Perspective

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Hydrogels for Therapeutic Delivery: Current Developments and Future Directions

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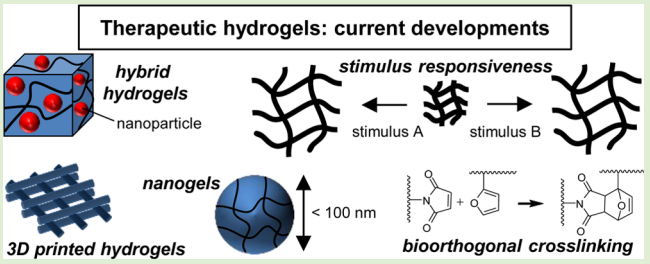
ABSTRACT: Hydrogels are attractive materials for the controlled release of therapeutics because of their capacity to embed biologically active agents in their water-swollen network. Recent advances in organic and polymer chemistry, bioengineering and nanotechnology have resulted in several new developments in the field of hydrogels for therapeutic delivery. In this Perspective, we present our view on the state-of-the-art in the field, thereby focusing on a number of exciting topics, including bioorthogonal cross-linking methods, multi-component hydrogels, stimuli-responsive hydrogels, nanogels, and the release of therapeutics from 3D printed hydrogels. We also describe the challenges that should be overcome to facilitate translation from academia to the clinic and last, we share our ideas about the future of this rapidly evolving area of research.



1. INTRODUCTION

Hydrogels are polymeric networks based on hydrophilic macromonomers that are able to retain large amounts of water.[1−5] Hydrogels generally exhibit excellent biocompatibility and their mechanical properties can be designed in such a way that they match those of many soft biological tissues. Furthermore, their soft and rubbery nature minimizes inflammatory reactions of the surrounding cells and tissues.[6] Hydrogels may be classified as natural, synthetic or hybrid, depending on the source of the constituting polymers. Hydrogels can be physically cross-linked by noncovalent interactions, chemically cross-linked by covalent bonds, or cross-linked by a combination of both. After their discovery in the 1960s by Wichterle and Lim, hydrogels were first successfully applied as contact lenses.[7] In the following decades, researchers also started to investigate the use of hydrogels for the localized and sustained release of therapeutic agents as a strategy to decrease the number of drug administrations, protect the drug from (enzymatic) degradation, and allow for therapeutic drug concentrations for prolonged times.[2] Initially, hydrogel research focused on relatively simple, chemically cross-linked networks of synthetic polymers, such as poly(2-hydroxyethyl methacrylate) (pHEMA), poly(ethylene glycol) (PEG), and poly(vinyl alcohol) (PVA), which straightforward network structures were also well-suited for fundamental characterization and modeling of various physicochemical hydrogel properties including swelling, solute diffusivity, and cross-link density. Hydrogels were mainly prepared either by polymerization of water-soluble monomers in the presence of a multifunctional cross-linker or by cross-linking of hydrophilic polymers. In the beginning of the 1970s the focus in hydrogel research shifted from simple, water-swollen macromolecular networks to hydrogels capable of responding to a change in environmental conditions such as pH or temperature to evoke specific events, including in situ gel formation and drug release. Initially these physical hydrogels, often based on PEG-polyester copolymers or poly(*N*-isopropylacrylamide) (pNIPAAm), were cross-linked via hydrophobic interactions, but from the 1990s onward other physical interactions, such as stereocomplexation and inclusion complexation, were also exploited as cross-linking methods that offered the possibility to enhance and finely tune the release properties. In the 21st century, advances in organic and polymer chemistry, bioengineering and nanotechnology have resulted in hydrogels that possess unprecedented levels of structural organization and novel properties, which can be tailored precisely for the desired delivery application.[2,5,8] In this Perspective, we present an overview of several recent developments that may, in our opinion, generate major steps forward in the field of hydrogels for therapeutic delivery. We highlight representative examples, identify challenges the field is currently facing and last, we give our view on the future directions of this rapidly advancing area of research. It should be noted that the choice of topics in this Perspective reflects our view on the state-of-the-art; as such, this manuscript does not pretend to provide a comprehensive overview of all current topics in therapeutic hydrogel research. The reader is therefore referred to several excellent reviews that focus on specific areas in research concerning hydrogels for therapeutic delivery.[8−17]

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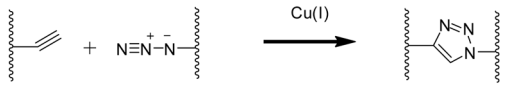
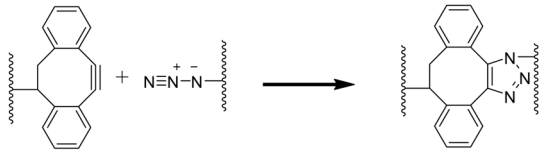
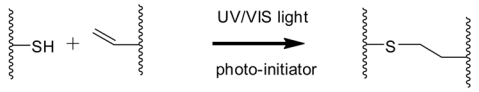
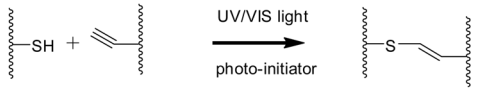
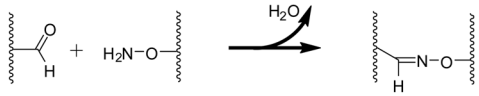
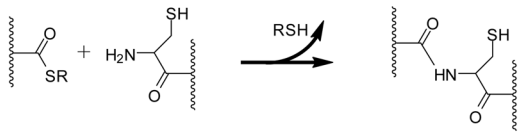
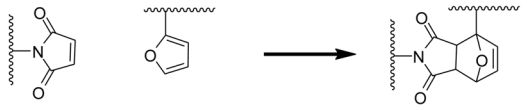
Table 1. Summary of Click Chemistry Strategies Which Have Been Used to Form Hydrogels (Adapted with Permission from Ref 37. Copyright 2014 Elsevier)

Click reaction	Features
Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition 	✓ Bioorthogonality × Toxic copper catalyst
Strain-promoted azide-alkyne cycloaddition (SPAAC) 	✓ No catalyst ✓ Bioorthogonality × Difficult synthesis of cyclooctynes
Thiol-ene reaction  Thiol-yne reaction 	✓ Spatiotemporal control ✓ Fast gelation × Potential toxicity from photoinitiators and radicals × Cross-reactivity with thiols
Oxime chemistry 	✓ No toxic catalyst × Cross-reactivity with amines
Native chemical ligation 	✓ No catalyst ✓ Bioorthogonality × Release of potentially toxic thiol byproducts
Diels–Alder cycloaddition 	✓ No catalyst ✓ Accelerated by H_2O ✓ Thermoreversible × Slow gelation

2. HYDROGELS FOR THERAPEUTIC DELIVERY: CURRENT DEVELOPMENTS

2.1. Bioorthogonal Cross-Linking Methods. For biomedical applications, the use of in situ forming hydrogels is preferred over preformed hydrogels since there is no need for surgical interventions as gelation can take place under physiological conditions upon injection. Furthermore, the initial fluidic nature of the precursor solution ensures proper shape adaptation and biological components can be incorporated in the hydrogel by simple mixing with the precursor polymer solution.[18,19] Physical cross-links, such as hydrophobic or ionic interactions, can be formed in situ under mild conditions, but the resulting hydrogels generally degrade or dissolve rapidly. Injectable chemically cross-linked hydrogels have been frequently prepared by photocuring polymers functionalized with vinylic groups.[2] Although the presence of photoinitiators and the generated radicals in the polymerization process has been associated with cytotoxicity, frequently good cell compatibility is found for cells encapsulated in this type of hydrogel.[20,21] However, important drawbacks of photo-cross-linkable systems include the limited penetration depth of UV radiation and its potentially damaging effects on living tissue. In situ forming hydrogels utilizing covalent cross-linking between polymers with complementary functional groups therefore received much attention in the last two decades. Early examples of chemical cross-linking reactions forming hydrogels include Schiff base formation between aldehydes and amines[22] and the Passerini and Ugi condensation.[23] However, remaining functional groups on polymers may react with functional groups present in biomolecules such as proteins, limiting the application of these hydrogels as in situ forming delivery systems. In this regard, the use of chemoselective, bioorthogonal cross-linking strategies, which do not interfere with biochemical processes or biomolecules, are very appealing. The

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most investigated chemoselective cross-linking reaction for hydrogels is the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of terminal alkynes and azides, commonly known as click chemistry. This term was originally coined in 2001 by Sharpless et al. for a number of new regiospecific linking reactions that give high yields and generally require no purification.[24] The first hydrogel cross-linked via click chemistry was described by the group of Hilborn and was based on PVA.[25] Although this category of hydrogels has been mainly used for tissue engineering applications, they have also been applied for the controlled release of therapeutics and different biomolecules, including doxorubicin[26] bovine serum albumin (BSA)[27] and pDNA.[28] Because "classical" alkyne−azide cycloadditions require the use of a copper catalyst, which may cause adverse effects in biomedical applications, the use of strain-promoted azide−alkyne cycloaddition (SPAAC) is attracting increasing interest. This reaction employs the inherent ring strain of cyclooctyne groups to achieve fast and efficient cross-linking without the need for catalysts or external stimuli such as UV light. Hermann et al. reported on PEG hydrogels for the delivery of bone morphogenetic protein (BMP) inhibitors to treat excessive postoperative bone regrowth (resynostosis) after surgical correction of premature fusion of cranial sutures (craniosynostosis) in children.[29] Mixing poly[(tetraethylene glycol methacrylate)-*co*-(azidotetraethylene glycol methacrylate)] with a 4-dibenzocyclooctynol PEG cross-linker resulted in the formation of hydrogels within 30 s, as demonstrated by oscillatory rheology. In vitro release experiments showed that the BMP inhibitor Gremlin1 remained bioactive when it was incorporated in the hydrogels by dissolution in the prepolymer solutions, confirming the bioorthogonal character of the SPAAC cross-linking method. When the prepolymer solutions were injected together with Gremlin1 in a surgically created cranial defect in weanling mice, a hydrogel formed in situ that was able to provide a controlled release of the inhibitor for 14 days. Following this approach, the authors were able to delay, but not completely prevent, postoperative bone regrowth in the cranial defect model. Since the ultimate goal of resynostosis therapy is to slow the postoperative bone growth for a period, but then allow the bone to regrow as the child ages, the reported approach holds promise as treatment after surgical intervention in craniosynostosis. Other emerging bioorthogonal strategies for the preparation of chemically cross-linked hydrogels include thiol−ene/yne reactions,[30,31] oxime chemistry,[32] native chemical ligation,[33,34] and Diels−Alder cycloadditions.[35,36] Table 1 presents a summary of several click chemistry strategies that have been used to form hydrogels, including advantages and disadvantages of each method.[37] Dove and co-workers recently reported on the simultaneous application of thiol−yne and Diels−Alder chemistry to independently create two interpenetrating networks in a one-step procedure (Figure 1).[38] A loose network was prepared from norbornene-functionalized chitosan and linear PEG-ditetrazine, while a dense network was constructed by cross-linking 4-arm PEG-tetraalkyne with linear PEG-dithiol. The resulting double network hydrogels displayed a high compressive stress of 15 MPa at 98% compression without fracture or hysteresis upon repeated load. The hydrogels were postfunctionalized with a thiol-containing fluorescent dye and tetrazine-modified biotin via reaction with unreacted alkyne and norbornene groups, respectively. The cross-linking reactions were suggested to be bioorthogonal because of the high viability of human mesenchymal stem cells,

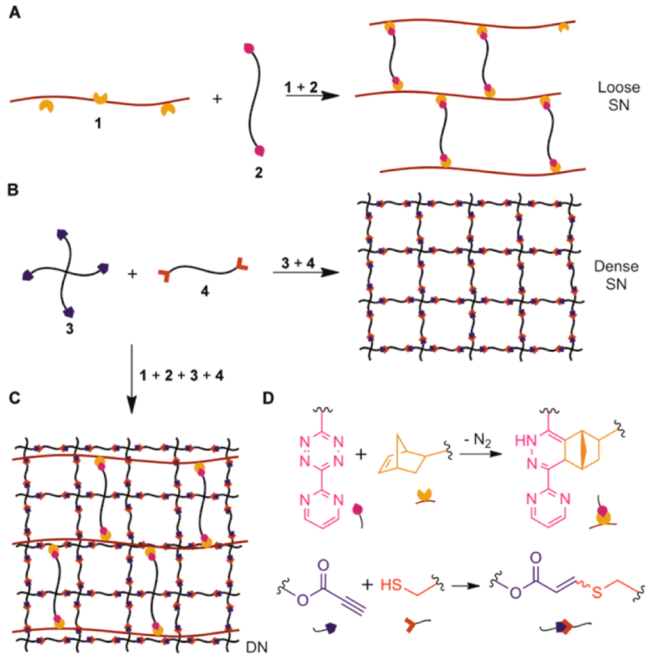

Figure 1. Schematic representation of double network PEG hydrogel fabrication. (A) Loose single network (SN) formed by norbornene-tetrazine addition. (B) Dense single network formed by nucleophilic thiol−yne addition. (C) Double network (DN) gel formed by mixing all four components. (D) Tetrazine-norbornene and nucleophilic thiol−yne addition chemistry used for cross-linking. Reprinted with permission from ref 38. Copyright 2015 American Chemical Society.

which were encapsulated by adding them to the prepolymer solutions prior to hydrogel formation. An extensive recent review concerning bioorthogonal chemistries for hydrogel cross-linking can be found in ref 37.

The above examples show that several bioorthogonal reactions have emerged as innovative and versatile strategies to construct new functional hydrogels due to their high coupling efficiency, chemoselectivity, and mild reaction conditions. Although many of the described cross-linking reactions are believed to be bioorthogonal, caution should still be used when hydrogels are cross-linked in the presence of biomolecules, most notably proteins. The group of Göpferich investigated the compatibility of a number of reactive groups present on polymers, including maleimide and thiol groups, with lysozyme.[39] Hydrogel preparation was simulated by using polymer/lysozyme mixtures and the possible conjugation of polymer chains to lysozyme was determined by SDS-PAGE analysis. Upon incubation with PEG-maleimide or PEG-thiol several protein modifications were indeed detected, which may affect the protein structure, adversely affect its activity and increase the risk of unwanted immune responses. This study indicates that side reactions between biomolecules and polymers, and possible strategies to prevent these, should always be considered when developing new hydrogels for therapeutic delivery and that the stability of each released biomolecule must be carefully evaluated for all cross-linking conditions in question. For example, cross-linking can be performed in slightly acidic solutions below pH 5 in order to decrease the nucleophilicity of amino groups by protonation and reduce their reactivity in Michael-type addition reactions. To protect proteins during radical polymerizations, Censi et al. managed to retain protein activity during photopolymerization

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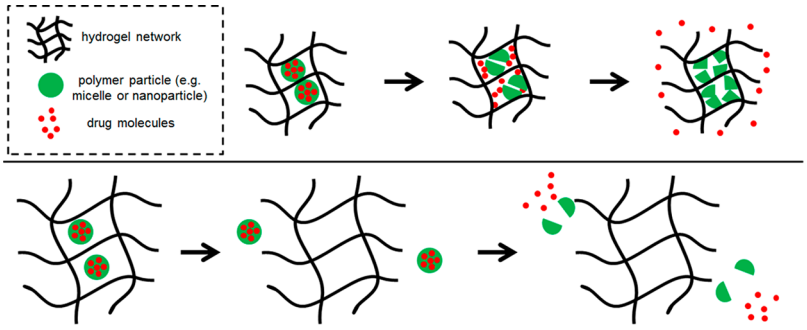

Figure 2. Schematic representation of two mechanisms for drug release from a particle embedded in a hydrogel network: release of the drug from the entrapped particles and subsequent drug diffusion through the polymer matrix of the hydrogel (top); release of drug-loaded particles from the hydrogel and subsequent release of the drug from the particles (bottom).

of methacrylate groups by separating hydrophobic polymerization sites from more hydrophilic protein-containing areas.[40]

2.2. Multicomponent Hydrogels. Early hydrogels were mostly based on a single (co)polymer and often designed to perform only one task. In the past few decades, research shifted increasingly to multicomponent hydrogels that better capture the multifunctional nature of native biological environments including living tissues. They also offer opportunities to selectively tailor materials properties, to diversify the functionality and to optimize the performance of hydrogels. Below we discuss recent approaches aimed at producing multicomponent hydrogels for therapeutic delivery, with descriptions of the use of particulate systems embedded in hydrogels, as well as the simultaneous use of synthetic and biologically derived macromolecules to impart desired properties.

Composites of Polymeric Micro- or Nanoparticles Embedded in Hydrogels. Hydrogels have proven very convenient for the controlled release of water-soluble compounds, but the hydrophilic nature of their matrices makes them particularly disadvantageous for direct entrapment of poorly soluble therapeutic agents.[41] Typically, such hydrophobic agents warrant encapsulation in micro- or nanosized particles, such as poly(ethylene glycol)-poly(lactide) (PEG−PLA) micelles or poly(lactic-*co*-glycolic acid) (PLGA) micro- or nanoparticles, to increase their solubility and to achieve controlled release in biological systems. In this respect, the development of a hybrid drug delivery system, comprising micro- or nanoparticles embedded in hydrogels, is an appealing approach.[42] This strategy has been used to provide a controlled and sustained delivery of poorly soluble drugs in hydrogel-based devices by incorporation of the drugs in particles, followed by loading of these particles in the hydrogel matrix. For example, the group of Allen reported on pHEMA-based hydrogels as a soft contact lens material for the release of ocular drugs.[43] The release of dexamethasone acetate (DMSA), a hydrophobic ophthalmic drug, was limited to three days when it was loaded directly in the hydrogel. To achieve a more sustained release, DMSA was loaded in poly(ethylene glycol)-poly(ε-caprolactone) (PEG−PCL) micelles, followed by incorporation of the micelles in the hydrogels. The incorporation of micelles enhanced the water content of the hydrogel, which was attributed to presence of the hydrophilic PEG corona on the surface of the micelles leading to an increase in the overall hydrophilicity of the construct. In vitro release of DMSA from the micelle/hydrogel hybrids, with varying drug loading levels, was observed for up to 30 days. Modeling of the drug release profiles revealed that diffusion of drug from the micelles was the rate-limiting step in the drug release process.

A composite drug delivery system may also address the problem of the relatively fast release of a loaded drug from particles alone: by incorporation of particles in the polymeric mesh of a hydrogel, a more sustained overall drug release may be achieved, either via the slow release of drug-loaded particles from the hydrogel and subsequent release of the drug from the particles (Figure 2, bottom), in reversed order via release of the drug from the entrapped particles and subsequent drug diffusion through the polymer matrix of the hydrogel (Figure 2, top), or via a combination of these mechanisms.[44] Which mechanism prevails will depend on various parameters, including particle size, hydrogel mesh size, and hydrophilicity of the incorporated drug, as well as the composition of the particle and hydrogel. Importantly, composite drug delivery systems comprising particles embedded in hydrogels may facilitate the simultaneous delivery of drugs with varying solubility. This is important to facilitate combination therapy in, for example, cancer therapy, which has shown several advantages (e.g., synergistic effects and lowering of drug resistance) and may prove more effective than single drug therapy.[45] For example, Zhong et al. prepared a dual-drug delivery system of poly(D,L-lactide) (PDLLA) microspheres embedded in alginate hydrogel beads.[46] In this system, glycyrrhetinic acid (GA, a hydrophobic drug) was encapsulated in the microspheres, while BSA as a hydrophilic model protein drug was loaded in the hydrogel. Following this approach, a prolonged GA release could be accomplished in vitro compared to GA release from PDLLA microparticles alone. In vitro release experiments demonstrated complete BSA release within two weeks, accompanied by a significant burst, whereas the delivery of GA was more sustained, with a release for several weeks. The release rate of BSA was largely accelerated by increasing the PDLLA/alginate ratio, whereas simultaneously, the release rate of GA decreased. However, independent control over the individual drug releases, which is essential for successful combination therapy, was not reported. In general, achieving independently controllable dual drug release from a hybrid system is challenging because the factors governing the release of each drug are often the same or closely related. Also, other polymeric particles such as hyperbranched polymers, liposomes, and cyclodextrins have been incorporated into hydrogels to exploit their hydrophobic drug encapsulation ability.[42]

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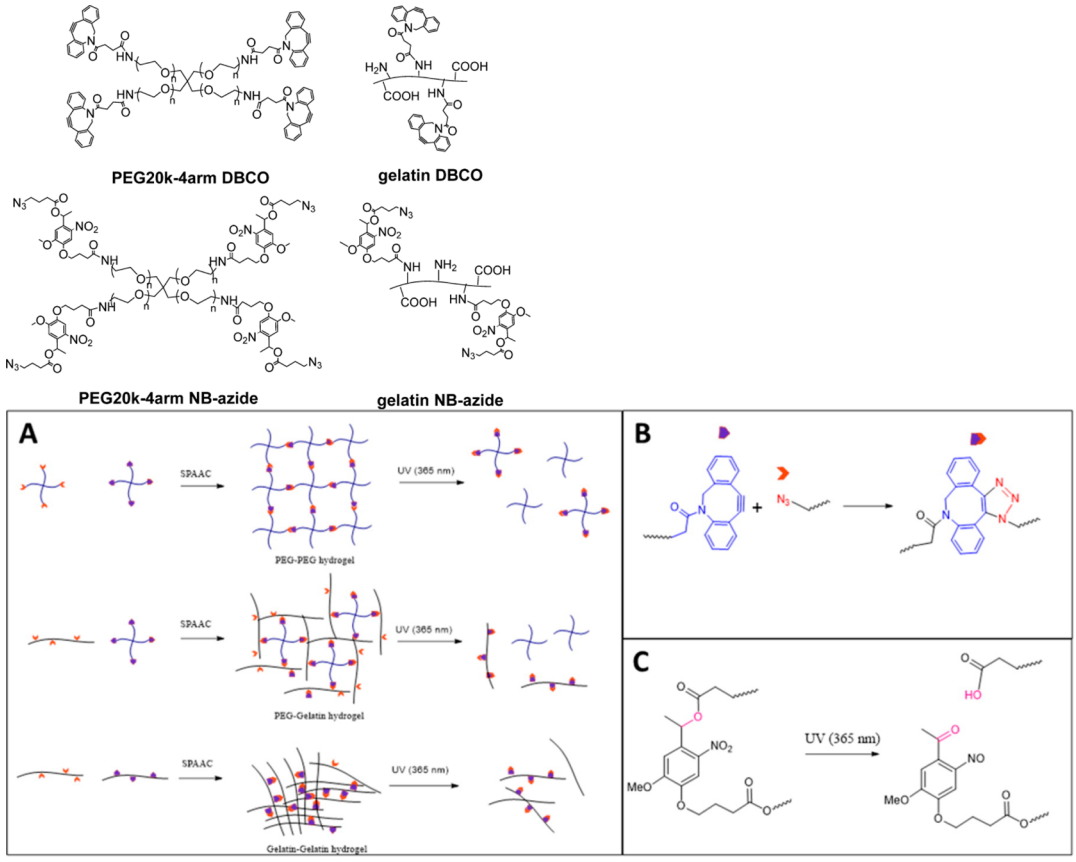

Figure 3. (A) Scheme for the formation and UV induced degradation of PEG−PEG, PEG−gelatin, and gelatin−gelatin hydrogels. (B) Reaction scheme for the SPAAC chemistry. (C) Reaction scheme for the photocleavage of the nitrobenzyl moiety. The chemical structures of the precursors used for hydrogel fabrication are shown at the top of the figure. Reprinted with permission from ref 55. Copyright 2015 American Chemical Society.

Hybrid particle/hydrogel systems have considerable potential as future therapeutic tools in tissue engineering. For example, the use of growth factor loaded microspheres embedded in a hydrogel structure is a promising approach to achieve multimodal protein delivery and to provide a desirable pore structure and porosity to potentially encapsulate cells.[3] Incorporation of small inorganic particles such as hydroxyapatite and calcium phosphate in hydrogels can both enhance the mechanical properties of the hydrogel and promote bioactivity, for example, mineralization for bone tissue engineering applications.[47] Ceramics−hydrogel composite materials have been investigated for drug delivery in tissue engineering, providing controlled scaffold porosity and, therefore, a tailored drug release.[48]

Although promising results have been obtained with composite drug delivery systems comprising polymeric particles embedded in hydrogels, no system exists yet that combines essential features such as biodegradability, mechanical robustness, injectability, and the possibility to independently control the release of two drugs of different aqueous solubility. This may be largely due to the relatively "simple" polymers used to date for the particles and the hydrogels such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO, commercially known as Pluronics or Poloxamers), PEG−PCL, and PVA, whose limited set of properties make it impossible to meet all the required specifications. In the future, emerging knowledge in organic and polymer chemistry will have to be applied to develop a system that meets all fundamental requirements of a particle-hydrogel composite delivery system and to fully exploit the potential of this promising concept.

Hybrid Hydrogels Combining Natural and Synthetic Polymers. In order to overcome the biological deficiencies of synthetic polymers and to enhance the mechanical properties of natural polymers, synthetic and natural polymers have been combined to create hybrid hydrogels that are bioactive, mechanically robust and possess highly tunable properties.[49] Further, by a proper design and selection of the building blocks, fully degradable hydrogels can be obtained. Early publications reported on nonchemically cross-linked blends of synthetic and natural polymers, such as collagen/PVA and hyaluronic acid/PVA hydrogels, which were used for the release of recombinant human growth hormone.[50] More recently, modern synthetic approaches have resulted in the introduction of various functional groups in natural and synthetic polymers and, consequently, the preparation of covalently cross-linked hybrid hydrogels, allowing for an even greater control over hydrogel properties and enhanced mechanical characteristics. For example, hybrid hydrogels have been prepared from methacrylated chitosan and PEG dimethacrylate via photo-cross-linking and were evaluated for insulin release.[51] Other functional group combinations that have been used to create hybrid hydrogels include, for example, isocyanates/amines,[52] hydrazides/aldehydes,[53] and amines/aldehydes. As an example of the latter, the group of Ding reported on an injectable hydrogel formed by Schiff's base reaction between amino groups of glycol chitosan (GC) and aldehyde groups of a multibenzaldehyde functionalized PEG analogue (poly(EO-*co*-Gly)).[54] The water uptake,

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mechanical properties, and network morphology of the GC/poly(EO-*co*-Gly) hydrogels could be modulated by varying the concentration of poly(EO-*co*-Gly). For example, the gelation time could be adjusted between 3.5 and 1.0 min by increasing the concentration of poly(EO-*co*-Gly). Both in vitro and in vivo testing confirmed the degradation of GC/poly(EO-*co*-Gly) hydrogels, and the degradation time of injected hydrogels in mice was greater than 3 months. Moreover, several researchers have explored bioorthogonal cross-link strategies for the preparation of hybrid synthetic/natural hydrogels. To illustrate this, the group of Forsythe prepared hydrogels from gelatin and PEG using SPAAC between dibenzylcyclooctyne and azide groups as cross-linking reaction (Figure 3).[55] By incorporating nitrobenzyl (NB) moieties between polymer chains and the azide functionalities, the obtained hydrogels were degradable upon irradiation with low intensity light of 365 nm. Fibroblasts encapsulated in these hydrogels were viable after 14 days and could be harvested using a light trigger, demonstrating the potential application of this hydrogel as a 3D culturing platform allowing for the capture and release of cells, as well as light-triggered cell delivery in regenerative medicine.

Truong et al. cross-linked water-soluble azide-functionalized chitosan with propiolic acid ester-functional PEG using copper-free click chemistry.[56] The resultant hydrogel materials formed within 5−60 min at 37 °C and exhibited storage moduli up to 100 kPa, which confirms that very robust mechanical properties can be achieved by covalently cross-linking natural and synthetic polymers into hybrid hydrogels.

Much of the research concerning hybrid hydrogels prepared by chemoselective cross-linking remains either fundamental or focuses only on tissue engineering applications.[57] However, this concept may offer tremendous possibilities for therapeutic delivery as well, either as dedicated drug delivery systems or in combination with tissue engineering applications. Since the number of reported polymers is relatively low for hybrid hydrogels cross-linked by chemoselective methods, a need exists for diversification of the polymer spectrum in order to more precisely tailor properties such as mechanical stability, degradability, and release kinetics.

Besides chemically cross-linked hybrid hydrogels, as discussed above, hybrid (semi)-interpenetrating polymeric networks (IPNs), which combine physically and covalently cross-linked networks of natural and synthetic polymers, have emerged as innovative hydrogels for drug delivery.[58] The resulting double network structures may exhibit very strong mechanical properties and lead to synergistic effects on different properties. In particular, the polysaccharides alginate and hyaluronic acid have been used to prepare (semi)-IPN hydrogels by combination with synthetic stimuli-responsive polymers to create new hydrogels that adjust their mechanical and drug release properties in response to an external stimulus. For example, the group of Muniz prepared IPNs of chemically cross-linked pNIPAAm, which shows temperature-responsiveness, and ionically calcium-cross-linked alginate.[59,60] The diffusion of the model compounds Orange II and BSA through hydrogel membranes was found to decrease upon a temperature increase above 35 °C, which was attributed to collapse of the pNIPAAm chains above their lower critical solution temperature (LCST) of 32 °C and subsequent shrinkage of the IPNs.

Besides IPNs, also hydrogels based on aqueous two-phase polymer systems (ATPS) have recently gained interest as method to tune drug release. As an example, dextran hydrogels containing PEG-rich droplets were prepared and pegylated-proteins resided mainly in these droplets. This localization of proteins in the droplets resulted in inhibition of burst release and moreover protein release kinetics could be tuned by the size of the PEG domains.[61,62]

Although there is a wide choice of polymers that can be combined with alginate, hyaluronic acid and dextran to obtain IPNs and ATPS to create "smart" hydrogels for therapeutic release, the biocompatibility and biodegradability of the used polymers and their networks remain important issues that must be addressed in order to effectively apply these systems in vivo and ultimately in clinical studies.

2.3. Stimulus-Responsive Hydrogels. As discussed in the previous paragraph, stimulus-responsive hydrogels undergo transitional changes in response to environmental triggers. They can swell, shrink, degrade, or exhibit a sol−gel phase transition upon changes in pH, temperature, solvent, pressure, ionic strength, light, and concentration of specific biomolecules such as enzymes. Their unique capability to accomplish specific functions, including drug release and in situ gel formation, in response to small changes in environmental conditions has made this class of hydrogels very useful for therapeutic delivery. In this section, we highlight some promising approaches that have been developed recently to achieve enhanced control over the responsive behavior and to improve the practical applicability of stimulus-responsive gels.

Multistimuli-Responsive Hydrogels. Most stimulus-responsive hydrogels to date have been designed to respond to a single trigger. For example, aqueous solutions of selected PEO-PPO-PEO triblock copolymers exhibit a phase transition from the sol to the gel state at low temperatures and from the gel to the sol state at higher temperatures when the concentration is above the critical gel concentration, which makes them suitable for in situ gel formation.[63] To achieve enhanced control over the responsive behavior, hydrogels responsive to multiple triggers, most often pH and temperature, have been developed. Hydrogels responding to both pH and temperature are generally prepared by cross-linking (co)polymers with pH-responsive moieties such as acrylic acid (AAc) and temperature-responsive moieties such as NIPAAm. In a recent publication, Cuginno et al. prepared chemically cross-linked hydrogels for oral or topical delivery by redox-initiated free radical polymerization of AAc and NIPAAm in the presence of *N*,*N*′-diallyltartradiamide as a cross-linker.[64] Adjusting the environmental pH above the pK_a of AAc (4.4) resulted in swelling of the hydrogel due to its charged state and as a result, the release of the basic model drug ofloxacin occurred significantly faster than at low pH. Increasing the environmental temperature above the LCST of pNIPAAm resulted in shrinking of the hydrogels due to partial network collapse, but any influence of temperature on the drug release was not reported. Interestingly, electrostatic interactions between the negatively charged AAc groups in the network and positively charged ofloxacin resulted in a high loading capacity of the hydrogel and sustained release profiles. Several other dual pH/temperature-responsive hydrogels have been described, for example, for the delivery of insulin[65] and the anti-inflammatory drug indomethacin.[66] To broaden the applicability of multi-responsive hydrogels, researchers have also started to explore other combinations of environmental triggers in order to create smart drug delivery systems, such as pH/reduction-,[67] pH/oxidation-,[68] and temperature/oxidation-responsive[69] hydrogels. In these systems, pH- or temperature-responsive polymers

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are combined or cross-linked with moieties such as disulfide or diselenide bridges that can be cleaved in aqueous media by redox agents such as dithiothreitol, glutathione, or hydrogen peroxide, resulting in disintegration of the network, swelling, and subsequent drug release. Reductant- and light-responsive hydrogels were prepared by cross-linking isocyanate-terminated PEG with disulfide-containing 3,3′-disulfanediyldipropane-1,2-diol, azobenzene, and β-cyclodextrin (β-CD; Figure 4).[70] UV irradiation increased the drug loading of the hydrophobic drug coumarin, which was attributed to isomerization of the azo group from the trans to the cis state, subsequent exclusion of the azo group from the β-CD cavity and increase of the network hydrophobicity due to the presence of the free *cis*-azo group. UV irradiation had little influence on the coumarin release, but the presence of the reductive tris(2-carboxyethyl)-phosphine in the release medium increased the drug release due to cleavage of disulfide linkages and the resulting decrease in cross-link density.

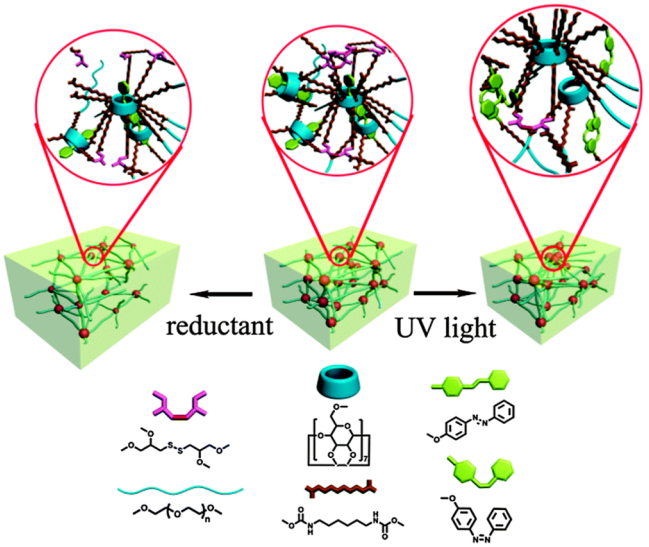

Figure 4. Responsive mechanisms of dual-responsive polyurethane hydrogels reported by Li et al.[70] UV light irradiation causes the azo group to dislocate from the β-CD cavity, resulting in azo group aggregation by hydrophobic interactions and an increase of the network hydrophobicity. The presence of reductant cleaves disulfide bonds, resulting in dissociation of cross-links. Reproduced with permission from ref 70. Copyright 2015 The Royal Society of Chemistry; http://pubs.rsc.org/en/Content/ArticleLanding/2015/TB/c5tb01702e#!divAbstract.

Another approach was adopted by Fu et al., who developed a prodrug gelation strategy for the sustained and dual stimulus-responsive release of doxorubicin from hyaluronic acid-based hydrogels.[71] Hyaluronic acid was functionalized with thiol groups as well as doxorubicin molecules. The drug molecules were attached to the polymer backbone via acid-labile hydrazone links. When exposed to air, the conjugate displayed the ability to self-gelate in aqueous solution resulting from intermolecular disulfide formation. The cumulative drug release was found to be significantly accelerated under acid and reductive conditions, mimicking the intracellular environments of cancer cells. In vitro cytotoxicity assays for human nasopharyngeal carcinoma cells incubated with various release media confirmed the effectiveness of this conjugate hydrogel for cancer cell inhibition.

Next to macroscopic hydrogels as highlighted above, also nanogels have been designed that can respond to multiple environmental triggers, which will be discussed in section 2.5.

The above examples demonstrate that multiresponsive hydrogels offer a superior level of control over their behavior compared to single-responsive constructs. The combined effects of multiple triggers on the network structure may result in increased drug release compared to the effects of a single trigger and hence improved therapeutic efficacy. Also, multi-responsive systems may offer a useful tool to optimize the control of drug release. For example, long-term release induced by one trigger and short-term release via another stimulus can be combined to control the drug release of dual-responsive

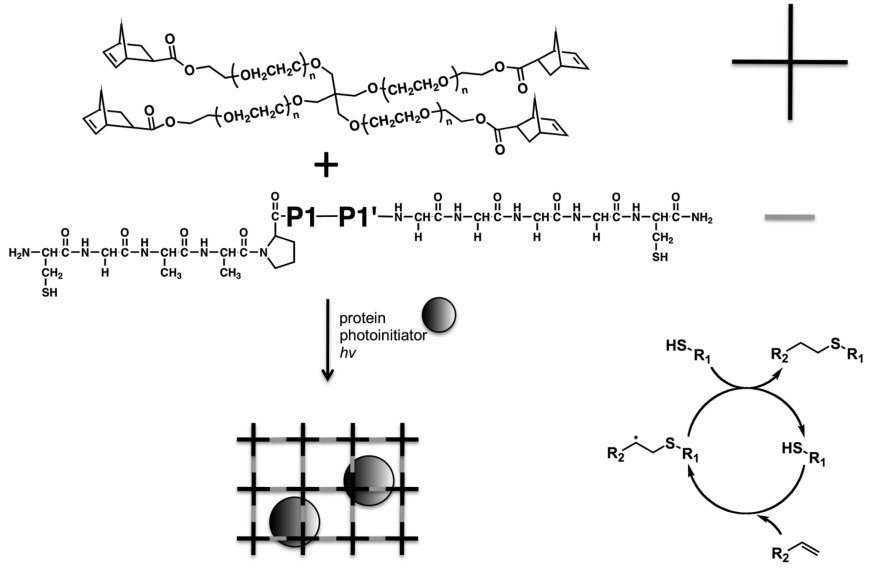

Figure 5. Design and formation of enzyme-responsive PEG hydrogels fabricated via thiol−ene photopolymerization (reaction mechanism included) for the controlled release of protein therapeutics. Enzyme cleavage occurs between the P1 and P1′ amino acid residues. Reproduced with permission from ref 77. Copyright 2009 Elsevier.

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hydrogels. Nonetheless, there is still a demand to continue the investigations of multiresponsive materials in the future. Most notably, a rapid responsiveness to the applied stimuli, a high magnitude of the resulting effects as well as a high level of reproducibility still represent challenges that need to be addressed in order to enhance the applicability of this promising class of hydrogels.

Enzyme-Responsive Hydrogels. Although pH- or temperature-responsive hydrogels have provided great advancements in the area of controlled release, it remains a challenge to reliably regulate the release profile based on the magnitude of these nonspecific, physicochemical stimuli beyond changes in diffusion rate.[72] An emerging area of drug delivery therefore focuses on localized, controlled release in recognition of a cellular response via the use of enzyme-responsive hydrogels. The most frequently employed methodology for the preparation of such hydrogels is by utilizing peptide sequences typically chosen to be cleaved by specific, cell-associated enzymes. The peptides may be incorporated as part of the polymer backbone, as cross-links within the hydrogel or as links between the polymer backbone and drug molecules. The latter strategy has been pioneered by Kopeçek and colleagues who, for example, developed hydrophilic polymers based on 2-hydroxyethyl methacrylate with doxorubicin attached via short oligopeptide sequences cleavable by the tumor-associated enzyme cathepsin B.[73,74] More recently, also macroscopic PEG hydrogel constructs employing a similar prodrug-like approach have been designed to selectively release active compounds such as chemotherapeutic platinum-based agents.[75] Although chemically linking a drug to the network via pendant enzyme-cleavable peptides may allow for a precise, on-demand release, the amount of drug that can be loaded within the hydrogel is often limited. The use of peptides as cross-linker rather than as a pendant linker, thus causing degradation of the hydrogel upon cleavage by the target enzyme, may facilitate higher drug loading by physical incorporation and may therefore result in enhanced and/or sustained therapeutic effects. Hubbell et al. prepared hydrogels by a Michael addition reaction between multiarm PEG vinyl sulfones and thiol-bearing peptides.[76] Incorporation of matrix metalloproteinase (MMP) cleavable sites allowed for MMP-mediated degradation and the release of physically entrapped BMP. Whereas these hydrogels were primarily used for tissue engineering, nowadays also systems focusing only on drug delivery have been developed. The group of Anseth reported on a PEG hydrogel platform, using thiol−ene photopolymerization, with human neutrophil elastase (HNE) sensitive peptide cross-links, which renders the gel degradable at sites of inflammation (Figure 5).[77] A controlled, zero-order release of BSA together with the smaller protein carbonic anhydrase was achieved in the presence of HNE, whereas no release was observed in the absence of HNE.

Gao et al. reported on small molecule hydrogels that formed via self-assembly of tyrosine-based tetra-peptide derivatives, which were prepared via solid phase peptide synthesis.[78] A transition from the gel to the sol state could be induced by the addition of tyrosinase, which converted tyrosine to quinone in the presence of oxygen, resulting in the loss of the cross-linking $\pi-\pi$ interactions between the aromatic phenol rings. The potential of the tyrosine hydrogels for controlled drug release was demonstrated by the incorporation of Congo Red, which was released upon the addition of tyrosinase. However, one of the primary limitations of using peptides is the difficulty of making large quantities of synthetic peptides, which results in high costs. The use of peptides as cross-linkers, rather than as an integral part of the hydrogel backbone, enables targeted degradability and drug release, while greatly reducing the amount of peptide required, making the cross-linking approach more cost-effective and therefore more likely to be clinically relevant.[79] An interesting approach in this respect is the preparation of hybrid, hydrogel-forming PEG-poly(alanine-*co*-phenyl alanine) block copolymers via ring opening polymerization of the N-carboxy anhydrides of alanine and phenyl alanine, as reported by the group of Jeong.[80] The hydrogel was stable in PBS, but gradually dissolved in subcutaneous tissue of rats, most probably due to polymer degradation by proteolytic enzymes, such as cathepsin B and elastase. A subcutaneous injection of an insulin formulation in diabetic rats created a hydrogel depot in situ via the temperature-induced sol-to-gel transition. The depot released the insulin and exhibited a hypoglycemic effect over a period of 18 days, most likely via a combination of diffusion and enzyme-induced hydrogel degradation.

Although enzymatically degradable hydrogels represent intelligent drug delivery systems capable of facilitating localized release in response to a cellular event, some issues need to be addressed for clinical translation. One challenge is the reproducibility of the performance of enzymatically degradable hydrogels, since the degradation kinetics and release of entrapped therapeutics depend on the concentration of the matching enzyme at the site of application, which may vary among patients as well as among parts of the body. Furthermore, some peptide sequences may induce an immune response, which hampers in vivo application of enzymatically degradable hydrogels containing these sequences.

Dual Gelling Hydrogels. In situ forming hydrogel systems are injectable fluids that can be introduced into any tissue, organ, or body cavity in a minimally invasive manner prior to gelation.[81−86] They offer several advantages over hydrogels that have to be formed into their final shape before implantation. To mention, there is no need for surgical procedures, their initially flowing nature ensures proper shape adaptation as well as a good fit with surrounding tissue, and biologically active species including living cells can be incorporated homogeneously in the hydrogel by simple mixing with the precursor polymer solution. Physically cross-linked injectable hydrogels may offer various advantageous properties, such as fast gelation under mild conditions and environmental responsiveness, but they are generally mechanically weak. Although chemically cross-linked hydrogels may yield mechanically robust networks after complete cross-linking, they often exhibit slow gelation kinetics, which may result in premature dissolution of the hydrogel. An interesting development in this respect is therefore the combination of physical and chemical cross-linking, which allows the administration of polymers as liquid formulations that undergo quick gelation due to physical interactions and are subsequently stabilized by chemical reactions. Most dual gelling systems reported to date are based on thermosensitive polymers, such as pNIPAAm, PEO-PPO-PEO, and poly(*N*-(2-hydroxypropyl)methacrylamide) (pHPMA) derivatives, which are modified with various chemical functional groups for postgelation reactions including Michael additions, Schiff base reactions, enzyme-mediated cross-linking, epoxy reactions with amines or phosphates, and photo-cross-linking. A limited number of these systems have been employed for the controlled release of therapeutic agents, as reviewed by Nguyen et al.[87] Recently, temperature-induced physical gelation has also

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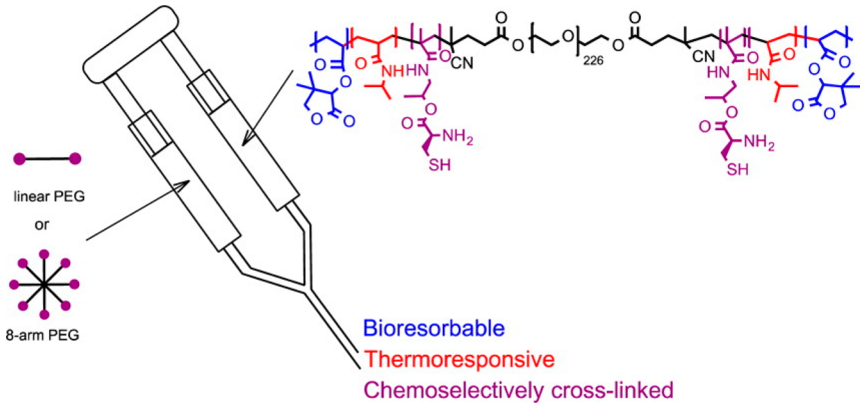

Figure 6. Schematic representation of the dual gelling system reported by Boere et al.[88] Thermoresponsive NIPAAm units (red) allow for initial, thermally induced gelation. Native chemical ligation between cysteine functionalized HPMA and thioester or NHS-functionalized PEG cross-linkers (purple) subsequently stabilizes the hydrogel. The hydrolyzable lactone ring in the dimethyl-γ-butyrolactone acrylate moiety (blue) facilitates bioresorbability. Reprinted with permission from ref 88. Copyright 2015 American Chemical Society.

been combined with native chemical ligation as a chemoselective cross-link strategy.[88] Triblock copolymers consisting of cysteine functionalities, thermoresponsive NIPAAm units, and degradable moieties were mixed with thioester or *N*-hydroxysuccinimide (NHS)-functionalized PEG cross-linkers (Figure 6). The combined physical and chemical cross-linking resulted in rapid network formation and mechanically strong hydrogels. Lysozyme was loaded in the gels, and after two days, more than 90% of the loading was released, confirming that the cross-linking reaction was chemoselective as the protein was not covalently grafted to the hydrogel network. Moreover, the degradation rates of these hydrogels under physiological conditions could be tailored from 12 days up to 6 months by incorporation of a monomer containing a hydrolyzable lactone ring in the thermosensitive triblock copolymer.

Gregoritza et al. prepared in situ forming hydrogels from furan- and maleimide-functionalized eight-armed PEG using the Diels–Alder reaction as a chemoselective cross-linking mechanism. Upon introduction of a 12-aminododecanoic acid spacer between the PEG backbone and the functional end groups, chemical gelation of the PEG hydrogels occurred twice as fast. This phenomenon was ascribed to improved association of macromonomers by hydrophobic interactions and subsequent enhanced chemical cross-linking via Diels–Alder chemistry. Due to the increased cross-linking density, the degradation rate of hydrophobic spacer-modified hydrogels decreased by a factor of 4, and the in vitro release of bevacizumab, a therapeutic antibody, sustained for almost 60 days.[89]

Among the dual gelling systems, an interesting approach was adopted by Hiemstra et al., who prepared in situ forming, yet robust hydrogels by combining stereocomplexation and photo-cross-linking of methacrylate-terminated enantiomeric PEG–PDLA and PEG–PLLA star block copolymers (Figure 7).[90] Stereocomplexation provided fast gelation at relatively low concentrations, while photopolymerization of the methacrylate groups yielded significantly increased mechanical properties and prolonged degradation times, but no release experiments were reported.

In summary, because both physically and chemically cross-linked hydrogels have their own limitations, dual gelling hydrogels are a promising solution, but the full potential of these systems for the controlled delivery of therapeutics particularly in vivo is yet to be explored.

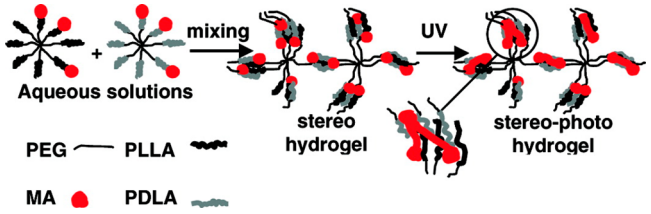

Figure 7. Schematic representation of the preparation of stereo-complexed and photo-cross-linked hydrogels based on methacrylate-terminated enantiomeric PEG–PDLA and PEG–PLLA star block copolymers. Reprinted with permission from ref 90. Copyright 2007 American Chemical Society.

The above examples demonstrate the major advances that have been achieved in the field of hydrogels undergoing dual gelation. We will conclude this section with an example of chemical gelation followed by on-demand chemical dissolution by Konieczynska et al., who developed a dissolvable hydrogel burn dressing for second-degree burn care.[91] The hydrogel dressing formed in situ after mixing aqueous solutions of a lysine-based dendron and a NHS-terminated PEG cross-linker containing thioester groups. In a second-degree burn wound model in rats, the hydrogel dressing completely covered the wound and acted as a barrier to bacterial infection. When a solution of cysteine methyl ester was applied to the dressing, the hydrogel dissolved due to thiol-thioester exchange between the thioester present in the hydrogel network and the exogenous thiolate solution, allowing for facile, atraumatic wound dress removal. This system also has potential for drug delivery since the gel can be retrieved after de-cross-linking once, for example, unfavorable effects of the loaded therapeutic are observed.

2.4. Release of Biologically Active Agents from 3D Printed Hydrogels. Tissue engineering applies the principles of biology and engineering to the development of functional substitutes for damaged tissue.[92] From a biological point of view, hydrogels are attractive candidates for tissue engineering constructs because they can provide an aqueous 3D environment for cells, simulating the natural extracellular matrix.[10,93] Also, viscoelastic properties of hydrogels can be tuned to influence cell fate and activity.[94] To mimic the complex nature of tissues, additive manufacturing has emerged as a valuable technology to generate bioengineered 3D structures. In this

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approach, termed biofabrication, biological structures for tissue engineering are created by a computer-aided manufacturing process for patterning and assembling living and nonliving materials with a prescribed 3D organization. The inks for biofabrication are typically based on hydrogels. The mild, biologically benign processing parameters that can be applied with this methodology facilitate the encapsulation of cells during the printing process, which has been reported in a number of cases, for example, by Melchels et al., who developed a bioink based on gelatin-methacrylamide.[95] It was shown that by adding gellan gum and tailoring salt concentrations, rheological properties such as pseudoplasticity and yield stress could be optimized toward gel dispensing for the additive manufacturing process (Figure 8). In the hydrogel formulation, salt was partly substituted by mannose to obtain isotonicity and maintain cell viability.

The next generation of 3D printed hydrogels should not only facilitate physical support for cells within a 3D microenvironment, but also be able to promote cell proliferation, direct cell differentiation, and induce extracellular matrix formation through covalent incorporation or the controlled release of cell-regulating factors. In this context, protein patterning is a promising technology, as demonstrated by the group of Tirrell. They used two bioorthogonal photochemistries to achieve reversible immobilization of bioactive full-length proteins within PEG-based hydrogels.[97] A photodeprotection-oxime-ligation sequence allowed precise quantities of proteins to be anchored within distinct subvolumes of a 3D matrix, and an *ortho*-nitrobenzyl ester photoscission reaction facilitated subsequent protein removal. By using this approach to pattern the presentation of the extracellular matrix protein vitronectin, reversible differentiation of human mesenchymal stem cells to osteoblasts was accomplished in a spatially defined manner. Apart from controlling cellular behavior, the incorporation and release of therapeutic agents may also be useful for other purposes. For example, anti-inflammatory drugs may be applied to control inflammatory responses following scaffold implantation, which still represents a challenge in tissue engineering.[98]

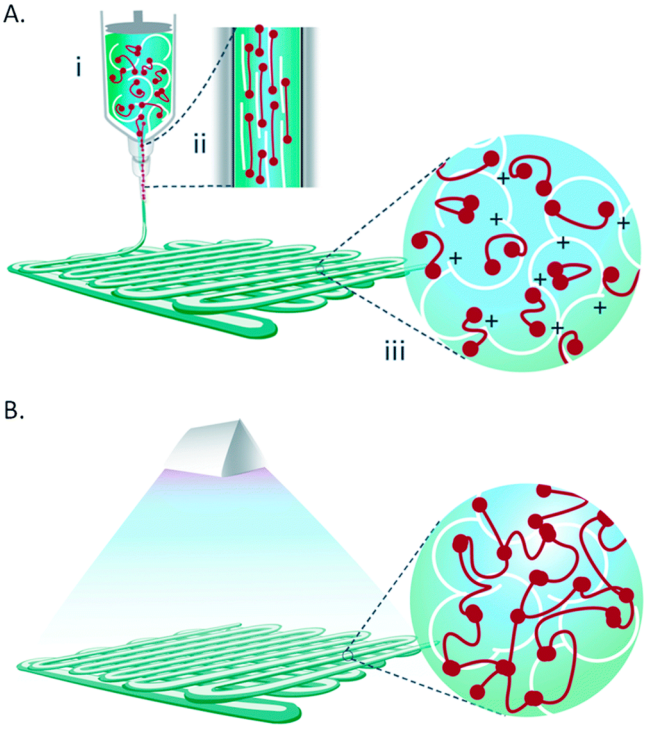

Figure 8. Schematic representation of gelatin-methacrylamide bioink dispensing (A) followed by UV curing (B). In the syringe, the gellan chains (in white) form a temporary network in the presence of cations, and induce gel-like behavior (i). Upon dispensing through a nozzle, the temporary network is broken up by shear forces and all polymer chains align resulting in a reduction of the viscosity (ii). Directly after removal of shear stress, the temporary network is restored and the plotted filament solidifies instantly (iii), after which the slower thermal gelation of gelatin-methacrylamide further increases the stability of the printed bars and struts. Upon UV exposure the gelatin-methacrylamide chains (in red) form a permanent polymer network (B). Reproduced with permission from refs 95 and 96. Copyright 2014 The Royal Society of Chemistry and Copyright 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim; http://pubs.rsc.org/en/Content/ArticleLanding/2014/TB/c3tb21280g#!divAbstract.

Although the number of publications on 3D printable hydrogels is rapidly increasing, as reviewed elsewhere,[96,99,100] research concerning drug and growth factor delivery from these constructs is still very limited. Mishra et al. prepared 3D hydrogels via layer-by-layer "writing" of cell-loaded PEG diacrylate prepolymer formulations using blue light induced photopolymerization.[101] Addition of silica fillers in the prepolymer formulation modified its rheological properties and made it easily dispensable for the fabrication of 3D hydrogels. The antibiotic tetracycline hydrochloride was incorporated in the 3D constructs by adding it to the prepolymer formulations prior to cross-linking. It was shown that the mode of transportation of the loaded solute was governed by Fickian diffusion and non-Fickian/anomalous transport for loosely and tightly cross-linked gels, respectively. In contrast, the water transport was controlled by a super case II mechanism, where processes other than diffusion, such as macromolecular relaxation and erosion of the polymer chains, also contribute to the swelling. The cytocompatibility of the 3D printed construct was demonstrated by the high viability and 3D growth of incorporated fibroblasts. Following a different approach, Poldervaart et al. used BMP-2 loaded cross-linked gelatin microspheres as a sustained release system, which was dispersed in an alginate prepolymer solution prior to hydrogel fabrication via layer-by-layer deposition and subsequent cross-linking with calcium ions.[102] Following this methodology, a prolonged BMP-2 release could be accomplished in vitro compared to direct incorporation of BMP-2 in the hydrogel. Importantly, the BMP-2 retained its bioactivity after 3D printing as evidenced by osteogenic differentiation of incorporated goat multipotent stromal cells in vitro, demonstrating the excellent protein-compatibility of this technology. The presence of BMP-2 in 3D printed hydrogels significantly increased bone formation in vivo, but the incorporation of BMP-2 in microspheres instead of direct BMP-2 incorporation did not influence the bone growth within the constructs.

In general, 3D printing is an attractive method to prepare hydrogels for therapeutic release because it enables the rapid production of constructs with a defined architecture and regional differences by computer-controlled deposition of the drug-loaded hydrogel, which potentially allows for a high level of spatial and temporal control over the release. The studies discussed in this paragraph, which focused on tissue engineering applications, show that controlled therapeutic delivery from 3D printed hydrogels is a feasible concept, but the field is still in its infancy. The further development of 3D printable hydrogels that facilitate the release of therapeutic agents will significantly

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advance this exciting and promising approach for tissue engineering, but only when the biological activity of the therapeutic agent can be maintained and the reproducibility and mechanical stability of the construct can be tailored and guaranteed.[103]

2.5. Nanogels. Whereas hydrogel research has mainly focused on macroscopic constructs in the 20th century, there is now an increasing interest in hydrogels exhibiting nanoscopic dimensions, termed nanogels.[104−107] They have properties similar to those of their macrogel counterparts, but nanogels have the advantage that they can be intravenously injected and reach areas of the body that are not easily accessed by macroscopic hydrogels exploiting the so-called enhanced permeability and retention (EPR) effect.[108] Moreover, because nanogels can be taken up by cells, they are excellent candidates for the intracellular delivery of therapeutic agents that need to be safely delivered into the cytoplasm of the target cell such as nucleic acid-based drugs and chemotherapeutics.[109−111] Various other nanocarriers have been described in literature, such as micelles, liposomes, and polymersomes, but in comparison with nanogels, these systems suffer from drawbacks such as a lack of controlled loadability, limitations in material composition, limitations in the type of biological cargo that can be loaded and released, and physical instability.[105] Nanogels should be prepared with a sufficiently high cross-link density, either via chemical or physical methods, to prevent premature leakage of the incorporated therapeutic compounds. Such nanogels generally release their cargo in a sustained manner due to hydrolytic degradation of the polymer network. However, this sustained release may lead to insufficient concentrations of the therapeutic agent at its site of action. To achieve enhanced control over the release and reach a higher drug concentration at the target site, our group recently prepared nanogels with tailorable degradation and release profiles under physiological conditions.[112] These nanogels were prepared via mini-emulsion photopolymerization of methacrylate-oligoglycolates-derivatized poly(hydroxypropyl methacrylamide) (pHPMAm-Gly-HEMA) and hydroxyethyl methacrylamide-oligoglycolates-derivatized poly(hydroxyethyl methacrylamide) (pHEMAm-Gly-HEMAm), in which methacrylate groups are coupled to hydrophilic pHPMAm and pHEMAm via labile, biodegradable glycolate esters. Since pHEMAm-Gly-HEMAm hydrolyzed more rapidly than pHPMAm-Gly-HEMA, pHEMAm-Gly-HEMAm nanogels showed faster degradation under physiological conditions. Hydrolysis studies of two types of nanogels with different cross-link densities revealed different degradation times from 24 h to over 4 d under physiological conditions (37 °C and pH 7.4). Release experiments with dextran blue, which was initially entrapped in the network mesh due to its large hydrodynamic radius, showed release times similar to the degradation times of the nanogels, indicating that the release is governed by degradation of the hydrogel network.

In recent years, nanogels have also been designed that rapidly degrade and swell in response to the physiological differences between the extracellular space and the intracellular environment and consequently release their payload inside the cell. An advantageous aspect of nanogels in this respect is their small size, which facilitates a rapid response to the various stimuli. Especially the low pH of the endo- and lysosomes as well as the low reduction potential inside cells have shown great potential as biological, intrinsic stimuli to trigger drug release from nanogel systems, as illustrated below.

The tripeptide glutathione is the most abundant reducing agent in mammalian cells having an intracellular concentration in the range of 2 to 10 mM, which is approximately 100−1000-fold higher than its concentration in the extracellular environment (2−20 μM). In a typical approach, therapeutics are physically entrapped in nanogels having disulfide linkages in their polymer network. Under reductive, intracellular conditions, the cleavage of disulfide bonds results in degradation of the gel and release of the drug. For example, Peng et al. developed redox-responsive nanogels for the encapsulation and release of enzymes.[113] The nanogel synthesis and encapsulation of the enzyme were performed simultaneously via in situ cross-linking of pyridyldisulfide-functionalized poly(vinylpyrrolidone) copolymers, employing a disulfide exchange reaction with multifunctional thiol-terminated cross-linkers. At physiological conditions, the nanogels were stable and encapsulated cellulase displayed little activity, as demonstrated with a fluorometric assay, which was ascribed to inaccessibility of the enzyme to the substrate. In the presence of 10 mM dithiothreitol (DTT), the nanogels degraded due to cleavage of the cross-links in the network resulting from competitive disulfide exchange with DTT and the enzyme activity rapidly recovered. Fluorescence resonance energy transfer (FRET) experiments revealed that the activity recovery was mainly due to the protein release induced by dissociation of the disulfide cross-linked network, instead of the enhanced substrate transport rate. In another strategy, an antigen was covalently attached to the nanogel network via a disulfide linkage that can be reduced intracellularly, as recently shown by our group.[114] A model antigen (ovalbumin, OVA) was covalently conjugated to the matrix of cationic dextran nanogels via disulfide bonds. Reversible immobilization of OVA in the nanogels was demonstrated by the observation that hardly any release of the protein occurred at pH 7 in the absence of glutathione, whereas rapid release of OVA was observed once the nanogels were incubated in buffer with glutathione. In a follow-up study, these nanogels proved to be very efficient delivery systems in mice, where the released OVA antigen was able to prevent tumor growth and reduce tumor size in a prophylactic and therapeutic vaccination setting, respectively.[115] In general, physical entrapment of the drug adds versatility to a nanogel system since a variety of drugs can be encapsulated in the same nanogel without the need for changing the drug attachment chemistry. On the other hand, covalently bound systems offer the advantage of greater drug stability and may prevent a burst release.

The slightly acidic environments in endosomes (pH 5.0−6.5) and lysosomes (pH 4.5−5.0) as compared to physiological pH of 7.4 in the blood have also been exploited to design nanogels for intracellular delivery of therapeutic agents. Nanogels have been prepared with acid-labile hydrazone linkages either in the polymer network[116] or between the drug and the polymer network.[117] Another frequently applied approach makes use of polymers with ionizable groups. Protonation of these groups in the intracellular compartments results in swelling or disassembly of the nanogels, leading to triggered release of the incorporated therapeutic agent. To exemplify this, the group of Yu prepared drug-loaded nanogels by mixing anionic sodium alginate with cationic doxorubicin, followed by in situ cross-linking with calcium ions under ultrasound (Figure 9).[118] In vitro release profiles showed a significantly higher doxorubicin release at pH 5.0 than at pH 7.4 due to protonation of the carboxylic acid groups on alginate,

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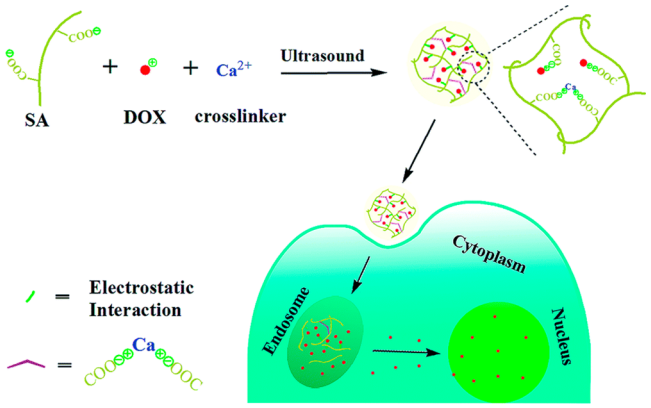

Figure 9. Illustration of the formation, cellular internalization, and intracellular drug release of doxorubicin-loaded alginate nanogels. Reproduced with permission from ref 118. Copyright 2015 The Royal Society of Chemistry; http://pubs.rsc.org/en/Content/ArticleLanding/2015/RA/C5RA13313K#!divAbstract.

resulting in dissociation of the Ca^{2+} cross-links as well as the disruption of the electrostatic interactions between doxorubicin and alginate. In vitro cytotoxicity tests demonstrated that doxorubicin-loaded gels were able to inhibit the growth of HeLa cells, displaying an IC_{50} value of 0.26 μg/mL, which is similar to that of free doxorubicin. Confocal laser scanning microscopy confirmed that the nanogels were internalized into HeLa cells through endocytosis and that doxorubicin was released into the cytoplasm and subsequently transferred to the nucleus.

However, a major concern regarding the use of polymers with ionizable groups in circulation is their hemocompatibility, as charged polymers have been reported to induce changes in blood coagulation, protein aggregation, red blood cells agglomeration, platelet activation, and complement activation. Narain et al. prepared galactose-based nanogels bearing net positive, negative, or neutral charges and evaluated them for their blood compatibility profiles.[119] The anionic and neutral nanogels exhibited high blood compatibility, whereas cationic nanogels showed increased blood coagulation time, fibrinolysis, and platelet activation unless the cationic content was covered by a protective neutral shell. This demonstrates that hemocompatibility should be taken into account as an important parameter when designing and evaluating pH-sensitive nanogels with ionizable groups for therapeutic delivery.

Recently, dual responsive nanogels were developed to prevent premature drug leaching and to achieve efficient intracellular drug release. For example, the group of Haag synthesized redox- and pH-responsive nanogels using hyperbranched polyglycerol.[120] The nanogels were prepared via an inverse nanoprecipitation method based on a thiol−disulfide exchange reaction resulting in disulfide-containing cross-links (Figure 10). A doxorubicin prodrug containing a hydrazone linkage was conjugated to the polymer matrix either in the nanogel interior or at its surface via a Michael addition reaction. The nanogels showed little drug leaching in the absence of any trigger or when only one stimulus was applied, but efficient release of the payload was observed in an environment that was both acidic and reductive (pH 5 and 10 mM DTT). Confocal laser scanning microscopy and flow cytometry experiments suggested that after internalization in tumor cells by endocytosis, the hydrazone bond was cleaved under increasing acidic conditions in the endosomes/lysosomes, changing the covalently bound prodrugs into physically encapsulated drugs. Doxorubicin was rapidly released as soon as the nanogels were degraded in the cytosol under reducing conditions, and the drug subsequently induced cell death by intercalation with DNA in the nucleus.

The different nanogel approaches, as discussed above, have demonstrated great potential for the encapsulation and efficient intracellular delivery of therapeutic agents. Particularly appealing is the design of nanogels that retain their payload in the extracellular environment but release it once they are internalized by target cells, in response to one or more intracellular triggers. However, little is known about the exact intracellular fate of these responsive nanogels, especially within endo/lysosomal compartments, due to the lack of direct evidence of cleavage of disulfide bonds and acid-labile linkages. Therefore, the further development of responsive nanogel systems requires a better understanding of their intracellular mechanism of action. Moreover, a need exists for in vivo data from nanogels in preclinical development, as there are few reports on their long-term accumulation and degradation profiles. To translate the nanogel concept into a viable therapeutic approach, it is necessary to further study toxicity, immunogenicity, and pharmacokinetics together with therapeutic effects in vivo.

3. CONCLUSIONS AND FUTURE PERSPECTIVES

This Perspective reflects the tremendous progress that has been achieved in the field of hydrogels for therapeutic delivery in the

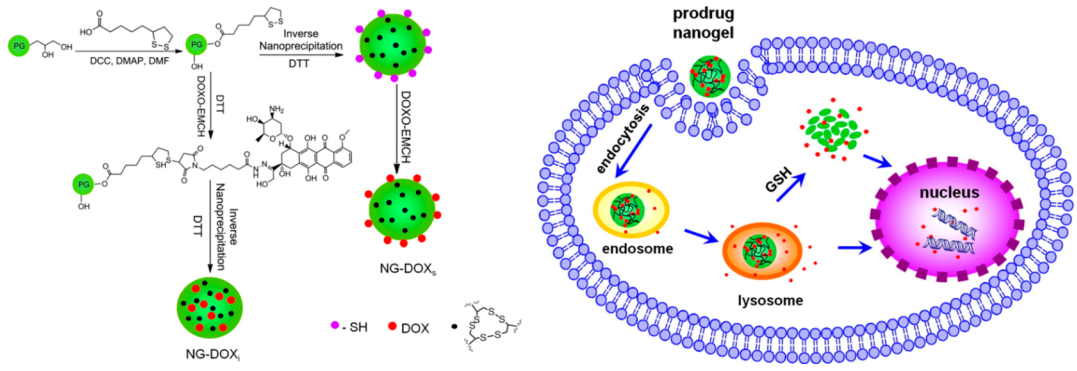

Figure 10. Synthetic pathways of redox- and pH-responsive nanogels with doxorubicin conjugated in the interior or at the surface (left); uptake of the nanogels followed by intracellular release of doxorubicin (right). Adapted with permission from ref 120. Copyright 2013 Elsevier.

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past 50 years. During this time, hydrogels evolved from relatively simple chemically or physically cross-linked networks for the prolonged release of a single component to today's complex multicomponent systems capable of releasing multiple therapeutics in a spatially and temporally controlled and triggered manner. We expect that in situ forming hydrogels will play an increasingly important role as controlled drug delivery systems due to their facile administration and the straightforward incorporation of active agents. In this respect, the use of tandem physically/chemically gelling hydrogels is an attractive approach because they combine injectability with mechanical robustness. Multiresponsive hydrogels allow for enhanced control over triggered drug release in response to environmental stimuli, while enzyme-responsive systems facilitate localized, controlled release in recognition of a cellular event. With improvements in bioinks and additive manufacturing techniques, 3D printed hydrogels increasingly mimic the complex biological and functional organization of native tissues. The incorporation and release of biologically active agents such as growth factors in 3D printed constructs, which may help to achieve enhanced control over cellular behavior, will undoubtedly further advance this promising approach for tissue engineering. Nanogels are particularly interesting for therapeutic delivery because they can be injected intravenously, unlike macroscopic hydrogels, and deliver drugs intracellularly. Their nanoscale dimensions facilitate a rapid response to environmental stimuli, which is attractive when aiming for triggered drug delivery in response to intracellular clues. Bioorthogonal cross-linking reactions play an important role in many of the new developments discussed in this Perspective. Although the functionalization of macromonomers with chemoselective groups may be challenging and elaborative in some cases, bioorthogonal cross-linking will increasingly replace "classical" cross-link reactions such as Schiff base reactions due to their fast reaction kinetics, high chemical selectivity and inertness toward native biological molecules.

Lastly, we provide a perspective on current basic demands for a hydrogel system that can encapsulate and deliver therapeutics successfully and efficiently. Although the design of each therapeutic hydrogel should be tailored to the targeted disease, the possibility of in situ formation can be regarded as a basic requirement. In this respect, depending on the applied cross-linking method, the use of bioorthogonal reactions (for chemical cross-linking) or a fast and significant response to environmental/physical gelation triggers (for noncovalent cross-linking) are considered essential. A second basic requirement is that each drug should be delivered at its optimal dose during specific periods in the treatment. Even though this may also be achieved with the relatively simple hydrogels developed in the 20th century, many of the new concepts discussed in this Perspective allow for modulation of the release after hydrogel administration, providing enhanced control in order to maintain an optimal drug concentration over time.

Some developments in this Perspective are still in an early stage, while for others, several issues need to be solved before clinical translation can be realized. Nonetheless, we believe that the new concepts, as highlighted in this Perspective, will significantly improve the safety, applicability, and performance of therapeutic hydrogels and, therefore, increase their role within the field of therapeutic delivery.

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ABBREVIATIONS

AAc	acrylic acid
BMP	bone morphogenetic factor
BSA	bovine serum albumin
β-CD	beta-cyclodextrin
DBCO	dibenzylcyclooctyne
DMSA	dexamethasone acetate
DTT	dithiothreitol
GA	glycyrrhetinic acid
HNE	human neutrophil elastase
IPN	interpenetrating polymer network
LCST	lower critical solution temperature
MMP	matrix metalloproteinase
NB	nitrobenzyl
NHS	*N*-hydroxysuccinimide
OVA	ovalbumin
PCL	poly(ε-caprolactone)
PEG	poly(ethylene glycol)
PEO-PPO-PEO	poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)
PDLLA	poly(D,L-lactide)
pHEMA	poly(2-hydroxyethyl methacrylate)
pHPMA	poly(*N*-(2-hydroxypropyl)methacrylamide)
PLA	poly(lactic acid)
PLGA	poly(lactic-*co*-glycolic acid)
pNIPAAm	poly(*N*-isopropylacrylamide)
PVA	poly(vinyl alcohol)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPAAC	strain-promoted azide−alkyne cycloaddition

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