronic acid (HA-PD) with a PEG dithiol. Mixing of these precursors at physiological conditions results in macroscopic gelation within 4–5 minutes. Fibroblasts, endothelial cells (HUVECs), and adult stem cells (pMSCs) were successfully encapsulated *in vitro* and shown to proliferate over several days, even in the presence of unreacted thiols and the low-molecular-weight pyridine-2-thione cross-linking by-product.

Diels–Alder cycloaddition

The addition of a conjugated diene to an activated double bond (dienophile) is referred to as a Diels–Alder cycloaddition. The most commonly used Diels–Alder reaction for cross-linking hydrogels involves the reaction between maleimide and furan functionalized precursors.^{123–125} Diels–Alder reactions occur spontaneously under physiological conditions and are highly selective and relatively biorthogonal, aside from free thiol groups present at relatively low concentration in proteins.¹²⁶ Although Diels–Alder adducts are considered dynamic covalent bonds, current reversibility of formed adduct requires elevated temperatures beyond physiological temperature.¹²⁷ However, hydrogel degradation can be promoted under physiological conditions if the diffusion of hydrogel degradation products shifts the equilibrium towards the reverse Diels–Alder reaction. An illustrative example of the use of Diels–Alder chemistry for PEG hydrogels was recently reported by the groups of Bowman and Anseth.⁴⁸ The hydrogel network was formed using thiol-Michael chemistry, cross-linking a 4-arm PEG maleimide with a 4-arm PEG thiol. The maleimide functional groups are cross-reactive in Diels–Alder chemistry and thus provide a tethering site for a furan-modified RGD peptide (see Fig. 3). By exploiting the reversible nature of the Diels–Alder bond and le Châtelier's principle, the peptide could be release from the hydrogel with a release rate that is simply controlled by the excess of unreacted maleimide groups in the formed hydrogel network. For example, doubling the amount of maleimide groups present in the hydrogel decreased the amount of peptide released from approximately 60% to about 40%. Consequently, this example elegantly demonstrates how two different cross-link chemistries (*i.e.* thiol-Michael and Diels–Alder) can be used to independently control the rate of hydrogel degradation as well as release.

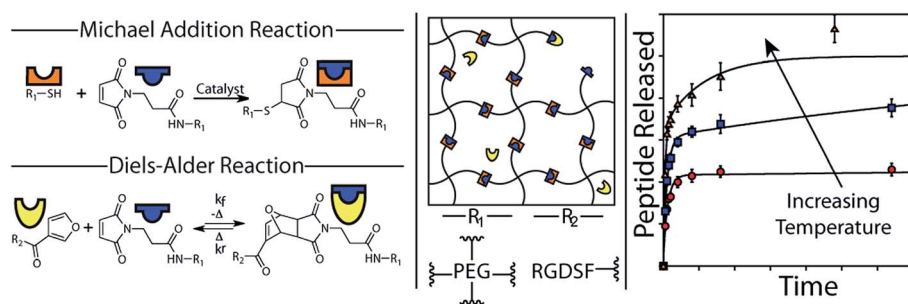


Fig. 3 The formation of PEG hydrogels using thiol-Michael addition and the functionalization with a releasable fluorescent RGD peptide using Diels–Alder chemistry. A schematic representation of the hydrogel network and the release kinetics as a function of the temperature are also shown. Reproduced from ref. 48 with permission from ACS publishing.

Schiff base formation

The nucleophilic addition of a nitrogen from an amine or hydrazide (or similar derivatives) to the carbonyl group of a ketone or aldehyde is referred to as a Schiff base or imine bond.¹²⁸ Schiff-bases are considered dynamic covalent bonds with the advantages of fast and tunable gelation kinetics¹²⁹ in the absence of a catalyst and complete reversibility. Conventional Schiff base formation between a carbonyl and a primary amine offers only limited hydrolytic stability and thus results in relatively fast hydrogel degradation.¹²⁸ Increased hydrolytic stability is achievable by increasing the nucleophilicity of the amine derivative, with the majority of the Schiff base cross-linked hydrogels reported have been based on hydrazone bond formation between a hydrazide and an aldehyde.^{28,73,130–133} An example of a PEG-based Schiff base hydrogel was reported by Saito and co-workers⁵² for the delivery of doxorubicin. A doxorubicin-loaded hydrogel was prepared by conjugating doxorubicin to a low molecular weight PEG dialdehyde (5 kDa) using Schiff base chemistry, followed by mixing with a solution of a high molecular weight poly(vinylamine) (PVAm) (Fig. 4). The precursor gelled quickly upon mixing and the resulting hydrogel showed sufficient hydrolytic stability at physiological pH for the targeted application. Prolonged doxorubicin was demonstrated up to 100 hours.⁵² However, the functional groups used in Schiff base formation (in particular aldehydes) are cross-reactive with functional groups on biomacromolecules (e.g. amines), such that care must be taken in designing the precursor polymers and gelation times in order to avoid potential toxicity.³⁷

Oxime chemistry

Oxime bonds form rapidly between an aldehyde or ketone and a hydroxylamine under physiological conditions and offer enhanced hydrolytic stability relative to Schiff base cross-linking strategies at the cost of requiring an acid catalyst in order to proceed at an appreciable rate. Grover and co-workers⁵¹ have used oxime chemistry to synthesize PEG hydrogels

through the reaction of an 8-arm aminoxy PEG with glutaraldehyde (Fig. 5). The mechanical properties, gelation kinetics, and water swelling ratios could be tuned according to the weight fraction of precursor polymers and the pH, and hydrogels modified with RGD successfully supported hMSC cell encapsulation. The *in vivo* potential of oxime cross-linked hydrogels formed between a 4-arm aminoxy PEG and a 4-arm PEG ketone was subsequently evaluated in myocardial tissue by introducing the hydrogel using a catheter.¹³⁴ While *in vitro* gelation rates ranged from 30 minutes at pH 4 to >50 hours at pH 7.4, *in vivo* gelation was observed within 20 minutes independent of the pH of the injection solution (pH 6.0–7.4). This effect can be attributed to the more complex biological environment *in vivo* relative to *in vitro* experiments.

Despite the obvious advantages of using PEG as a precursor polymer for the preparation of injectable hydrogels (specifically, its controlled molecular weight and protein repellent properties), PEG also suffers from a number of limitations. As PEG can only be selectively modified at the α,ω -hydroxyl chain ends, relatively expensive multi-arm PEG precursors must be used as precursor polymers to promote network formation at lower PEG concentrations. Furthermore, incorporation of specific functionalities (e.g. RGD peptide or other biomolecules) effectively competes with cross-linking since these functional tethers need to be incorporated at the same reactive sites; in this sense, the number of cross-links and functional tethers is inherently limited by the number of chain ends on the PEG starting materials, significantly limiting the mechanical strength of the resulting hydrogel. Consequently, we have started exploring injectable PEG analogue polymers that can be synthesized from free-radical polymerization as a potential system to improve upon PEG-based hydrogel designs.

4 Poly(oligoethylene glycol methacrylate)-based hydrogels

In order to address challenges with injectable PEG hydrogels and PEG more broadly as a biomaterial, poly(oligoethylene glycol methacrylate) (POEGMA) is now being investigated as an alternative to PEG.^{68,135} Unlike PEG, which is typically prepared *via* ring-opening polymerization and thus cannot readily be co-polymerized with other functional comonomers, POEGMA is synthesized from conventional (or controlled/living) free-radical polymerization,^{136–138} offering excellent control over polymer composition, functionality, architecture and molecular weight distribution with a range of functional monomers to impart desired functionalities to the polymers. POEGMA can also be made thermoresponsive *via* the statistical copolymerization of oligo(ethylene glycol) methacrylate (OEGMA) monomers with varying lengths of ethylene oxide (EO) side chains (n).¹³⁸ For example, statistical copolymers of diethylene glycol methacrylate (M(EO)₂MA, $n = 2$) and OEGMA with an average molecular weight of 475 g mol⁻¹ (OEGMA₄₇₅, $n = 8–9$) display a cloud point in water according to a linear correlation between the cloud points of the respective PM(EO)₂MA (~23 °C) and POEGMA₄₇₅ (~90 °C) homopolymers.¹³⁸ The reversible temperature

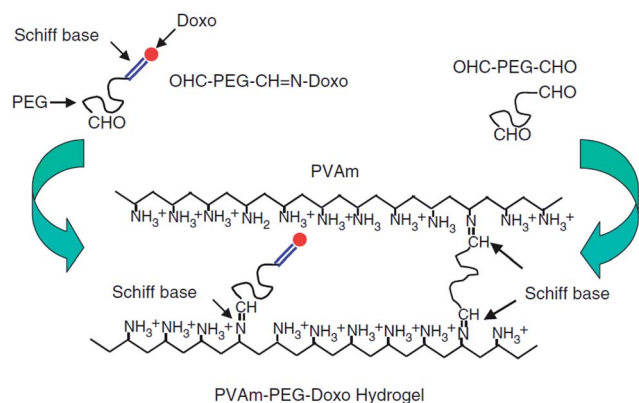


Fig. 4 Schematic representation of the formation of poly(vinylamine) (PVAm)-poly(ethylene glycol) (PEG)-doxorubicin (doxo) hydrogels using Schiff base chemistry. Reproduced from ref. 52 with permission from SAGE journals.

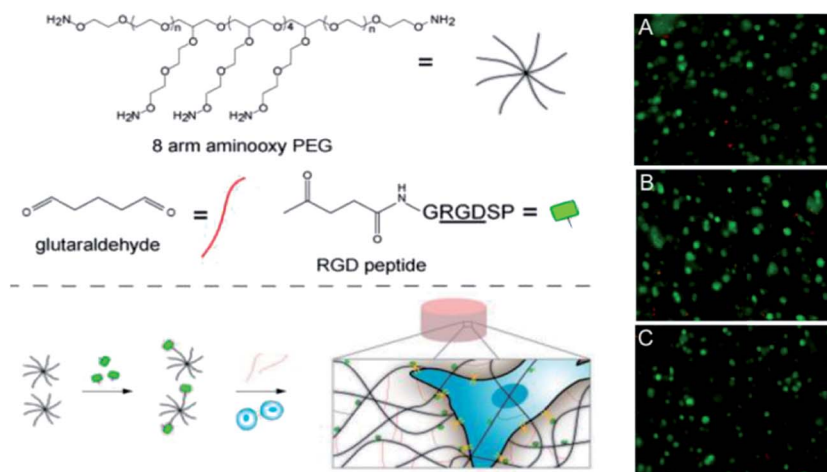


Fig. 5 Synthesis and encapsulation of MSCs within RGD-functionalized oxime-cross-linked PEG hydrogels. Reproduced from ref. 51 with permission from the American Chemical Society.

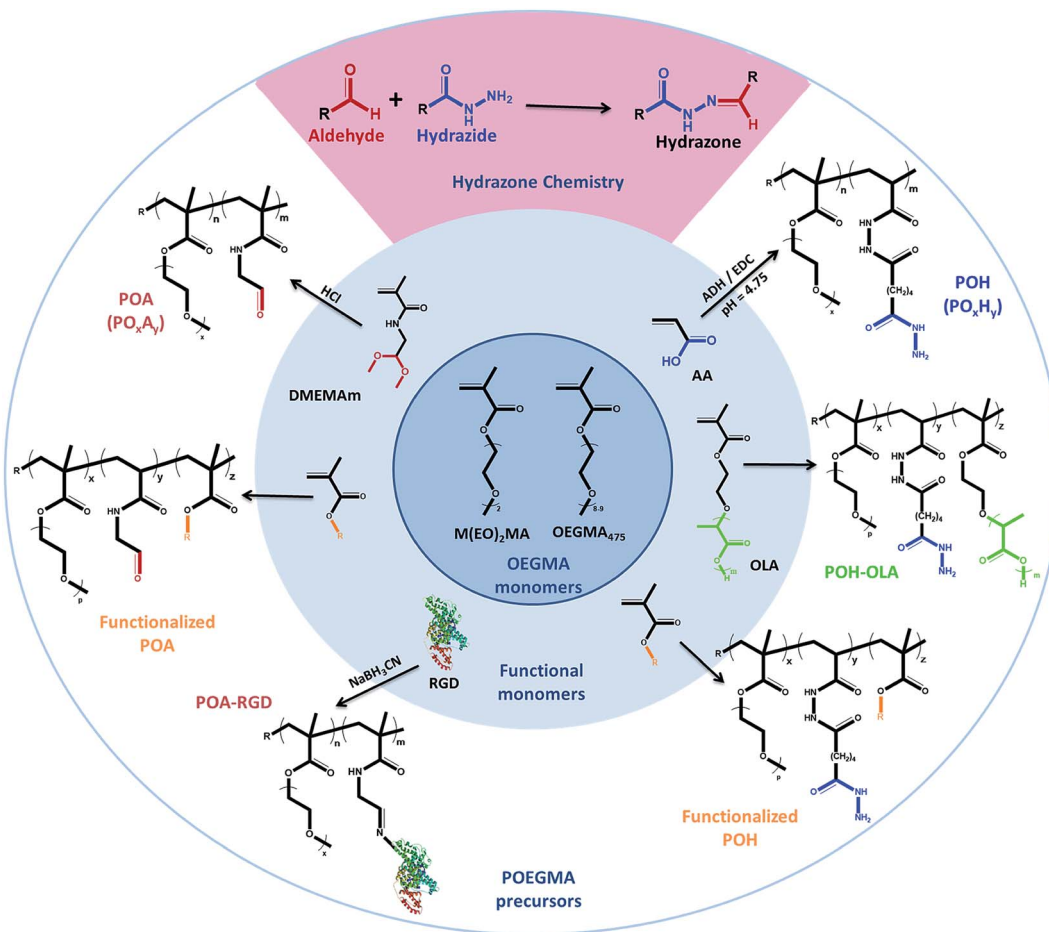
transition shows little hysteresis¹³⁹ when compared to other thermoresponsive synthetic polymers such as *N*-isopropylacrylamide (NIPAAm) due to the lack of a hydrogen-bond donor.¹⁴⁰ This property is of potential interest both for the design of “smart”, environmentally-responsive biomaterials for on-demand drug delivery, triggerable cell adhesion, *etc.* as well as more generally as PEG-based materials with significantly improved bioadhesion in the absence of functional peptide tags. At the same time, POEGMA has been demonstrated to display similar bio-inert (“stealth”)^{141,142} and non-cytotoxic properties¹⁴³ when compared to PEG. Consequently, we and others have considered POEGMA a promising platform for the synthesis of PEG-analogue hydrogels with improved control over the chemical, physical, and biological properties of the hydrogels.

A number of POEGMA-based hydrogels have been reported to-date,^{144–148} with the majority prepared *via* conventional free radical polymerization in the presence of a di(meth)acrylate cross-linker to promote gelation. However, with the exception of a 4-arm PEG-*b*-POEGMA polymer reported by Fechler, which undergoes physical gelation at the physiological temperature of 37 °C,¹⁴⁹ none of these previously hydrogels are either injectable or degradable *in vivo*, which severely limits their potential clinical application.

In response, we have done extensive work in our laboratory on the design of injectable hydrogels based on dynamic covalent chemistry,^{127,150} with a particular focus on Schiff-base chemistry between aldehyde and hydrazide functional groups (see Scheme 2). Use of this cross-linking chemistry for injectable hydrogels was first proposed by Bulpitt and Aeschlimann¹⁵¹ and has been further developed by Langer’s group^{73,130} and others^{91,131,152,153} to a broad range of biopolymer, synthetic and composite hydrogel systems. Our choice of hydrazone chemistry for the preparation of injectable hydrogels was driven by the fast gelation kinetics and hydrolytic reversibility of the hydrazone bond. Hydrazide and aldehyde functional groups are also readily introduced onto a range of synthetic and natural

polymers using facile and few step copolymerization and post-polymerization modification strategies. The desired physicochemical and biological hydrogel properties can consequently be “dialed-up” by selecting and modulating different hydrogel precursors. Furthermore, hydrazide and aldehyde functionalized precursors provide sufficient bio-orthogonality (even in the presence of potential Schiff-base formation between the pendant aldehydes and available amines on native proteins) to in our experience avoid causing any significant adverse reactions *in vitro* and *in vivo*.^{41,42} We attribute this result to the high reversibility of the aldehyde-amine Schiff base pair (*i.e.* a hydrazone bond will form preferentially to minimize free energy), the fast gelation kinetics which rapidly consume a large fraction of the free aldehyde groups before proteins can diffuse into the hydrogel, and the high local concentration of hydrazides used during injection which ensures the majority of aldehyde groups are reacted following gel formation.

Hydrazide-functionalized POEGMA precursors (POH) are synthesized from conventional free-radical copolymerization of M(E_o)₂MA, OEGMA₄₇₅ and acrylic acid (AA) in the presence of thioglycolic acid (TGA) to limit the molecular weight below the kidney cut-off, followed by post-polymerization modification using the carbodiimide-mediated coupling of adipic acid dihydrazide (ADH).⁴¹ The aldehyde-functionalized POEGMA precursors (POA) are synthesized from the copolymerization of M(E_o)₂MA, OEGMA₄₇₅ and *N*-2,2-diethoxyethyl methacrylamide (DMEMAm) in the presence of TGA, followed by the acid-catalyzed deprotection of the acetal to the aldehyde (see Scheme 2).⁴¹ The aldehyde and hydrazide reactive precursors are labeled as PO_xA_y and PO_xH_y respectively, where *x* denotes the mole percentage of OEGMA₄₇₅ among the OEGMA monomers used and *y* denotes the overall mole percentage of DME-MAm or AA in the synthesis recipe. We have exploited the synthetic versatility of the POEGMA platform to synthesize precursor polymers that differ in (i) OEGMA composition (0 ≤ *x* ≤ 100, and thus phase transition temperature),⁴² (ii) reactive group content (20 ≤ *y* ≤ 40, and thus theoretical cross-link



Scheme 2 Library of hydrazide and aldehyde-functionalized poly(oligoethylene glycol methacrylate) (POEGMA) hydrogel precursors prepared for designing hydrogels.

density),⁴¹ or (iii) the presence of specific functional moieties (e.g. hydrophobic oligo(lactic acid) (OLA)¹⁵⁴ or RGD cell-signaling peptide to direct internal domain formation, cell adhesion, *etc.*)⁴¹ (Scheme 2). Since the POH and POA polymers represent both the hydrogel precursors as well as degradation products,⁴¹ the design of these polymers with a M_n below the renal clearance-limit of ~ 32 kDa should facilitate elimination of the precursors *via* the kidneys following hydrogel degradation.

POEGMA hydrogels (labeled as $PO_{x,y}$, where x indicates the mole fraction OEGMA₄₇₅ among the OEGMA monomers) are generally prepared by co-extruding POH and POA precursor solutions of equal concentration (most typically $150 \text{ mg mL}^{-1} = 13 \text{ w/w\%}$) and comparable degree of functionality ($y = 30 \text{ mol\%}$). Despite this similarity in precursor concentration and reactive group content, hydrogel properties vary considerably as a function of the precursor composition (x) (Fig. 6). The gelation time, defined as the time point at which the hydrogel visually no longer flows on the time scale of test tube rotation (5 s) and cross-link density decrease with increasing mole fractions of OEGMA₄₇₅ ($n = 8-9$); for example, PO_0H_{30} and PO_0A_{30} gel within 5 s and form a stiff hydrogel ($G' \sim 10 \text{ kPa}$) whereas $PO_{100}H_{30}$ and $PO_{100}A_{30}$ require 20 min to gel and form a relatively weaker hydrogel ($G' \sim 0.8 \text{ kPa}$). We hypothesize this trend is attributable

to the steric hindrance of the longer PEG side chains in OEGMA₄₇₅ ($n = 8-9$) that reduces the availability of the reactive aldehyde and hydrazide groups for cross-linking. This difference in gelation kinetics also has a direct impact on the underlying morphology of the hydrogel network formed. A small-angle neutron scattering (SANS) study on these hydrogels revealed that the fast gelling PO_0 precursors form an inhomogeneous hydrogel network with a small mesh size, whereas the slow gelling PO_{100} precursors form a much more homogeneous network with a large mesh size.¹⁵⁵

The volume phase transition temperature (VPTT) of POEGMA hydrogels aligns well with the cloud point (or lower-critical solution temperature, LCST) reported for analogous statistical POEGMA copolymers of similar $M(\text{EO})_2\text{MA-OEGMA}_{475}$ composition.¹³⁸ Although the cloud points of the POH and POA precursors are well over 50°C in phosphate-buffered saline (PBS) due to the hydrophilicity of the hydrazide and aldehyde functional groups (key to avoiding local precipitation and subsequent inflammation as well as to further promote clearance of the precursor polymers after the gel has degraded),⁴² once cross-linked the formed hydrazone bond does not significantly alter the VPTT of the POEGMA hydrogels *versus* what would be predicted from a statistical copolymer of the two

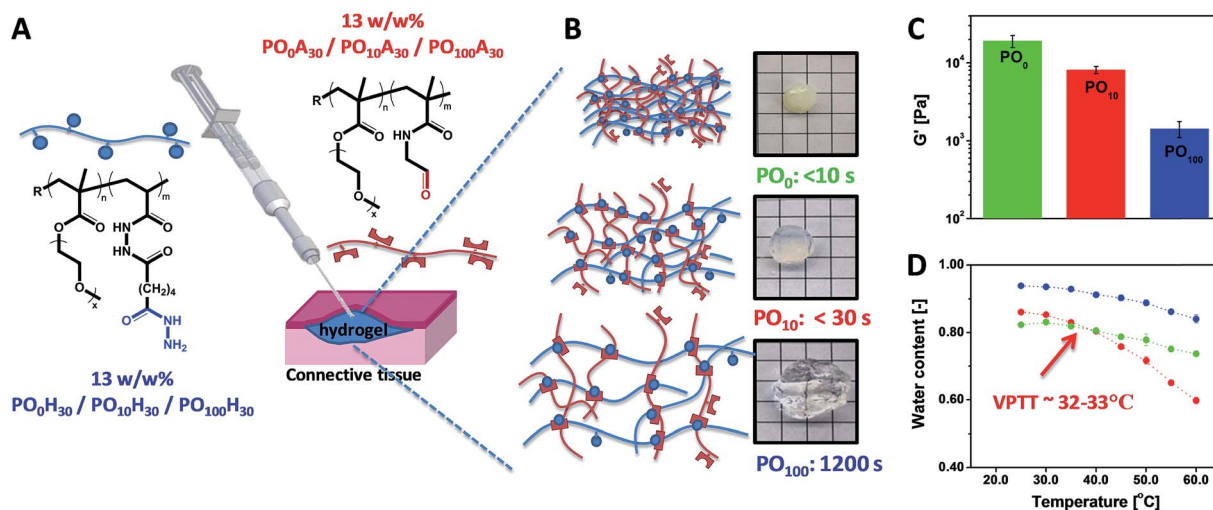


Fig. 6 (A) Preparation of POEGMA hydrogels using a double-barrel syringe. (B) Physical appearance and gelation time for PO_0 , PO_{10} and PO_{100} hydrogels. (C) Hydrogel mechanics as measured by the shear storage modulus (G'). (D) Thermoresponsive properties of PO_0 , PO_{10} and PO_{100} hydrogels. Data reproduced from ref. 42 with permission from Elsevier.

OEGMA comonomers used.⁴² Consequently, we have been able to prepare POEGMA hydrogels that are completely collapsed at 23°C (PO_0), display a VPTT at $\sim 33^{\circ}\text{C}$ (PO_{10} , comparable to PNIPAAm), or do not display a VPTT up to 60°C (PO_{100} , comparable to PEG) (Fig. 3D). These hydrogels show remarkable differences in swelling kinetics at physiological temperature due to these different temperature dependencies; PO_0 and PO_{10} hydrogels prepared at $13 \text{ w/w}\%$ in PBS (water content = 87.0%) de-swelling at 37°C to plateau water contents of $80 \text{ w/w}\%$ and $78 \text{ w/w}\%$, respectively while PO_{100} hydrogels prepared at the same precursor concentration swell to a plateau water content of $91 \text{ w/w}\%$ at the same temperature.

Our modular approach to designing hydrogels allows for (i) the incorporation of building blocks with specific functionalities that cannot be attained using synthetic polymers using simple mixing (e.g. superparamagnetic iron oxide nanoparticles)⁴⁰ and (ii) facile tuning of the hydrogel properties by mixing hydrogel precursors with different properties, avoiding the often trial-and-error optimization procedures conventionally used to synthesize hydrogels with targeted physical properties.¹⁵⁶ Building on this concept, we have exploited our library of POA and POH precursors to prepare “mixed precursor” POEGMA hydrogels that demonstrate this principle, using 2 POA and 2 POH precursors with different x values (i.e. different LCST values) as the precursor units; the high and low transition temperature precursor polymer pairs are mixed at different ratios to tune the gel properties.¹⁵⁷ Important macroscopic physiochemical properties such as the gelation time, equilibrium water content, and hydrogel elasticity of these “mixed precursor” hydrogels can be predicted according to the simple rule of mixtures based on the corresponding properties of “single precursor” POEGMA hydrogels (i.e. hydrogels prepared using only 1 aldehyde and 1 hydrazide precursor of the same x) (Fig. 7). Consequently, on a macroscopic scale, desired hydrogel properties can be “dialed-up” simply by mixing

different precursor polymers, avoiding the laborious synthesis and characterization required to optimize a hydrogel using trial-and-error synthesis. On a microscopic scale, however, “mixed precursor” hydrogels are less homogeneous than “single precursor” hydrogels and contain large inhomogeneous domains that cause an opaque appearance as well as alter the interactions between the hydrogel with its environment (e.g. protein binding, cell adhesion, drug uptake).¹⁵⁵

POEGMA hydrogels with associative hydrophobic domains have also been prepared by synthesizing a $\text{PO}_{100}\text{H}_{30}$ precursor polymer containing a functional oligo(lactic acid) methacrylate macromonomer (OLA_m , where m represents the lactic acid chain length).¹⁵⁴ Co-extrusion with $\text{PO}_{100}\text{A}_{30}$ results in the formation of POEGMA hydrogels that are both chemically (via formation of hydrazone bonds) and physically (via self-association of hydrophobic OLA chains) cross-linked, with both the chemical and physical cross-links degradable over time via hydrolysis. Hydrogel swelling, degradation and elasticity can be independently tuned using POH–OLA precursors according to the OLA chain length ($m = 4, 8, \text{ or } 16$) and concentration ($z = 10, 20, \text{ or } 30 \text{ mol}\%$). SANS analysis of the hydrogel microstructure revealed the presence of hydrophobic associative domains as well as the effect of the competition between physical and chemical cross-linking on the mesh size, which can be directly tuned to create hydrogels with targeted properties and well-defined nanodomains.

Like PEG hydrogels, POEGMA hydrogels hold significant promise in biomedical applications like controlled release and cell scaffolding. Similar to conventional PEG hydrogels, unmodified POEGMA hydrogels show little affinity for hydrophobic therapeutics and generally show significant burst-release and a short therapeutic release window. In an attempt to improve the affinity of PEG-based hydrogels for hydrophobic therapeutics, we explored the release of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) from

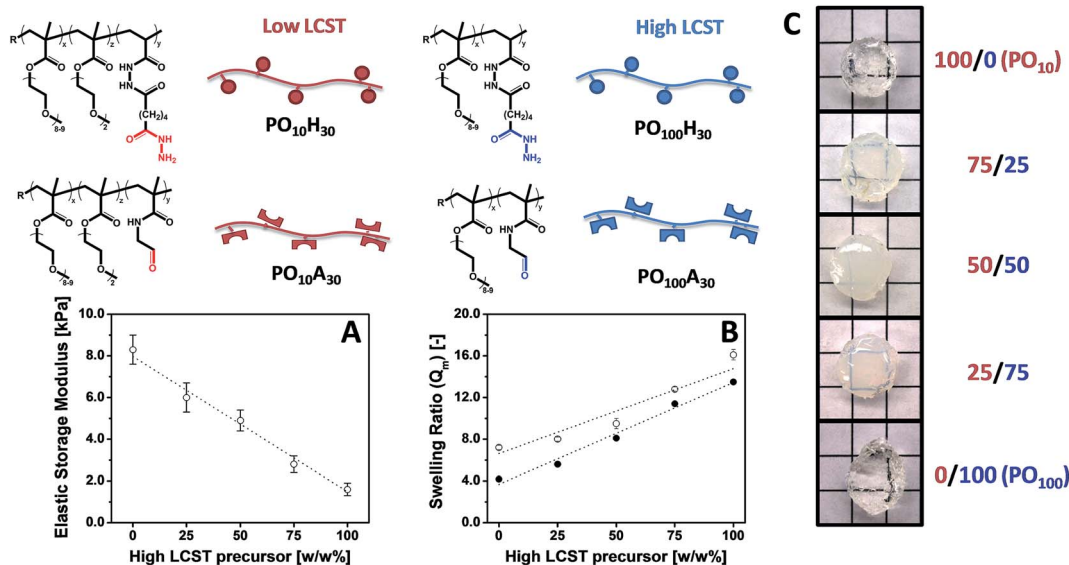


Fig. 7 Tuning hydrogel properties by mixing POEGMA precursors of different LCST. (A) Elastic storage modulus (G'), (B) mass-based swelling ratio (Q_m) and (C) physical appearance of POEGMA hydrogels containing 100/0, 75/25, 50/50, 25/75 and 0/100 w/w% PO₁₀H₃₀/PO₁₀₀H₃₀ and PO₁₀A₃₀/PO₁₀₀A₃₀. Data reproduced from ref. 155 and 157 with permission from Wiley-VCH and ACS.

“mixed precursor” as well as POEGMA–OLA hydrogels. The inhomogeneous microscopic network structure of “mixed precursor” hydrogels offered somewhat improved control over FITC-BSA burst release but did not significantly extend the therapeutic release window *versus* conventional, more homogeneous “single precursor” hydrogels. In comparison, the incorporation of oligo(lactic acid) (OLA) pendant side chains into POH precursors resulted in significantly lower burst release as well as moderate control over the duration of the following sustained release.¹⁵⁴ However, further optimization of these PO–OLA hydrogels is required to attain release profiles that are clinically relevant.

In a biological setting, POEGMA hydrogels show similar non-cytotoxicity and bio-inert properties to conventional PEG hydrogels, even in the case of thermoresponsive hydrogels with lower VPTT values (Fig. 8). None of the POH or POA precursors showed any decrease in cell viability up to 2000 $\mu\text{g mL}^{-1}$ in MTT assays on 3T3 mouse fibroblasts,⁴² and the PEG-analogue PO₁₀₀ hydrogels exhibited very low adsorption of bovine serum albumin (BSA) and fibrinogen (Fib) on par or better than reported PEG-based materials.^{158–162} Interestingly, while POEGMA hydrogels above their VPTT also demonstrated extremely low protein adsorption relative to most biomaterials, they were still capable of supporting moderate adhesion of 3T3 mouse fibroblasts.⁴² Furthermore, the thermoresponsivity of the lower VPTT POEGMA hydrogels can be exploited to turn cell adhesion to the hydrogels effectively on and off; for example, lowering the temperature of a 3T3 fibroblast-adhered PO₀ hydrogel to 4 °C ($T < \text{VPTT}$) for just 15 minutes results in significant (~25%) delamination of cells from the gels for cell recovery without the need to use harsher enzymatic methods such as trypsinization (Fig. 8B).⁴² Consequently, POEGMA-based hydrogels can be applied in tissue engineering applications not readily served by conventional PEG hydrogels.

Alternately, analogous to the several examples of PEG-based hydrogels cited previously,^{48,89,134,163} POA precursors can be also functionalized with RGD to prepare POEGMA hydrogels that selectively promote cell adhesion.⁴¹ Subcutaneous injection of these soft materials caused a relatively mild acute inflammatory response (2 days) and little to no chronic inflammation (30 days) (Fig. 8C). Furthermore, *in vivo* experiments showed that the POEGMA hydrogels could fully degraded under physiological conditions without any obvious signs of local tissue toxicity.⁴²

The results to date demonstrate the potential of POEGMA-based injectable hydrogels for biomedical applications such as controlled release and cell encapsulation. The physicochemical properties of the hydrogels (governed by the network structure) can be easily controlled by the choice of OEGMA and functional monomers during the precursor polymer synthesis. The biological properties of these hydrogels are generally similar to PEG, provided that the VPTT is above physiological temperature. However, the presence of a VPTT also extends the range of applications that can be considered for POEGMA-based hydrogel materials, and the ease of POEGMA modification (particularly relative to PEG) opens up possibilities for specifically tuning these materials to targeted biomedical applications. We are currently exploring the biocompatibility of these POEGMA-based hydrogels in various biological environments as well as the more detailed cell responses (in terms of cell spreading and differentiation) to the various types of hydrogel structures accessible using this approach to assess the applicability of these materials for both *in vivo*, clinical applications as well as *in vitro* cell culture-based applications.

5 Summary and perspectives

PEG and PEG-analogue materials hold tremendous potential for the design of both *in vivo* scaffolds for drug delivery or

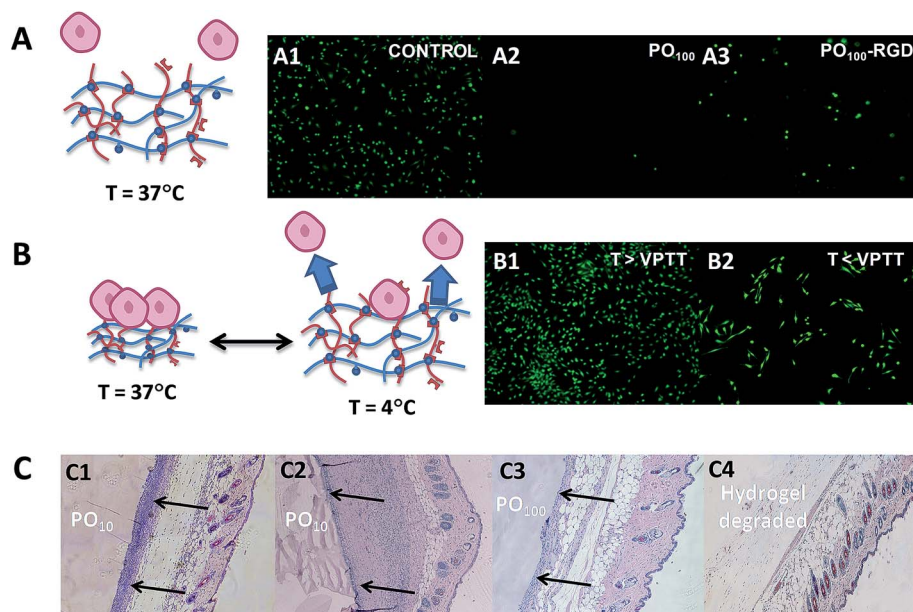


Fig. 8 Overview of the biological properties of POEGMA hydrogels. (A) Bio-repellent properties of unmodified POEGMA hydrogels (A2) and controlled adhesion of cells using RGD-functionalized POEGMA hydrogels (A3) relative to a tissue culture polystyrene control (A1). (B) Switching cell adhesion "on" above the VPTT (B1) and "off" below the VPTT (B2). (C) Histological sections following subcutaneous injection following acute (C1 and C3) and chronic (C2 and C4) time points. Complete degradation of a PO₁₀₀ hydrogel is shown in C4. Images reproduced from ref. 41 and 42 with permission from the Royal Society of Chemistry and Elsevier.

tissue engineering as well as *in vitro* for cell culturing applications. Injectable analogues of such hydrogels offer even more promise both for effective 3D scaffolding of cells in these hydrogels by simple mixing as well as non-invasive administration *in vivo*, reducing the complexity of hydrogel use in the body as well as opening up potential uses of hydrogels in areas generally disfavored for routine surgical procedures if they are avoidable (*e.g.* the eye). Newer methods to incorporate multiple functional tethers (both physical and biological) on PEG-based hydrogels to direct cell responses in more precise and, in some cases, externally triggered ways suggest the potential use of PEG-based hydrogels in next-generation tissue engineering approaches. However, in our view, the significant synthetic challenges associated with functionalizing PEG, particularly in a manner that is independent of the cross-link density of the ultimate PEG-based hydrogel network, make PEG analogues such as POEGMA of increasing significance along the path of synthetically replicating (to the extent possible) natural cell microenvironments. To this end, an improved understanding of the similarities and differences between the well-established tissue responses and clearance mechanisms of PEG and those of POEGMA is essential to establish whether or not the synthetic versatility offered by POEGMA-based hydrogels can truly be exploited in a broad range of potential biomedical applications.

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