CHEMICAL REVIEWS

Review

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Hydrogels for Protein Delivery

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1. INTRODUCTION

1.1. Pharmaceutical Proteins and Delivery Issues

Once a rarely used class of therapeutic agents, pharmaceutical proteins have increased remarkably in number and frequency of use since the introduction of the first recombinant protein therapeutic, human insulin, 30 years ago.[1] With more than 130 FDA (US Food and Drug Administration) approved products and many more in development, protein therapeutics have gained a significant role in almost every field of medicine, like cancer, inflammatory diseases, vaccines, and diagnostics.[2] The increasing use of pharmaceutical proteins can be explained by some advantageous properties as compared to small-molecule drugs. Proteins serve a highly specific and complex set of functions—i.e., catalysis of biochemical reactions, formation of membrane receptors and channels, transport of molecules within a cell or from one organ to another, and intracellular and extracellular scaffolding support—that can hardly be tackled by small synthetic compounds.

Importantly, with the advent of hybridoma and recombinant DNA technologies, the limitations associated with the extraction and purification of pharmaceutical proteins from animal sources can be circumvented. Protein therapeutics have become mass-scale products, manufactured using bacteria, yeast, mammalian cells, and transgenic plants. These biotechnological routes yield proteins with improved safety profiles, being generally well-tolerated and less immunogenic, as compared to animal-extracted proteins. Protein therapeutics derive their specificity and function from their amino acid-based primary, secondary, and tertiary structure. For example, somatostatin owes its biological activity to a characteristic hairpin-loop structure, which is present in both the 14 and 28 amino acid active forms.[3] Since this discovery, the production of smaller therapeutically active synthetic analogues (i.e., octapeptide octreotide), all sharing the hairpin loop pattern, became possible. However, the delicate three-dimensional structure of proteins is also a major limitation to the use of

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pharmaceutical proteins, as they suffer from poor stability, due to proteolytic and chemical degradation as well as physical unfolding and aggregation.[4−6] This instability leads to loss of activity and often elicits an immune response.[7,8] Because of their fragile nature, oral administration of proteins is a particularly challenging route due to the high proteolytic activity and low pH of the stomach, which destabilize and degrade the protein structure, resulting in loss of biological activity. However, the capability to protect the active compound from the harsh conditions of the gastrointestinal (GI) tract is not the only challenge. Bioavailability is another major issue associated with oral administration. The large molecular size as well as the hydrophilicity of protein therapeutics makes their absorption through biological membranes difficult, and consequently, oral and transdermal administrations are ineffective. Therefore, to date protein drugs are almost without exception administered parenterally. But, because of the first-pass hepatic metabolism, the fast renal clearance, and consequently the short half-lives of many proteins (i.e., growth hormone, insulin, oxytocin, paratyrhoid hormone, vasopressin have half-lives lower than 25 min),[9,10] frequent injections or infusions, which limit patient's comfort, convenience, and compliance, are required to obtain a therapeutic effect.[11] The mentioned drawbacks represent an immense challenge to modern medicine, as they restrict the widespread acceptance and applications of proteins as therapeutics by patients and physicians but also represent a tremendous opportunity for the drug delivery field. Among the approaches implemented to enhance protein's pharmacokinetic and pharmacodynamic properties, while preserving its native form and improving patient's compliance, scientists centered their focus mainly on the following strategies: (a) development of needle-free administration routes with high bioavailability, such as pulmonary, oral, and nasal delivery,[12,13] (b) extension of circulation time and masking immunogenicity of protein drugs by conjugation of the protein with macromolecules like poly(ethylene glycol) (PEG) or (polysialic acid),[14,15] and (c) development of injectable controlled release delivery systems, including liposomes, polymeric micro- and nanoparticles, and hydrogels.

Generally speaking, all of these approaches aim to achieve the following benefits: (a) Maintaining plasma protein-drug concentration within the therapeutic window over an extended period of time, (b) protecting the active therapeutic from premature degradation, (c) enhancing drug efficacy, while reducing side effects, and (d) avoiding frequent administration and lowering drug dosage.

This review describes injectable polymeric delivery systems used for the controlled release of pharmaceutical proteins, with special focus on hydrogels. We shortly overview the currently available technologies other than hydrogels, which include lipid-based delivery systems, nano/microparticles based on degradable hydrophobic polymers, highlighting their rationale, characteristics, and shortcomings in protein delivery. In this review, hydrogels are extensively described, from their general features to recent advances in synthesis and pharmaceutical and clinical applications. The discussion covers injectable systems based on nano- and particularly microparticles as well as physical and chemical cross-linking methods used for in situ gelling systems and environmentally responsive hydrogels and their use for protein release. Some limitations of hydrogels in protein delivery (e.g., burst and incomplete release, protein damage due to encapsulation) are also discussed and ways to tackle these are provided. Emerging techniques to study release from hydrogels are illustrated and current applied hydrogel systems are discussed. Finally, hydrogel/protein formulations that have reached clinical trials are summarized and discussed.

1.2. Particulate Protein Delivery Systems

1.2.1. Microspheres and Nanoparticles. Nanoparticle- and microsphere-based drug delivery systems are advantageous because of their injectability and the possibility to achieve prolonged release.[16] Biocompatibility and biodegradability are necessary criteria for selecting the drug carrier. A variety of synthetic and naturally occurring biodegradable polymers have been investigated in the past 30 years for the preparation of nano- and microspheres.[16] A few examples of natural polymers used for the preparation of protein formulations include chitosan, used for vaccination purposes;[17,18] alginate-based microparticles, for the pulsatile release of insulin;[19] and polymerized serum albumin beads, for vaccine delivery.[20] However, the development of polymer-based micro- and nanospheres based on natural polymers has been overshadowed by the advances made in synthetic polymer technology. Among the synthetic polymers, aliphatic polyesters, polyanhydrides, polyorthoesters, polyphosphazenes, and polyamino acids are the most relevant and most frequently studied polymers for protein delivery.[21−23] Aliphatic polyesters, in particular poly(lactic-*co*-glycolic acid) (PLGA), dominate the field with a number of marketed formulations (i.e., Lupron Depot, Nutropin Depot, Decapeptyl, etc.),[24,25] because of the demonstrated biocompatibility and degradation into toxicologically acceptable products.[26] Despite the many advantages of PLGA microspheres, they also showed some inherent shortcomings, such as polymer hydrophobicity; acidic microenvironment during bioerosion,[27,28] leading to protein denaturation and aggregation;[29] burst and incomplete release;[30−32] and chemical reactions between proteins and polymers.[33,34] Therefore, microparticle-based delivery systems are still awaiting major clinical successes, and extensive research is being conducted to improve the current technology.[35] For example, approaches to reduce the burst release and increase loading efficiency in microspheres comprise optimization of preparation method parameters (typically the double emulsion technique) as well as the use of excipients to counteract the drop in pH during degradation of the polymer matrix and to prevent protein/peptide acylation.[35−40] Furthermore, the synthesis of novel hydrophilic polymers such as poly(lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) as an alternative to PLGA for the preparation of microspheres has been reported.[41,42] Protein and peptide delivery using nanoparticles is still in its infancy, but the interest in this field is increasing and some examples of nanodelivery systems for proteins have become available. Early developments on nanoparticulate systems for protein delivery were reviewed by Couvreur et al.,[43] while more recent advances were discussed by Pinto Reis et al.[44] The major advantage of using this approach resides in the possibility to achieve site-specific release of the drug by passive or active targeting and, in contrast to microparticles, to accomplish intracellular protein delivery. Targeting drugs to the desired site of action would not only improve therapeutic efficiency but also permits a reduction in the dose of drug administered and thereby minimizing unwanted toxic effects.[45,46]

1.2.2. Lipid-Based Delivery Systems. Liposomes, many emulsions, and solid lipid nanoparticles are all examples of lipid-based delivery systems for proteins. Proteins and peptides

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can be incorporated in the internal phase of water-in-oil emulsions and delivered in a controlled fashion upon administration.[47,48] Some studies have demonstrated in vivo efficacy of these delivery systems, releasing, for example, aprotinin for a prolonged period of time[48] or inducing immune response upon oral antigen delivery.[49] Despite these encouraging results and the possibility to modify to some extent their release behavior[50] by varying disperse phase volume fraction and particle size, formulation and protein stability issues as well as low encapsulation efficiency, incomplete release, and poor control over release kinetics limit the practical use of these protein delivery systems.

Liposomes, consisting of one or more phospholipid bilayers separated by internal aqueous compartments, are well-established and extensively investigated particulate carrier systems that have been successfully employed for the controlled release and site specific delivery of low molecular weight drugs.[51,52] With the possibility to vary their dimensions, composition, surface charge, and structure, liposomes have also demonstrated to be suitable for encapsulation of enzymes and proteins. An advantage of liposome-encapsulated enzymes/proteins is their potential ability to enter the cytoplasm or lysosomes of cells. In the past 20 years, extensive literature on their application for the intracellular release of encapsulated or surface-associated proteins and peptides[53−55] has been published (reviewed by Torchilin[56] and Tan[57]). Unlike emulsions, liposomes can be lyophilized and administered upon reconstitution[58,59] and they have been shown to be able to protect therapeutics from degradation and to slowly release them when liposome destabilization takes place. However, difficulties to achieve tailorable controlled release by liposomal formulation along with the risk for opsonization in humans still represent obstacles for the use of liposomes as protein and peptide delivery systems.[60,61]

Finally, a novel class of particles (based on lipid components other than phospholipids), described for the first time by Müller et al. are solid lipid nano- and microparticles.[62] Incorporation of proteins into solid lipid nano- and microparticles is a relatively new research area. Nevertheless, some examples of prolonged in vitro release and in vivo efficacy are available and allow concluding that these types of formulation hold potential as protein carriers.[63−66] However, optimization of the solid lipid particle formulation aimed to overcome burst and incomplete release is needed.[64,67,68] Moreover, the possibility to tailor drug release has not been investigated in detail yet.

1.3. Hydrogels for Sustained Delivery—General Features

Hydrogels are cross-linked networks of hydrophilic polymers capable of retaining large amounts of water yet remaining insoluble and maintaining their three-dimensional structure. Since their discovery and application in the biomedical field by Wichterle et al. in the early 1950s,[69] an immense number of hydrogels have been developed, and they have been studied for a wide range of biomedical and pharmaceutical applications, including contact lenses,[70] tissue engineering,[71−73] diagnostics, drug delivery,[74,75] vascular prostheses,[76] and coating for stents and catheters.[77] The hydrophilic polymers used to create hydrogels need to be physically and/or chemically cross-linked to prevent their dissolution. Hydrogels can be prepared from natural and synthetic polymers and they can consist of homopolymers, copolymers, and interpenetrating or double polymeric networks. Hydrogels can be made biodegradable by a proper selection of their building blocks as well as the applied cross-linking strategy.[78−80]

Hydrogels are generally regarded as biocompatible materials because their high water content and soft nature render them similar to natural extracellular matrices and minimize tissue irritation and cell adherence.[81] Furthermore, their porous structure, along with their water content, are extremely suitable properties to accommodate high loads of water-soluble compounds, like therapeutically active proteins and peptides. Unlike other delivery systems (microparticles, emulsions, etc.), where preparation conditions are sometimes detrimental to proteins (i.e., use of organic solvents and protein denaturating processes, like homogenization, exposure to interfaces, and shear forces, etc.), hydrogel preparation procedures are beneficial in preserving protein stability, as very mild conditions (aqueous environment, room temperature) are normally adopted. Finally, proteins have a limited mobility or are immobilized in the hydrogel network, which is favorable for preservation of their mostly fragile 3D structure.

All these unique properties of hydrogels have raised increasing interest in their use as reservoir systems for proteins that are slowly released from the hydrogel matrix in a controlled fashion to maintain a therapeutic effective concentration of the protein drug in the surrounding tissues or in the circulation over an extended period of time.

Proteins can be physically incorporated in the hydrogel matrix, and their release is governed by several mechanisms, such as diffusion, swelling, erosion/degradation, or a combination of these mechanisms. Hydrogels allow fine-tuning of the protein release by tailoring their cross-link density via changes in polymer architecture, concentration, molecular weight, or chemistry. Other strategies to tailor drug release from hydrogels exist and they rely on reversible protein−polymer interaction or encapsulation of the protein in a second delivery system (e.g., micro- or nanoparticles) dispersed in the hydrogel network.[82,83]

Preformed, macroscopic hydrogels have to be administered by surgical intervention. This is costly and rather inconvenient for the patient, and therefore, nowadays, attention is focused on injectable hydrogels that can be administered in a minimally invasive manner. Injectable hydrogels can be in the form of clear polymer solutions prior to administration and that turn into a viscoelastic system at the site of administration upon injection. They jellify in response to external stimuli (like temperature, pH, ionic strength, solvent) or by means of other physical and chemical cross-linking methods (stereocomplexation, inclusion complexation, photopolymerization, Michael addition, etc.) as discussed in section 2 of this review.

Moreover, as mentioned by appropriate design of the hydrogel building blocks as well as the applied cross-linking methods, biodegradability and bioresorption can be ensured. Biodegradation is defined as conversion of materials into water-soluble intermediates or end-products that can be eliminated from the body without harmful effects.[84] In general, it can be accomplished via dissociation of the polymer chains or by enzymatic and/or hydrolytic degradation pathways.

2. CROSS-LINKING METHODS FOR IN SITU FORMING HYDROGELS

2.1. Physical Cross-Linking

Physical cross-linking between polymers can be obtained by using several noncovalent interactions, such as hydrophobic interactions, ionic interactions, hydrogen bonding, host−guest

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interactions, or combinations of these. The most frequently exploited interactions for building physically cross-linked hydrogels are hydrophobic interactions, because they are strong interactions in aqueous environment and hydrogels can simply be prepared by using amphiphilic block copolymers, as discussed in more detail in the Smart Hydrogel section. This section will focus on more specific interactions that have been used recently to prepare hydrogels for protein delivery.

2.1.1. Inclusion Complexes. Inclusion complexes of β-cyclodextrins (βCD), which are cyclic oligosaccharides with an internal hydrophobic pocket, and complementary low molecular weight guest molecules have been used in a cross-linking method for the design of in situ gelling networks.[85] Yui et al. reviewed several aspects of supramolecular self-assembling systems based on rapidly responsive hydrogels from polymeric hosts and low molecular weight guests.[86] βCD and cholesterol end-functionalized star-shaped PEG polymers have been synthesized and used as gelators for the preparation of hydrogels aimed at protein delivery.[87−89] Upon hydration of a mixture of star PEG−βCD and star PEG−cholesterol, hydrogels are formed (Figure 1). The hydrogels are assembled

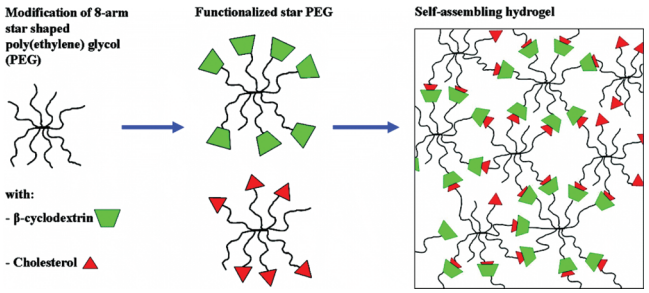

Figure 1. Self-assembling poly(ethylene glycol) hydrogel system based on inclusion complexes between β-cyclodextrin (βCD) and cholesterol. Hydrogels are formed after hydration of a mixture of star-shaped eight-arm poly(ethylene glycol) (PEG) end-modified with βCD groups and the same star-shaped PEG end-modified with cholesterol moieties. Reprinted with permission from ref 87. Copyright 2008 American Chemical Society.

by formation of βCD/cholesterol inclusion complexes driven by hydrophobic and van der Waals interactions. The hydrogels exhibited thermosensitive behavior, being completely reversible upon cooling and heating steps. In particular, at low temperature viscoelastic behavior, due to slow dynamics, was observed, while at higher temperatures a viscous system, due to a reduced number of βCD/cholesterol complexes and faster chain relaxation processes was obtained.[87] A study of hydrogels based on cholesterol- and βCD-modified PEGs of different architecture (linear, four-arm star, and eight-arm star) revealed that the eight-arm star based mixtures yielded the strongest viscoelastic network.[88] Importantly, the mechanical properties could be tuned easily by choosing the PEG architecture, and thereby the strength of the gels can be matched to the desired application. The eight-arm star PEG-based hydrogels were used to study release of model proteins and a quantitative and nearly zero-order release of entrapped proteins was shown. The release was governed by surface erosion, which depended on the network swelling stresses and initial cross-link density of the gels.[90]

Other supramolecular-structured hydrogels, displaying a gel−sol phase transition, were prepared by inclusion complexation between poly(ethylene glycol) grafted dextrans and α-cyclodextrins (αCDs) in aqueous solution. The gel−sol transition was based on the supramolecular assembly and dissociation, and the transition was reversible and controllable by the polymer concentration and the PEG content of the graft copolymers, as well as the ratio between guest and host molecules. Thermosensitive behavior was also observed, as at high temperatures the network dissociated reversibly.[91] To add pH responsiveness to the described thermosensitive hydrogel based on inclusion complexes, poly(D-lysine) (PL), a cationic polymer, was grafted onto dextran and used for inclusion complexation with αCDs. Transition from a phase-separated structure of hydrated dextrans and hydrophobically aggregated inclusion complexes in buffer solution at pH 10 (where the primary amines of PL are deprotonated, allowing the CDs to be threaded onto the PL chain) was observed. The hydrogels showed thermoreversible gel−sol transitions as well as pH-sensitive phase transitions.[92]

2.1.2. Stereocomplexation. Stereocomplexation is defined as cocrystallization of two enantiomers. (Enantiomers are stereoisomers that are mirror images of each other that are "nonsuperposable" (not identical)). This physical interaction has been exploited as a cross-linking method for the preparation of injectable hydrogels. The enantiomers mainly employed for the preparation of in situ forming hydrogels are poly(L-lactide) (PLLA) and poly(D-lactide) (PDLA) that when combined in a 1:1 racemic mixture are able to form stereocomplex crystals.[93,94] Other specialized reviews are focused on these stereocomplexed hydrogels, from their synthesis, to crystallization mechanism, degradation, and general applications;[95,96] therefore, just a few systems are discussed in this secton. When PDLA and PLLA are coupled to hydrophilic polymers like dextran and mixed in an aqueous medium, a hydrogel is formed. Proteins can be loaded in the hydrogels by dissolving them in the solution of the hydrogel precursors (Figure 2). This gelation mechanism was first described by De Jong et al.[97,98]

The degradation of dextran−lactate gels involved multiple processes: swelling of the network and hydrolysis of ester bonds present in the lactic acid grafts, finally resulting in dissolution of the gel. The length of the lactic acid grafts as well as the degree of substitution highly influenced the total degradation time. Although stereocomplexed high molecular weight PLA has been reported as highly resistant toward hydrolytic degradation, the dextran−lactate stereocomplex gels degrade within a few weeks under physiological conditions.[99,100] The application of these hydrogels as controlled drug delivery systems was described.[101] Release of model proteins (lysozyme and IgG)[101] as well as the therapeutically relevant protein (recombinant human interleukin-2, rhIL-2)[75] from dextran−lactate hydrogels was studied in vitro and in vivo. Lysozyme was released from 30 wt % polymer hydrogels in 5 days by diffusion, while the bigger protein IgG was released in 8 days by a combination of diffusion and swelling/degradation of the matrix. RhIL-2 was initially rapidly released in vitro and in a later stage a slower release was observed. In vivo studies were done by injecting RhIL-2 loaded hydrogels intraperitoneally into tumor-bearing mice. Placebo hydrogels and RhIL-2 bolus injections were used as controls. The same therapeutic effect of one injection of RhIL-2 loaded hydrogels was achieved as with five consecutive Rh-IL2 bolus injections. In vivo biocompatibility studies showed only a mild foreign body reaction, most likely due to degradation of the polymer.[102]

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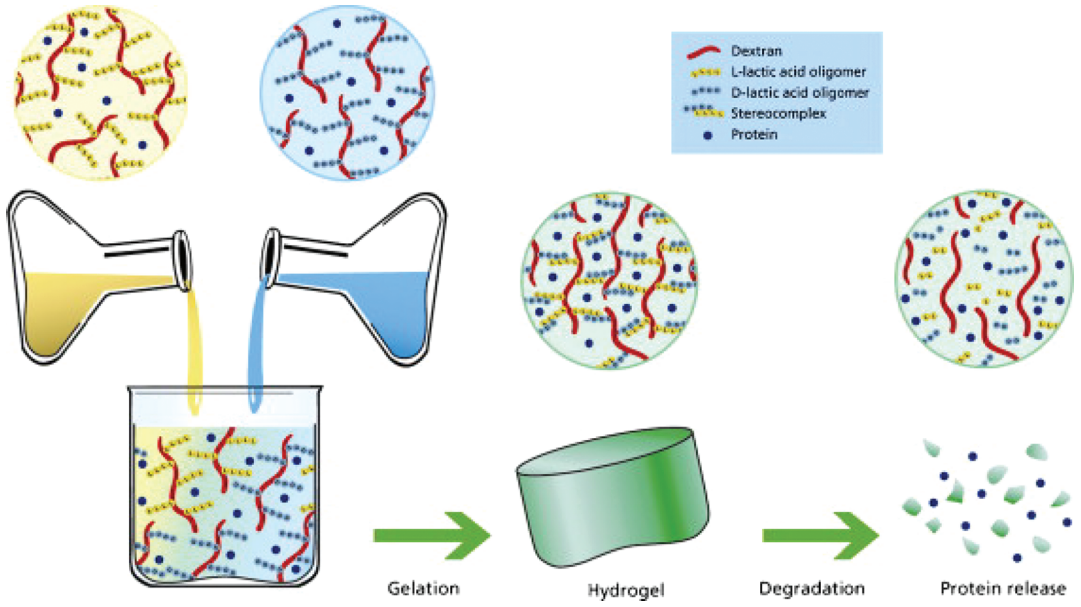

Figure 2. Schematic presentation of the self-assembling mechanism of stereocomplexed dextran hydrogel. Reprinted with permission from ref 97. Copyright 2000 American Chemical Society.

2.1.3. Peptide Interactions. Polypeptides are important biodegradable and biocompatible polymers having a variety of conformations, such as α-helix, β-sheet, and random coil, and they lend themselves as building blocks or cross-linking units for hydrogels with potential biomedical applications.[103,104] Many synthetic polymer/peptide hybrid structures have been synthesized in the past decade, and their self-assembling properties have been widely studied because of the options to design complex architectures.[105] Because of seemingly unlimited possibilities to tailor the design of hybrid polymers (e.g., by incorporation of non-natural amino acids), the impact of the development of new peptide-based hydrogel materials cannot be fully recognized yet.

Tirrell et al. introduced a polymer consisting of a leucine zipper terminated protein flanking a central, flexible, water-soluble polyelectrolyte segment. Formation of coiled-coil aggregates of the terminal domains in near-neutral aqueous solutions triggers formation of a three-dimensional polymer network, with the polyelectrolyte segment retaining solvent and preventing precipitation of the chain.[106] Kopeček et al. reported on a hybrid hydrogel system assembled from water-soluble synthetic polymers and a coiled-coil protein-folding motif. These hydrogels underwent temperature-induced collapse owing to the cooperative conformational transition of the coiled-coil protein domain.[107] Figure 3 shows the design of this hybrid peptide hydrogel that makes use of Ni^{2+} coordination chemistry for attachments of the peptide units. The same group further developed this hybrid system in which the peptide sequences are covalently attached to the polymer backbone.[108]

The inherent ability of certain amino acids to complex metal ions has been used by more scientists lately for the preparation of hydrogels.[109,110] Schneider et al. developed a metal-responsive peptide-based hydrogel that upon addition of zinc ions self-assembled into a β-sheet-rich fibrillar hydrogel.[111] Metters et al. introduced metal ion chelating monomers (methacrylated iminodiacetic acid, GMIDA) during the photopolymerization of PEG-diacrylate, and protein release was tailored by nickel or copper chelation of histidine-tagged proteins.[112] Both ligand concentration and the choice of metal ion determined the release rate. The same monomer could be used to introduce negative charges into the gel network and thereby also tailor the release of nontagged positively charged proteins such as lysozyme. In this way, a dual protein delivery system was obtained with independent release profiles for positively charged proteins and His-tagged proteins (Figure 4).[113]

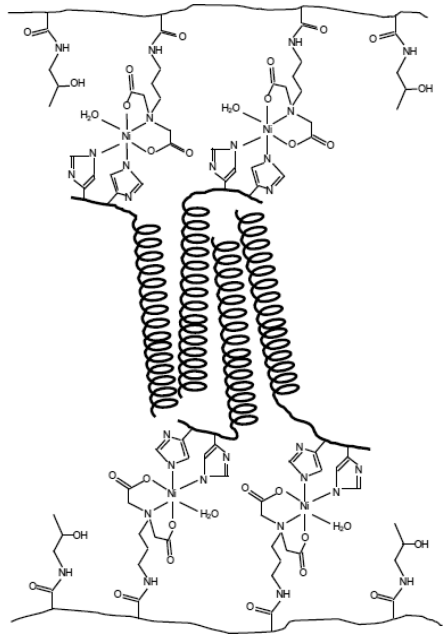

Figure 3. Structural representation of the hybrid hydrogel primary chains and the attachment of His-tagged coiled-coil proteins. Poly(HPMA-*co*-DAMA) is shown here as the primary chains. The pendant iminodiacetate groups form complexes with transition-metal ions, such as Ni^{2+}, to which the terminal histidine residues of the coiled coils are attached. A tetrameric coiled coil, consisting of two parallel dimers associating in an antiparallel fashion, is shown here as an example of the many possible conformations. Reprinted by permission from ref 107. Copyright 1999 Macmillan Publishers Ltd.

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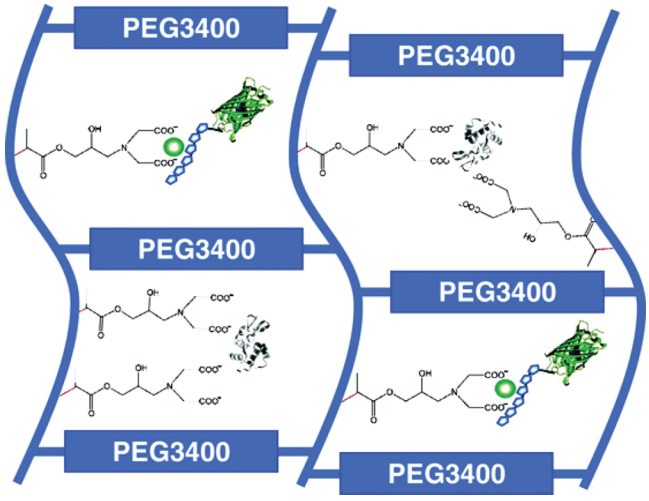

Figure 4. Schematic representation of the proposed monolithic affinity hydrogel system for dual-protein delivery. The affinity hydrogels are prepared from one-step photopolymerization of PEGDA and methacrylated affinity ligands, GMIDA(Ni^{2+}) and GMIDA, which reversibly complex with hisGFP and lysozyme, respectively. Reprinted with permission from ref 113. Copyright 2008 American Chemical Society.

An amphiphilic diblock copolypeptide that assembles into a gel both by supramolecular and thermal association was reported by Nowak et al.[114] Jeong et al. developed L/DL-poly(alanine) (PA) end-capped poly(propylene glycol)−poly(ethylene glycol)−poly(propylene glycol) (PLX) PA−PLX−PA polymers that in aqueous solutions underwent a sol-to-gel transition at increasing temperature due to an increase in β-sheet content of PA and dehydration of PLX. This system was stable in vivo for over 15 days.[115] Physically cross-linked poly(amino acid) hydrogels, formed by a sol−gel transition of amphiphilic poly(*N*-substituted α/β-asparagine)s in an aqueous solution was described by Tacheuchi et al.[116], while recently Jeong et al. described amphiphilic polymers consisting of a hydrophilic poly(*N*-vinylpyrrolidone) (PVP) block and a hydrophobic PA block that formed micelles in water that aggregated as the temperature increased to yield gels. They also demonstrated the use of PVP as an alternative to PEG to design reverse thermogelling biomaterials.[117]

Besides the well-known peptide−peptide interactions (coiled-coil, β-sheets, etc.), also specific peptide−polysaccharide interactions can be used to build supramolecular hydrogels.[118] Kiick et al. showed an elegant example making use of the interaction between low molecular weight heparin and heparin binding peptide sequences both coupled to star PEGs. These gels were able to bind and release heparin-binding growth factors in a controlled fashion.[119]

2.2. Chemical Cross-Linking

Chemical cross-linking will yield covalent bonds between different polymer chains, and the resulting hydrogel network is in general more resistant to mechanical forces than physically cross-linked networks. Many conventional coupling reactions have been used to obtain cross-linked polymers, but in recent years especially "click chemistry"[120] (azide−alkyne cycloaddition) and also "native chemical ligation" (ligation of a C-terminal thioester to an N-terminal cysteine residue)[121] are becoming more popular due to their ease of use and high conversion. In this section, we focus on techniques that are used for in situ gelation and therefore most interesting for protein delivery at the moment.

2.2.1. Photopolymerization. Photopolymerization is a form of radical polymerization that allows the formation of in situ formed hydrogels by means of UV or visible light, in the presence of a photosensitive compound, called a photoinitiator. This chemical cross-linking reaction is initiated by decomposition of the photoinitiator upon exposure to UV or visible light, leading to the formation of radicals. In the presence of hydrogel precursors bearing polymerizable groups, such as acrylate or methacrylate moieties, a gel is formed. As UV irradiation can be applied in vivo in a minimally invasive manner, by means of laparoscopic devices, catheters, or transdermal illumination,[122,123] photopolymerization is a suitable method for the preparation of in situ gelling viscoelastic systems. Since Hubbell et al. introduced this cross-linking method,[124] photopolymerization has been used for a number of biomedical applications, as it offers as series of advantages over other types of cross-linking methods. The photocuring process is fast, taking usually only seconds to minutes to complete, can be conducted at room or body temperature without the use of organic solvents, and offers the advantage of spatial and temporal control.[125−127] In 2002, Nguyen et al. reviewed photo-cross-linked hydrogels for potential tissue engineering applications, describing the available photoinitiators and photopolymerizable compounds,[71] and Van Tomme et al. provided a more recent update on the progress of photopolymerized hydrogel systems, as well as other types of in situ cross-linking methods.[95]

As mentioned, Hubbell et al. pioneered the field of photopolymerized hydrogels for biomedical applications, designing a polymer composed of a central PEG chain and lateral oligomeric blocks of a hydrolyzable α-hydroxy acid or other degradable moieties. The hydrogel precursors were synthesized by reacting dihydroxy polyethylene glycol with D,L-lactide using stannous octoate as a catalyst. This polymer was then reacted with acryloyl chloride to couple an acrylate unit at each end. PEG molecular weight was varied to tune the permeability as well as the physical properties of the hydrogel, while the length of the α-hydroxy acid oligomer blocks modulated the degradation of the hydrogels. The photoinitiator 2,2-dimethoxy-2-phenylacetophenone was dissolved in *N*-vinylpyrrolidone and added to the polymeric precursor solution in which also proteins were dissolved to be released upon formation of the gels. Upon UV curing, a rapid gelation of the solution was observed. The release of several proteins of different molecular weight was investigated (discussed in section 4.2.).[124,128] One of the drawbacks of photopolymerization is the possible degradation of proteins during photopolymerization, as the UV light, as well as the developed radical species upon photoinitiator decomposition, might be detrimental to the encapsulated therapeutic. However, it was demonstrated that UV light of selected wavelength and low intensity preserves protein stability.[123] Furthermore, several papers, where enzymes were encapsulated by photopolymerization in hydrogel networks, demonstrated that both the biological activity and the protein structure were retained.[128] Pescosolido et al. confirmed the protein compatible nature of photopolymerization by demonstrating that the enzymatic activity of horseradish peroxidase, encapsulated in and released from interpenetrating networks composed of ionically cross-linked Ca^{2+}−alginate combined with photopolymerized methacrylated dextran, was preserved.[129] In a photopolymerized

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thermosensitive hydrogel based on methacrylated poly(*N*-(2-hydroxylpropyl)methacrylamide lactate)−PEG−poly(*N*-(2-hydroxylpropyl)methacrylamide lactate) [p(HPMAm-lac)−PEG−p(HPMAm-lac)] it was demonstrated that lysozyme retained its enzymatic activity and secondary structure after photopolymerization and release. The self-assembly mechanism of the hydrogel played a beneficial role in the stabilization of the protein. The hydrogel was prepared by dissolving polymer, photoinitiator, and enzyme in an aqueous medium, and the resulting solution was heated to body temperature before photopolymerization to mimic the physiological situation. By this procedure, hydrophobic domains of self-assembled thermosensitive chains and hydrophilic PEG-rich pores were formed. The hydrophobic photoinitiator has affinity for the hydrophobic domains, while the protein resides most likely in the hydrophilic PEG pores. The phase separation between the two species confined the radical cross-linking reaction in the hydrophobic domains, minimizing potential damage to the protein, which is mainly present in the PEG-rich domains.[130] However, protein stability and photopolymerization is a controversial topic, where generalized principles cannot be applied and protein stability must be assessed for each specific polymer, photoinitiator, and protein therapeutic. For example, the hydrogel developed by Hubbell et al., of which the preparation was not associated with detectable modification of encapsulated proteins, adhered to surrounding tissues when photopolymerized in vivo, most likely as a result of chemical reactions between polymer and extracellular proteins. In contrast, tissue adherence was not observed when the hydrogels were photopolymerized ex vivo and subsequently implanted.[131] The choice of photoinitiator can also play a crucial role in the successful application of photopolymerizable systems in the field of tissue engineering and protein release. Besides protein stability, the cyto- and biocompatibility of the initiator used to carry out the polymerization have to be taken into account. Bryant et al. performed a comparative cytocompatibility study on several photoinitiating systems, including 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651), 1-hydroxycyclohexyl phenyl ketone (Irgacure 184), 2-methyl-1-[4-(methylthio)-phenyl]-2-(4-morpholinyl)-1-propanone (Irgacure 907), and 2-hydroxy-1-[4-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959); camphorquinone (CQ) with ethyl 4-*N*,*N*-dimethylaminobenzoate (4EDMAB); and triethanolamine (TEOA) and the photosensitizer isopropyl thioxanthone. Both UV and visible light were used, and a fibroblast cell line, NIH/3T3, was exposed to the photoinitiators at varying concentrations from 0.01% (w/w) to 0.1% (w/w) and studied before and after exposure to the initiating light. The results demonstrated that at low photoinitiator concentrations [≤0.01% (w/w)] all photoinitiators were cytocompatible with the exception of CQ, Irgacure 651, and 4EDMAB. At low light intensity, Irgacure 2959 at concentrations ≤0.05% (w/w) was among the most promising cytocompatible UV and visible light initiating compounds, and this initiator is nowadays used in most photopolymerizable biomaterials.[123] Nevertheless, I2959 needs light within a wavelength range (<360 nm)[132] that is regarded as poorly compatible with living cells. An alternative was proposed by Anseth et al., who used a water-soluble lithium acylphosphinate salt that was able to initiate photopolymerization of PEG-diacrylates using visible light exposure at a faster rate as compared to I2959.[133]

However, Lin et al. showed that photopolymerization was the cause of the incomplete release of BSA from cross-linked PEG-based networks because of grafting of the protein to the polymeric network during the gelation reaction. The fraction of immobilized protein decreased with increasing initial loading.[134] The covalent coupling between BSA and hydrogel precursors was studied recently by Valdebenito et al., who demonstrated that BSA can act as chain transfer agent in radical reactions.[135]

Another critical factor to be evaluated in photopolymerized hydrogels is their biodegradability. Although the polymer precursors are designed to be biodegradable, a third polymeric species is formed during polymerization, namely, polyacrylic or polymethacrylic acid in case of acrylate and methacrylate bearing prepolymers, respectively. The solubility of these polyorganic acids and hence their possibility to be excreted by renal filtration highly depends on their molecular weight. Metters et al. carried out a detailed investigation of the bulk degradation phenomenon of photopolymerized hydrogels based on PEG and poly(lactic acid), previously developed by Hubbell and co-workers.[136] They developed a mathematical model, where the molecular weight of the degradation products and, more in general, the kinetics of the bulk degradation phenomenon in chemically cross-linked matrices are predictable, allowing consequently a priori design of degrading hydrogels. They showed in a first and simpler model that the mass loss from the chemically cross-linked network depends on network parameters such as the number of cross-links per backbone chain and the mass fraction of the network contained in the backbone as opposed to the rest of the network. Model predictions versus degradation time also depended on reaction parameters such as the order of the hydrolysis reaction and the value of the kinetic rate constant.[137] In a subsequent paper, an extension of this model to other aspects of the network degradation, where inclusion of partially reacted polymer and varying number of lactic acid repeating units were included, was elaborated. This model allowed a more realistic representation of the bulk degradation of cross-linked hydrogels.[138]

Inspired by the work initiated by Hubbell, many other scientists designed photopolymerizable materials, such as acrylated four-arm PEG, methacrylated dextran, methacrylated dextran−HEMA−dimethylaminoethyl (dex−HEMA−DMAE), and methacrylated eight-arm PEG−poly(lactic acid) (PEG−PLA) star block copolymers.[95,139,140] Smeds and colleagues reported on the use of two methacrylate-modified polysaccharides, alginate and hyaluronan, that, upon photopolymerization, formed viscoelastic gels for tissue engineering.[141] Similarly, Leach et al. reported on photo-cross-linkable hyaluronic and PEG-based hydrogels for protein delivery and tissue engineering.[142] Anseth et al. studied PEG- and poly(vinyl alcohol)-based polymers containing acrylate or methacrylate functionalities for the in situ generation of photopolymerized networks.[143]

2.2.2. Michael Addition. Many of the known addition reactions are carried out in organic solvents and performed using rather toxic compounds. Therefore, generally speaking, addition reactions are not suitable for in situ gelling systems, as all traces of unreacted compounds and solvents have to be removed. However, Michael addition reactions are suitable for the preparation of injectable hydrogels, because this chemical reaction occurs in aqueous medium, at room temperature, and at physiological pH. This reaction involves the addition of a nucleophile or activated olefin to a carbon−carbon double bond on alkenes.[144] The Michael addition reaction is recently emerging as an advantageous cross-linking method for the

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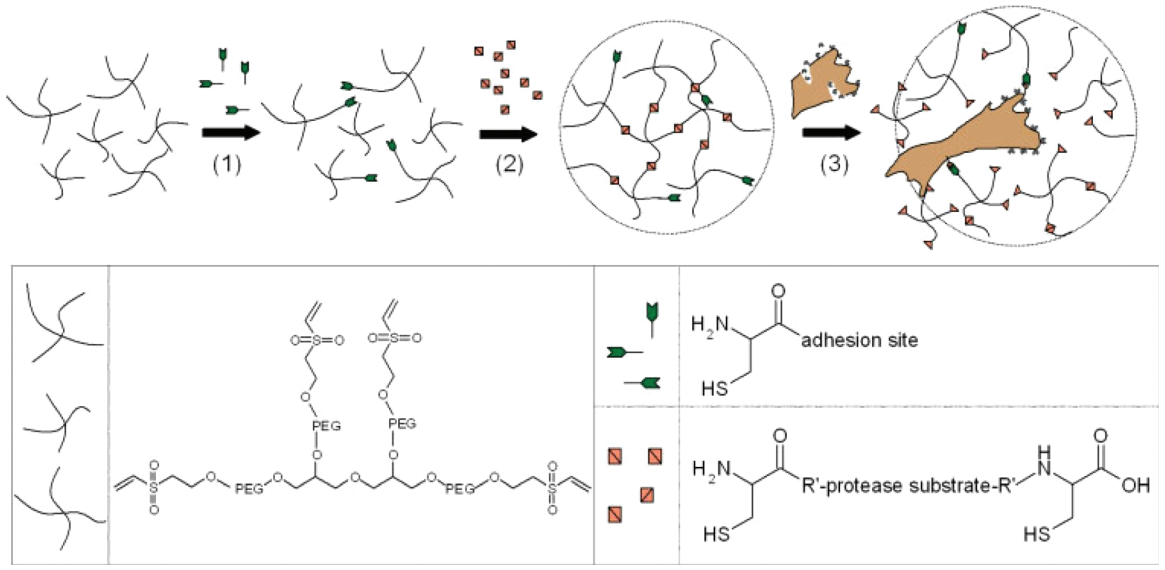

Figure 5. (1) Vinyl sulfone-functionalized PEGs were modified via Michael-type addition reaction with monocysteine adhesion peptides and (2) bis-cysteine MMP substrate peptides was used to form gels from aqueous solutions in the presence of cells and $VEGF_{105}$. (3) These elastic networks were designed to locally respond to protease activity at cell surface. Reprinted with permission from ref 150. Copyright 2003 Wiley-VCH Verlag GmbH & Co. KGaA.

development of injectable biomaterials, especially when thiol groups are used as Michael donor species. The benefits of this reaction originate from the high selectivity of Michael acceptors for thiols as compared to amines.[145] This means that in the presence of proteins, either encapsulated in the hydrogel network or present on cell surfaces or in extracellular matrices, the hydrogel precursors hardly show cross-reactivity with amine groups of proteins. Moreover, using Michael-type addition, the formation of a polyacrylic or methacrylic acid of uncontrolled molecular weight is avoided, in contrast to photopolymerization, because the donor and the acceptor molecule react in a 1:1 stoichiometry. Hubbell and co-workers introduced this type of reaction for the preparation of injectable matrices by reaction of PEG−dithiol with PEG−acrylates in aqueous medium at physiological pH and room temperature. Solid particles of bovine serum albumin were mixed with the gel precursor solution, and upon Michael addition curing, the protein-encapsulating hydrogels were formed in approximately 15 min. The different hydrogels reached equilibrium swelling in 24 h and degraded in 5−25 days, depending on the PEG functionality (PEG−triacrylate degraded faster than PEG−octaacrylate) by hydrolysis of ester groups. Albumin was released during 5−12 days, and importantly, complete release of the protein was observed, demonstrating the self-selectivity of the Michael addition of acrylated PEG for the thiol-modified PEG, rather than for the disulfide bonds (S−S), free thiol (SH), or amine groups of the protein. It was reasoned that S−S and SH groups are normally located in hardly accessible pockets, limiting the cross-reactivity of the protein with the hydrogel precursors.[146] In a subsequent study, Hubbell and co-workers used a similar approach to cross-link hydrogels by combining Michael addition donors such as pentaerythritol tetrakis 3′-mercaptopropionate (QT) and addition acceptors such as poly(ethylene glycol) diacrylate (PEGDA), pentaerythritol triacrylate (TA), and poly(propylene glycol) diacrylate (PPODA). The reactions were carried out both in phosphate buffer solutions of physiological pH and in emulsions to facilitate the dissolution of poorly water-soluble compounds. Gels were obtained on a time-scale between 5 and 10 min with complete conversion of thiols and acrylates, when the two species were combined in a 1:1 ratio. This indicates that side reactions, such as disulfide formation, are negligible on the time scale of the gelation. These cross-linked materials (at 75 wt % solid) showed compression moduli of 1.8 and 6.7 MPa and deformations up to 37%, depending on the preparation method (dispersion vs emulsion). In contrast to the highly water-swollen hydrogels designed for protein delivery, these materials exhibited much higher mechanical strength and they were therefore proposed for load-bearing applications, such as augmentation of collagenous or cartilaginous tissues.[147]

PEG bis(vinyl sulfone)s were used in combination with cysteine-functionalized recombinant proteins containing sequences for integrin receptor ligation for the preparation of cell-adhesive hydrogels aimed at tissue repair applications. Nondegradable and degradable networks were designed, with the latter containing protease cleavable units.[148] The cross-linking kinetics of these hydrogels could be controlled by pH and the presence of charged amino acid residues in close proximity to the cysteine residue, which modulated the pK_a of the thiol group.[149] In a follow up paper, Hubbell et al. synthesized hydrogels comprising multiarm vinyl sulfone-terminated PEG, a monocysteine-containing adhesion protein, and a bis(cysteine) metalloprotein substrate protein (MMP). These hydrogels were studied for tissue engineering purposes, and both cells and vascular endothelial growth factor ($VEGF_{105}$) were encapsulated in the hydrogel. Figure 5 shows the stepwise formation of the hydrogel containing the adhesion proteins and the MMP substrate proteins as well as the attack on the substrate protein by a metalloproteinase.[150]

Michael addition reaction was also used as a curing method for the preparation of hyaluronan (HA) hydrogels. Jin et al. showed that solutions of HA conjugates containing thiol functional groups (HA−SH) and PEG vinyl sulfone (PEG−VS) macromers reacted with each other under physiological conditions via Michael addition to form a 3D network. Gelation times varied from 14 min to less than 1 min, depending on the

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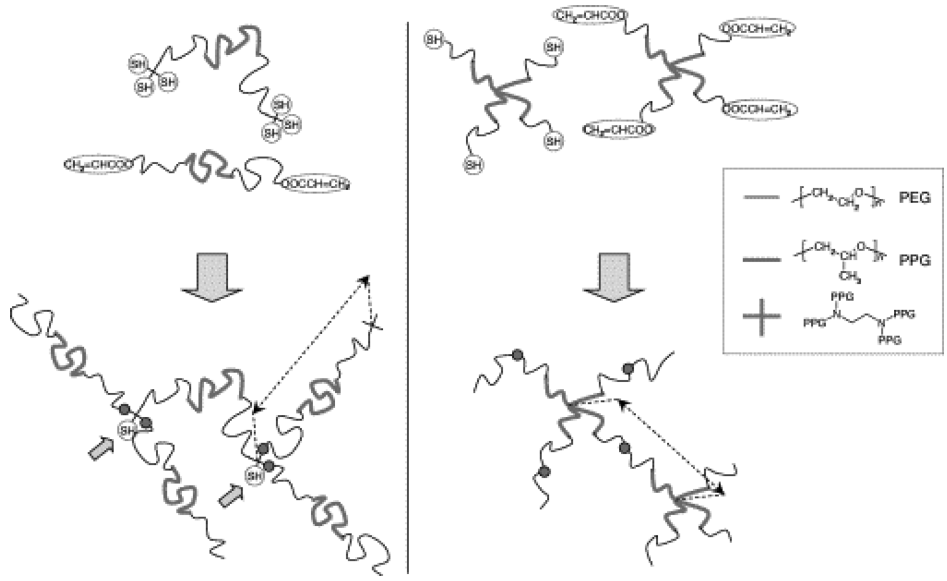

Figure 6. Linear (on the left) and tetramer (on the right) triblock copolymers of pluronic (PEG−PPG−PEG) were functionalized with two groups of clustered thiols or with two acrylates, respectively. Reprinted with permission from ref 157. Copyright 2004 Elsevier.

molecular weights of HA−SH and PEG−VS, degree of substitution (DS) of HA−SH, and total polymer concentration. Good chondrocyte viability and differentiation was shown in these gels.[151]

A novel sustained release formulation for erythropoietin (EPO) was developed by Hahn et al. using hyaluronic acid hydrogels cross-linked by Michael addition. Adipic acid dihydrazide grafted HA (HA−ADH) was synthesized and subsequently converted into methacrylated HA (HA−MA) by reaction with methacrylic anhydride in aqueous medium and isolated after ethanol precipitation. EPO was loaded into the hydrogels during their preparation by reacting HA−MA with two different cross-linkers, dithiothreitol (DTT) and a thiol group containing peptide linker. The gelation times were approximately 30 and 180 min for the peptide linker and DTT, respectively. The faster reaction kinetics of HA−MA with the peptide linker was ascribed to the positive charges of the amino acids adjacent to the thiol groups, which accelerated the Michael addition due to their electrostatic interaction with the negatively charged HA−MA, as also observed by Lutolf et al.[152] Approximately 90% of EPO was released in vitro from both hydrogels degraded by hyaluronidase SD (HAse SD), and the kinetics showed a rapid phase of release during the first two days, followed by a slower phase for the next 7 days. In vivo release tests of EPO from HA−MA hydrogels cross-linked with the peptide linker confirmed an elevated plasma concentration of EPO for 7 days.[153,154]

Cellesi et al. described the simultaneous thermal gelling and Michael addition cross-linking of both linear and tetra-arm Pluronics, which exhibit a reverse thermal gelation in aqueous solutions at physiological temperature and pH for the design of a synthetic substitute of alginate. Pluronics were derivatized with thiols or electron-poor olefins, and when these two polymers were combined, a simultaneously physically and chemically cross-linked hydrogel was formed. The physical interactions caused gelation at 37 °C and provided hardening kinetics similar to that of alginate. With slower kinetics, the chemical cross-linking then developed an irreversible and elastic gel structure, which in turn determined the transport/release properties of loaded compounds.[155] Figure 6 shows the gelation chemistry of the described hydrogel. The hydrogels showed very high diffusivity, and these materials were also investigated for the preparation of beads and liquid-core hydrogel-based nanoparticles for drug encapsulation and release.[156]

Injectable physically and chemically cross-linked hydrogels using Michael addition were developed by Lee et al. A thermosensitive copolymer of *N*-isopropylacrylamide (NIPAAm) and hydroxyethyl methacrylate (HEMA) with an LCST of 32 °C was synthesized and converted to poly(NIPAAm-*co*-HEMA-acrylate) by reaction of some of the hydroxyl groups with acryoyl chloride. When the obtained poly(NIPAAm-*co*-HEMA-acrylate) was mixed with pentaerythritol tetrakis 3-mercaptopropionate (QT) stoichiometrically in a PBS solution of pH 7.4, at 37 °C, a physical hydrogel was formed due to the thermosensitivity of poly(NIPAAm-*co*-HEMA-acrylate) that was stabilized through a Michael-type addition reaction. The hydrogel had a low swelling and showed improved elastic properties at low frequency compared to the control physically cross-linked gels.[158] More recently, Robb et al. copolymerized NIPAAm with *N*-acryloxysuccinimide (NASI) via free radical polymerization. The synthesized poly(NIPAAm-*co*-NASI) was further modified to obtain poly(NIPAAm-*co*-cysteamine) through a substitution reaction of NASI with the amine group of cysteamine. In addition to thermoresponsive physical gelling due to the presence of NIPAAm, this system also chemically gels via a Michael-type addition reaction when mixed with poly(ethylene glycol) diacrylate. The presence of both physical and chemical gelation resulted in material properties that were substantially improved in comparison to the corresponding physical gels.[159,160] Similarly, poly(NIPAAm-*co*-PEG-acrylate)-based hydrogels that simultaneously physically and chemically cross-link (Michael addition) were developed by Vernon et al.[161] Very recently, Wang et al. described the preparation of thermosensitive Michael addition cross-linked injectable thiol- and vinyl-modified poly(*N*-isopropylacrylamide) (PNIPAAm)-based copolymer hydrogels for DNA delivery.[162]

The synthesis, characterization, and peptide release behavior of an in situ physically and chemically cross-linking hydrogel was recently reported by Censi et al. (Meth)acrylate bearing

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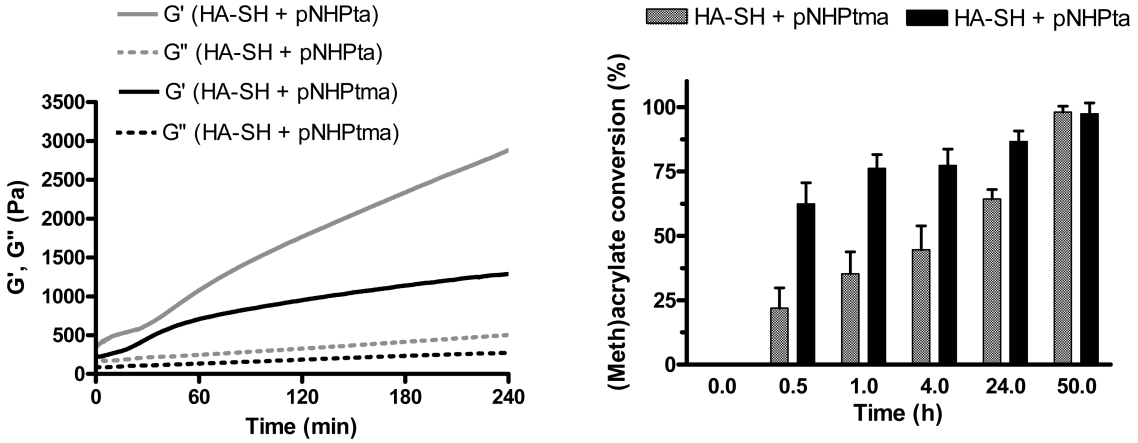

Figure 7. Kinetics of hydrogel formation at 37 °C and pH 7.4, studied by rheological analysis and (meth)acrylate conversion. On the left, storage (G') and loss (G'') moduli at 37 °C as a function of time directly after mixing of HA−SH with pNHPtma and pNHPta, respectively. On the right, (meth)acrylate conversion in time (polymer concentration 20 wt % pNHPt(m)a + 5.4 wt % HA−SH; ratio thiol/(meth)acrylate groups = 1/1). Reprinted with permission from ref 163. Copyright 2010 American Chemical Society.

ABA-triblock copolymers consisting of a PEG middle block, flanked by thermosensitive blocks of random *N*-isopropylacrylamide (PNIPAm)/*N*-(2-hydroxypropyl)methacrylamide dilactate (PHPMAm-lac$_2$) and exhibiting lower critical solution temperature behavior in aqueous solution were synthesized. Physical gelation occurred at 37 °C and the obtained gels were cured by Michael-type addition reaction with thiolated hyaluronic acid (HA−SH) to yield biodegradable but structurally stable and biocompatible hydrogels. A higher reactivity of acrylate bearing polymers with thiol groups, as compared to methacrylate analogues, resulted in a faster gel formation (Figure 7). Because of the presence of hydrolytically sensitive ester bonds in the cross-links, as well as in the lactate side chains and between PEG and thermosensitive blocks, the hydrogels were biodegradable at physiological conditions and complete dissolution of the gels within 3−4 months was observed. Methacrylated polymer gels loaded with a model peptide (bradykinin) showed a diffusion-controlled release of this peptide, tailorable by the polymer concentration.[163]

2.2.3. Native Chemical Ligation. Native chemical ligation is an even more chemoselective coupling reaction when compared to Michael addition reactions. Native chemical ligation was described for the first time in 1994 by Dawson et al. and involves the reaction of a thioester and an *N*-terminal cysteine resulting initially in a thioester-linked compound[164] that undergoes rapid rearrangement, yielding a native peptide bond as shown in Figure 8. In the past decade, this coupling method has been used mainly for the synthesis of large peptides, moderate sized proteins, and peptide-based dendrimers.[165,166]

Recently, native chemical ligation has been investigated as cross-linking mechanism for hydrogels.[167,168] Native chemical ligation is attractive for in situ hydrogel formation because of its chemoselectivity and its ability to react under physiological conditions, while avoiding the use of toxic reagents or catalysts. Messersmith et al. mixed four armed PEG macromers either functionalized with a thioester or *N*-terminal cysteine peptide to yield strong hydrogels. The thiol groups that become available after the rearrangement step can be used for further biofunctionalization of the hydrogels, making them attractive for biomedical applications. The authors did not study these hydrogels for delivery of therapeutic proteins, yet. Nevertheless,

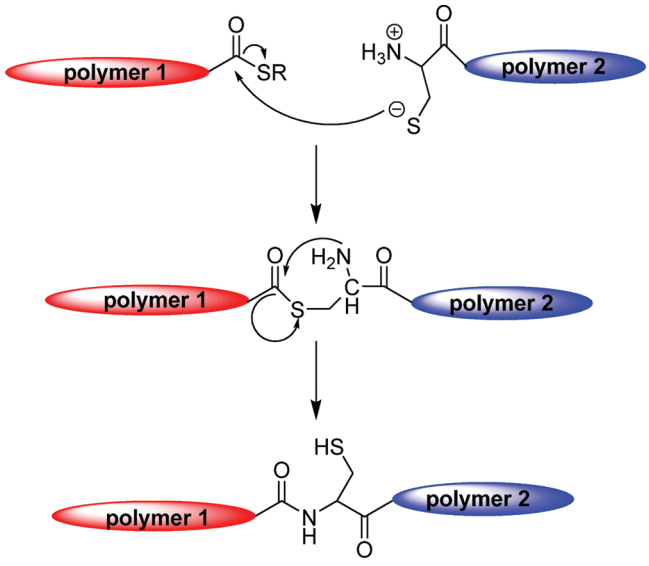

Figure 8. Mechanism of native chemical ligation applied for coupling of two polymers.

as the method is very chemoselective, the cross-linking method is expected to be highly compatible with proteins or other biomolecules, and more publications exploiting this cross-linking method for the design of protein releasing hydrogels are expected in the near future.

2.2.4. Click Chemistry. Over the past 5 years also the nowadays popular "click reaction" between azides and acetylenes has gained interest as polymer cross-linking method for construction of hydrogel networks.[169] The advantages of this copper(I)-catalyzed reaction include quantitative conversion, high specificity, and lack of side reactions with functional groups of biomolecules. A more controlled distribution of the cross-links over the network and therefore a better control over the mesh size distribution can be obtained using this cross-linking method when compared to radical or photochemical cross-linking methods.[169] The reaction kinetics of this click reaction are fast under physiological conditions and typically gel formation is found within minutes up to half an hour.[169,170] A disadvantage for biomedical applications, however, is the need for a Cu(I) catalyst to reach fast reaction

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kinetics and complete conversion. Cu(I) is a rather toxic ion and needs to be removed before the hydrogel can be administered to a patient, making injectable formulations based on Cu(I)-catalyzed click chemistry not feasible at this time.[171,172] However, recent advances have already resulted in in situ cross-linkable hydrogels using a Cu(I)-free click reaction.[173] However, reaction rates of copper-free click reactions are much slower than for the copper-catalyzed reactions and are in the range of hours up to days to reach completion, limiting the applicability as hydrogel systems for protein delivery for now. Progress in copper-free reaction kinetics for hydrogel formation is to be expected in the near future using strain-induced azide−alkyne cycloaddition[174] or by introduction of functional moieties influencing the electron deficiency or electron density of alkynes or azides, respectively.[173]

3. HYDROGELS BASED ON NATURAL POLYMERS

Many hydrogels with natural polymers as building blocks have been developed. These natural polymer networks display multiple advantages over synthetic polymer gels for biomedical applications with respect to their often inherent biocompatibility, biodegradability, and good cell adhesion properties. Biopolymer-based hydrogels have been investigated extensively for cell encapsulation for regenerative medicine.[72,175−177] The natural extracellular matrix has more in common with these biopolymer gels as compared to synthetic polymer hydrogels, generally resulting in better cell survival and differentiation. Besides cell encapsulation, also growth factors are often incorporated to enhance the performance of these artificial tissues. This chapter mainly focuses on the protein and/or growth factor release from hydrogels based on natural polymers used for tissue engineering applications.

3.1. Polysaccharides

Polysaccharides are in general very hydrophilic polymers and are therefore very suitable for the design of hydrogels. The most commonly used polysaccharides in recent hydrogel research aimed at protein delivery are chitosan, alginate, hyaluronic acid, and dextran (Figure 9).[178,179] Hydrogels

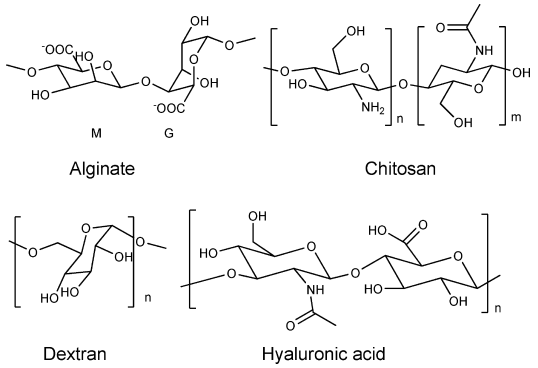

Figure 9. Most commonly used polysaccharides for hydrogel preparation for biomedical applications (M = mannuronic acid, G = guluronic acid).

based on these polysaccharides are discussed below with respect to their use for protein delivery. Besides the polymers mentioned above, other polysaccharides such as cellulose, heparin, and pullulan have also been studied for the development of hydrogels.[180]

3.1.1. Alginate. Alginate is an unbranched polysaccharide consisting of 1−4′-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) moieties in varying compositions. The physical properties of alginate depend largely on the composition and sequence of the M and G units. G-rich blocks of the polymer are able to bind to divalent cations of which Ca^{2+} is frequently used for cross-linking alginate to obtain hydrogels. Ca^{2+} ions bind to the G units of different polymer chains cooperatively in a so-called egg-box arrangement. This polysaccharide is unique because without further derivatization it is capable of forming strong gels by simple addition of certain metal ions to an aqueous solution of sodium alginate. This mild method of preparation makes these gels very suitable for encapsulation of living cells and for controlled release of entrapped fragile biomolecules. Therefore, alginate hydrogels are often used for tissue regeneration while simultaneously releasing growth factors or cytokines.[181−183] Several reviews have been published on the use of alginate for protein delivery and other biomedical applications.[184−187]

As the mechanical properties and pore size of alginate hydrogels depend only on the type and concentration of the used alginate, the possibilities to tailor the release rate of proteins is limited. Many researchers investigated several modified alginates to overcome these limitations.[188,189] An elegant example is the simple sulfonation of the uronic acids in alginate that specifically bind heparin binding proteins, including several growth factors, to obtain a sustained release of these proteins and enhanced therapeutic activity in a murine hindlimb ischemia model in rats.[190,191] Alginate is also often mixed with other polymers to obtain either interpenetrating networks (IPN's) or covalently linked mixed polymer systems to improve stability of the gels and control the release of entrapped proteins.[129,192,193]

3.1.2. Chitosan. Chitosan is essentially a copolymer of glucosamine and *N*-acetylglucosamine and is derived from natural chitin by partial deacetylation. The polymer is positively charged at low pH's and uncharged and insoluble at neutral and high pH's. Due to its cationic nature, chitosan has mucoadhesive properties, and therefore, it has been studied extensively in mucoadhesive drug delivery systems.[194,18,195] Besides, chitosan can act as a permeation enhancer by opening epithelial tight junctions.[196] Recently, a comprehensive and complete review on chitosan hydrogels was published concerning their drug/protein delivery aspects.[197]

3.1.3. Dextran. Dextran is a hydrophilic polysaccharide that consists of α-1,6-linked D-glucopyranoses with some degree of 1,3-branching. Several methods have been exploited to cross-link dextran to obtain hydrogels.[198,199] Besides for the design of hydrogels, dextran has also been used as a carrier system for many therapeutic and contrast agents.[200,201] The high number of available hydroxy groups presents many options for derivatization of dextran for subsequent physical or chemical cross-linking. In 2007, Van Tomme et al. published an overview of dextran-based hydrogels for protein delivery.[202] An elegant example of recent developments in dextran-based hydrogels involves peptide cross-linking resulting in an enzyme-dependent degradation mechanism.[203] In this way, cell-secreting enzymes determine the rate of degradation and thereby mimic the degradation processes of natural extracellular matrix.

3.1.4. Hyaluronic Acid. Hyaluronan or hyaluronic acid (HA) is a linear glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and *N*-acetylglucosamine.[204−206] HA is a negatively charged, naturally occurring

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polysaccharide with high molecular weights up to 10^7 Da. It is found mainly in the extracellular matrix (ECM) and in the synovial fluids of joints, where it reduces the friction of bones due to its unique viscoelastic properties.[207] Because of HA's biocompatible and attractive physical properties, biomaterials based on HA have mainly been developed for tissue engineering purposes.[208,209] However, also within this field, the delivery of proteins such as growth hormones is an important issue.[209−213] A few HA gels have been studied for their protein delivery possibilities including photopolymerized HA, HA−tyramine conjugates cross-linked using an oxidation reaction, HA−SH cross-linked using disulfide bond formation, and HA cross-linked by Michael addition.[214−219] For the preparation of gel networks, usually the carboxylic groups of hyaluronan are derivatized to obtain cross-linking functionalities in the polymer chains. Since HA is negatively charged at physiological pH, the protein release rate will be affected by the charge of the protein. Nevertheless, complete release can be obtained by enzymatic degradation of HA by hyaluronidase, which is present in biological tissues.[215,218]

3.2. Protein-Based Hydrogels

3.2.1. Collagen and Gelatin. Collagen can be found predominantly in connective tissues in the body and has been investigated extensively as biomaterial due to its advantageous properties such as high tensile strength, good cellular interactions, and biodegradability.[220] Gelatin is produced by partial hydrolysis of collagen, yielding a polymer that is widely used in food industry.[221] Because of the good cellular interactions, collagen and gelatin hydrogels have mainly been investigated within the tissue engineering field and for the release of growth factors to promote tissue formation.[176,222,223]

Collagen and gelatin hydrogels are mostly cross-linked using gluteraldehyde or water-soluble carbodiimides.[224] These cross-linking methods are not suitable for protein-releasing gels, because the applied agents also react with the proteins loaded in the gels. However, also noncovalently cross-linked fibrillar collagen can be used to create hydrogels by entanglements of collagen fibers.[225] The mesh sizes of these entangled collagen fibers are quite large and therefore diffusion of only very large proteins can be controlled. Nevertheless, the protein release rate may still be lower than expected on the basis of diffusion coefficients due to weak interactions between collagen and loaded proteins.[225]

Nowadays, gelatins can be produced in yeast cells recombinantly leading to slightly different polymer properties. The advantage is that properties such as molecular weight, amino acid sequences, and isoelectric points can be tailored precisely. Degradation times, swelling properties, and ultimately also drug release kinetics are affected by the design of recombinant gelatin. Only a few recombinant gelatin hydrogels have been investigated for their protein delivery properties.[226,227]

3.3. DNA-Based Hydrogels

Another unique biopolymer, DNA, has gained the interest of scientists for constructing materials over the past 2 decades.[228] For hydrogel preparation a few routes are applied. Natural DNA chains can form a gel by physical entanglements or they can be stabilized by chemical cross-linking.[229,230] Recently, the molecular design of DNA strands opened opportunities to make use of the recognition of cDNA strands to build three-dimensional networks. Hybrid materials have been designed in which cDNA strands act as cross-links between synthetic polymers.[231−233] Branched motifs of double-stranded DNA were prepared having three or four complementary sticky ends that could self-assemble and were stabilized by enzyme-catalyzed ligation.[234,235] Different designs of the branched motifs yield hydrogels with tunable mechanical properties and drug or protein release profiles.[234] High protein encapsulation efficiencies and sustained release of insulin was shown for these DNA hydrogels.

The slow ligation step, however, is not necessary to prepare DNA hydrogels using branched motifs. Liu et al. have shown that increasing the number of complementary base pairs in the "sticky ends" resulted in fast and stable gel formation just by self-assembly, as shown schematically in Figure 10.[236] These

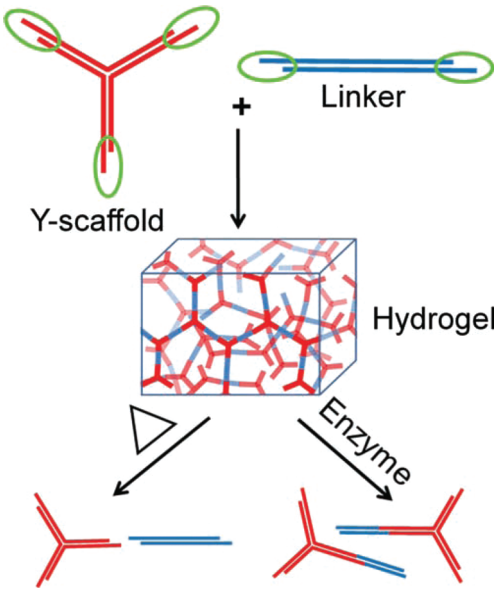


Figure 10. Schematic representation of DNA hydrogel formation. The Y-scaffold and linker are designed to cross-link by hybridization of their sticky ends (emphasized by green circles). By tailoring these sticky ends and inserting restriction sites in the linker sequences, the DNA hydrogels show thermal and enzymatic responsive properties. Reprinted with permission from ref 236. Copyright 2011 Wiley-VHC.

gels showed thermoresponsive behavior and by the introduction of a restriction site in the designed DNA hydrogels, they underwent a gel−sol transition upon addition of the matching restriction enzyme.

It can be debated whether these fully designed DNA hydrogels can still be regarded as natural materials. Nevertheless, they possess many advantageous features of natural DNA, such as biodegradability and biocompatibility. Moreover, due to the synthetic design they have a very high tunability with respect to mechanical properties, release kinetics, and responsiveness toward external triggers and they are not expected to show immunogenicity.

4. SMART HYDROGELS

Smart hydrogels are defined as materials able to undergo transitional changes in response to environmental stimuli.[237,238] They can rather abruptly swell, shrink, degrade, or undergo a sol−gel phase transition when exposed to external physical or chemical triggers, for example, changes in pH, temperature, solvent, pressure, ionic strength, light, and concentration of specific biomolecules.[19,239−242] Environmental triggers can be

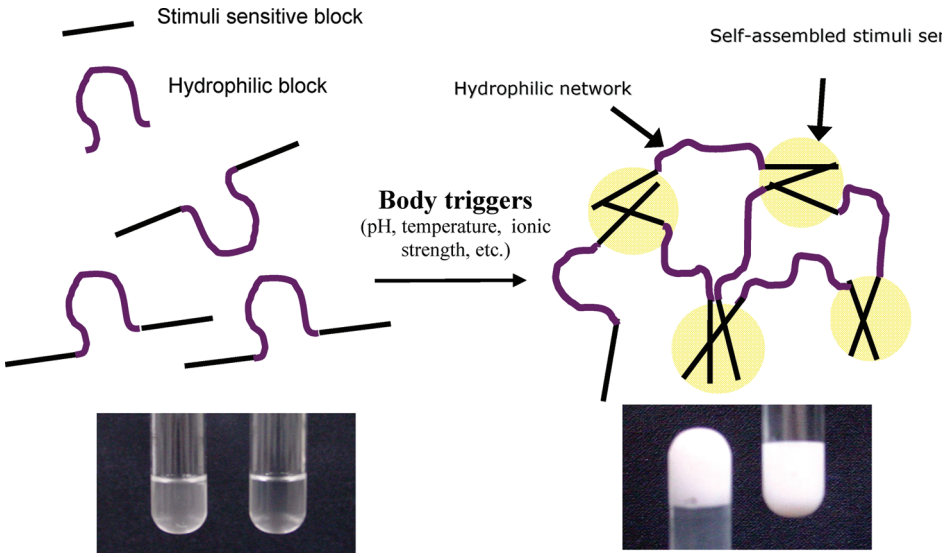
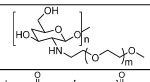
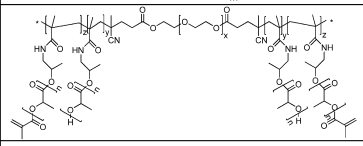
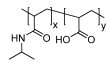
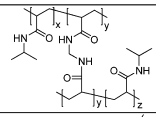
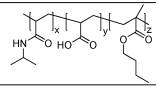
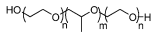
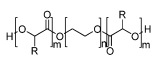
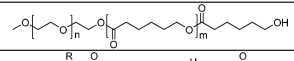
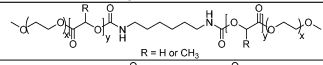
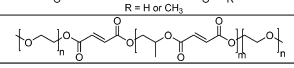
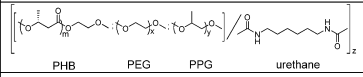
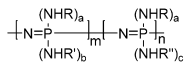


Figure 11. Self-assembly mechanism of stimuli-sensitive hydrogels. Photographs show polymer solutions of thermosensitive polymers below and above the gelation temperature, respectively.[247]

Table 1. Thermosensitive Hydrogels Applied as Protein-Releasing Materials

Name	Structure	Behavior	Protein release/application	
PEG-g-chitosan		LCST	BSA	256
Methacrylated pHPMAlac-PEG-pHPMAlac		LCST	Lysozyme, BSA, IgG	130
pNIPAm-co-AA		LCST	Artificial pancreas (islets of Langerhans encapsulated and secreting insulin)	263, 264, 266
pNIPAm cross-linked with BIS		LCST	BSA, insulin	267
Terpolymers of NIPAM, BMA, AA		LCST/pH	insulin	268
Pluronics PEO-PPO-PEO		LCST	Insulin, urease, interleukin-2, several growth factors	279
PLG-PEO-PLG		LCST	Cytochrome-c, ovalbumin, BSA, tetanus toxoid	295
MPEG-PCL		LCST	BSA	308
PEG-PLGA-PEG		UCST & LCST	TGF-β, insulin	300, 303
PEG-PPF-PEG		LCST	tissue engineering	310
Poly (PEG/PPG/ PHB/ urethane)		LCST	BSA	312
polyphosphazenes		LCST	HSA, artificial pancreas (islets of Langerhans encapsulated and secreting insulin	325

exploited to accomplish specific functions, such as drug release, protein separation, and muscle activity, or to design in situ gelling systems. Section 4.1 reviews stimuli-sensitive polymeric systems used for the preparation of in situ gelling hydrogels,

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while section 4.2 provides some examples of environmentally responsive hydrogels used for other biomedical purposes. Section 4.3 highlights the recent interest in nanogels having specific applications due to their size related properties. Many reported nanogels have similar "smart" properties as the described macrogels.

4.1. Stimuli-Sensitive Polymers for in Situ Gelling Systems

Stimuli sensitivity has been widely applied for the design of injectable in situ forming hydrogels, with pH and temperature responsive systems being the most attractive representatives. In the past 10−15 years, research has shifted its interest from the area of implantable materials to the fast-developing field of injectable in situ gelling systems. In situ forming hydrogels (Figure 11) exist as viscous but still liquid aqueous formulations prior to administration but abruptly turn into gels upon administration.[243−246] In contrast to permanent networks formed by chemical cross-linking, stimuli-sensitive hydrogels are transient physical networks that can be reversibly transformed into solutions by varying the environmental conditions. The advantages of these delivery systems, able to form macroscopic drug-encapsulating gels at the site of injection, include improved patient compliance, cost reduction compared to surgical intervention, and the ability to overcome the limitations associated with drug postloading techniques.

4.1.1. Temperature-Sensitive Hydrogels. Temperature is the most widely used stimulus in environmentally responsive hydrogels. Temperature-responsive polymers are characterized by a critical gelation temperature in aqueous solutions, where self-assembly of the polymer chains occurs due to hydrophobic interactions and thus phase separation is observed. Especially polymers exhibiting lower critical solution temperature (LCST) behavior with ideally a transition between room and body temperature are attractive as injectable formulations, since they are soluble at low temperature and gels are formed upon administration, e.g. by subcutaneous injection. Polymers can also display upper critical solution temperature (UCST) behavior, when the polymer solution is phase-separated below a specific temperature. Thermosensitive behavior of polymers is generally viewed as a phenomenon governed by the balance of hydrophilic and hydrophobic moieties on the polymer chain.[248,249] Most of the thermosensitive polymers studied for biomedical applications exhibit LCST behavior, for example, natural polymers (e.g., gelatin) and polysaccharides (e.g., agarose or cellulose derivatives).[250−252] Table 1 gives an overview of the thermosensitive polymers described in this section.

Only a few natural polymers display LCST behavior in the range between room and body temperature. Some cellulose derivatives [methyl and hydroxypropyl methylcellulose (HPMC)] at low concentrations (1−10 wt %) in water are liquid at low temperature but jellify upon heating. However, their gelation temperature is far above body temperature, representing a limitation of this material as an in situ gelling system.[253] Chemical and/or physical modification can be adopted to lower the gelation temperature, for example, by addition of NaCl or decreasing the degree of hydroxypropyl substitution of HPMC.[253,254] However, no studies on protein release with these systems have been published to date. Chitosan has been reported by Chenite et al. to form a gel close to body temperature and at physiological pH when combined with glycerol phosphate disodium.[255] Bhattarai et al. developed a chitosan−PEG copolymer (chitosan-*g*-PEG) based injectable, thermoreversible gel that utilized intermolecular chitosan chain interactions for gelation. This hydrogel was used as a depot system for sustained protein release.[256] This type of thermosensitive gelation has also been observed in cellulose derivatives grafted with hydrophilic moieties.[257]

Synthetic polymers offer many more opportunities as compared to natural polymers for the design of injectable hydrogels. The most frequently studied synthetic thermosensitive polymer for biomedical and pharmaceutical applications is poly(*N*-isopropylacrylamide) (PNIPAm), because its LCST in water is 32 °C, thus suitable for in situ gelling (Figure 12).[249]

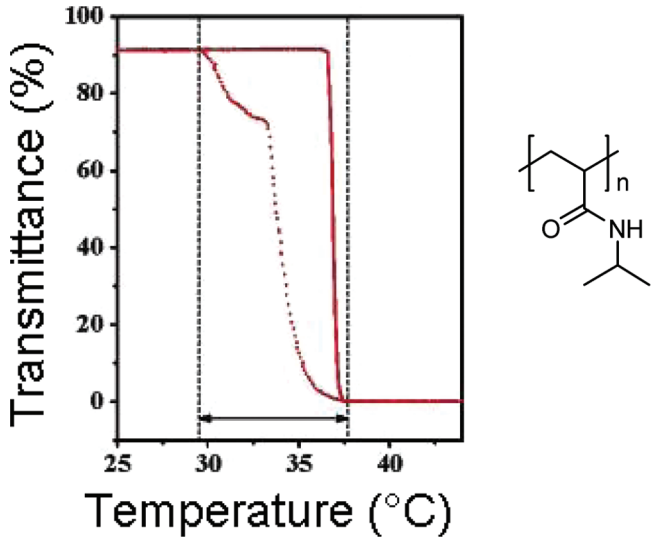

Figure 12. Thermosensitive behavior of PNIPAm with a LCST close to body temperature. Plot of transmittance as a function of temperature measured for an aqueous solution (3 mg/mL). Solid line, heating cycle; dotted line, cooling cycle. Reprinted with permission from ref 262. Copyright 2006 American Chemical Society.

The incorporation of hydrophilic monomers in PNIPAm increases the LCST, whereas more hydrophobic units decrease it.[258] Similar behavior was observed by Vermonden et al., who reported a decrease in LCST of poly(hydroxylpropyl methacrylamide lactate) (PHPMAm-lac) upon introduction of hydrophobic methacrylate moieties in the polymer lactate side chains.[259] Similarly, the gelation behavior of poly(D,L-lactide-*co*-glycolide)−poly(ethylenglycol)−poly(D,L-lactide-*co*-glycolide) (PLGA−PEG−PLGA) was influenced by the hydrophobicity of end-caps (hydroxy, acetyl, propionyl, and butanoyl groups); an increase in the hydrophobicity of the copolymer lowered the transition temperature.[260] The same finding was obtained for cholesterol end-capped star PEG−PLLA copolymers.[261]

Physically cross-linked PNIPAm-based hydrogels were described for the first time by Han et al.,[263] who synthesized poly(*N*-isopropylacrylamide-*co*-acrylic acid) [p(NIPAm-*co*-AA)] to prepare thermosensitive matrices that were used in follow-up studies for biomedical purposes, particularly as synthetic matrices in refillable bioartificial pancreas. Encapsulated Langerans islets showed good viability, and the cell-laden artificial matrices showed insulin release.[264−266] Similarly and more recently, PNIPAm networks were cross-linked using *N*,*N*′-methylenebisacrylamide (BIS) and used for bovine serum albumin (BSA) and insulin release studies in vitro. The release of the protein was not complete and a strong interaction

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between polymer and protein was proposed as the reason for the nonretrieved protein.[267] Kim et al. described the use of pH/thermosensitive polymeric beads based on terpolymers of NIPAm, butyl methacrylate (BMA), and acrylic acid (AA) (pH-sensitive) to modulate the release of insulin. A high loading efficiency was accomplished (90−95%), and while no release of insulin was observed at pH 2.0 and 37 °C, the drug was released at physiological pH.[268] The release rate and mechanism depended on the molecular weight (MW) of the polymer: low MW terpolymers eroded very quickly and released insulin within 2 h by an erosion-mediated mechanism, while in case of high MW polymers, which had a better stability, the gels showed a release of insulin for 8 h that was governed by swelling/diffusion.[268] As observed for many other thermally assembled polymers, the stability of such hydrogels is rather poor and represents a major limitation in the use of these materials for pharmaceutical purposes. Therefore, in recent years, strategies to improve the stability of thermosensitive networks by chemical cross-linking methods, suitable for in situ gelling, have been exploited. Examples of such methods are photopolymerization or the Michael addition reaction discussed in sections 2.2.1 and 2.2.2, respectively.[156,158,159,163,259,269,270]

PNIPAm-based hydrogels self-assembling in a thermoreversible fashion and displaying improved hydrophilicity, thus with enhanced capability to retain water within the hydrogels matrix, were synthesized by grafting NIPAm to permanently hydrophilic polymers like poly(ethylene glycol) (PEG), for example via Ce^{+}/OH redox initiated free radical polymerization.[271,272] A series of polymers with different architectures were synthesized [AB, BAB, $A(B)_4$, and $A(B)_8$ linear and star-shaped block copolymers with PEG as A block and PNIPAm as B block] and characterized for gelation mechanism and exploited for chondrocyte immobilization.[273] Several other copolymers of NIPAm with 2-methacryloyloxyethyl phosphorylcholine (MPC) were synthesized and characterized.[274,275]

Other nondegradable thermosensitive polymers exhibiting hydrophilic−hydrophobic transitions at temperatures close to body temperature are poly(vinyl ether)s (PVEs); their derivatives and copolymers[276] are excellently reviewed elsewhere[277] and are beyond the scope of this review, as data on pharmaceutical and biomedical applications to date are lacking.

A series of polymers, namely Pluronic (BASF), based on poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) triblock copolymers (PEO−PPO−PEO), with varying PEO/PPO molecular weights and contents, exhibit LCST behavior below body temperature[278] and have been extensively investigated for their physical−chemical and thermodynamic properties, as well as for pharmaceutical applications.[279−282] Pluronics have been extensively used as in situ forming drug delivery matrices, and the possibility to prolong to some extent the drug pharmacokinetics by using Pluronic-based hydrogels was demonstrated. For example, monoamino-terminated Pluronic (mainly Poloxamer PF127) was coupled to poly(acrylic acid) (PAA) using dicyclohexyl carbodiimide (DCC), and graft copolymers of poly(acrylic acid)-*g*-Pluronic of different MW were synthesized via chain transfer reactions.[283] These graft copolymers gave improved gelation and mechanical properties as compared to the corresponding Pluronic, due to the presence of pH-sensitive moieties (PAA) that affect ionization and chain expansion of the polymer. Pluronic-based copolymers were widely studied for the delivery of protein and peptide therapeutics, like insulin, interleukin-2, urease, epidermal growth factor, and endothelial cell growth factor. Sustained release over several hours was observed with the possibility to tailor the release kinetics by the polymer concentration or addition of excipients.[284−288] However, Pluronics, as well as PNIPAm, are not ideal biomaterials for in vivo applications. Besides toxicity issues, observed with Pluronics after intraocular implantation,[289] their main disadvantage is their nonbiodegradability, which makes surgical intervention necessary to remove the delivery system from the body after the drug has been released. In addition, weak mechanical strength and stability, as well as high permeability for entrapped compounds, are further limitations associated with the use of these polymers. Some of the listed drawbacks were partially overcome. To mention, Cohn et al. copolymerized PEG and PPO segments using two synthetic pathways: (1) chain extension of native Pluronics with hexamethylene diisocyanate (HDI) and (2) covalent binding of PEG and PPO chains using phosgene as the connecting molecule. The multiblock copolymers synthesized displayed remarkably improved mechanical properties as compared to Pluronic; moreover, an extension of the drug release time as compared to self-assembled Pluronic hydrogels was found.[290] However, biodegradability issues still exist.[291] Many block copolymers of Pluronics with aliphatic polyesters (PLA and PCL) were also reported.[292−294]

The most advanced thermosensitive delivery systems for proteins rely on biodegradable polymers, which is very advantageous for in vivo applications. Kissel et al. synthesized triblock copolymers based on poly(DL-lactic acid-*co*-glycolic acid) and PEG and prepared microspheres for protein delivery from these polymers in 1990s.[295] Shortly afterward, biodegradable and biocompatible PEG/polyester block copolymer hydrogels, initiated by Kim and co-workers,[296] were introduced as a novel class of biodegradable thermosensitive matrices. ABA-type PEG−poly(L-lactide)−PEG triblock copolymers (PEG−PLLA−PEG) were first synthesized by ring-opening polymerization of L-lactide (LLA) using the monomethoxy PEG (MPEG) as macroinitiator, and subsequently, PEG−PLLA−PEG triblock copolymers were obtained by coupling MPEG−PLLA using hexamethylene diisocyanate (HMDI). These polymers exhibited UCST behavior; therefore, the drug loaded hydrogels were prepared at 45 °C and then gelation was induced by lowering the temperature below 37 °C. The release of fluorescein isothiocyanate (FITC) labeled dextran (a model compound for pharmaceutical proteins) was studied and it was demonstrated that 12 days sustained release was achieved for 35 wt % hydrogels. Formulations of lower polymer content showed burst release that could be decreased by increasing the polymer concentration. Also a series of star-shaped PLLA−PEG block copolymers were synthesized by coupling star PLLA with monocarboxy-MPEG using DCC coupling reaction.[297] The main disadvantages of this system are the long degradation time due to PLLA crystallinity and the need for high temperatures for the preparation of the hydrogels, as the polymer exhibits UCST behavior. Under these conditions the structure of labile protein molecules, along with their activity, might be adversely affected.

The next generation of PEG/polyesters hydrogels was based on PEG−PLGA−PEG triblock copolymers.[298] These materials displayed both LCST and UCST behavior and were processable avoiding the use of high temperatures to dissolve the polymer. It was shown that upon subcutaneous injection in vivo (rat model) the hydrogels were stable for 1 month.[299] TGF-β1 was loaded into these hydrogels and used as a reservoir

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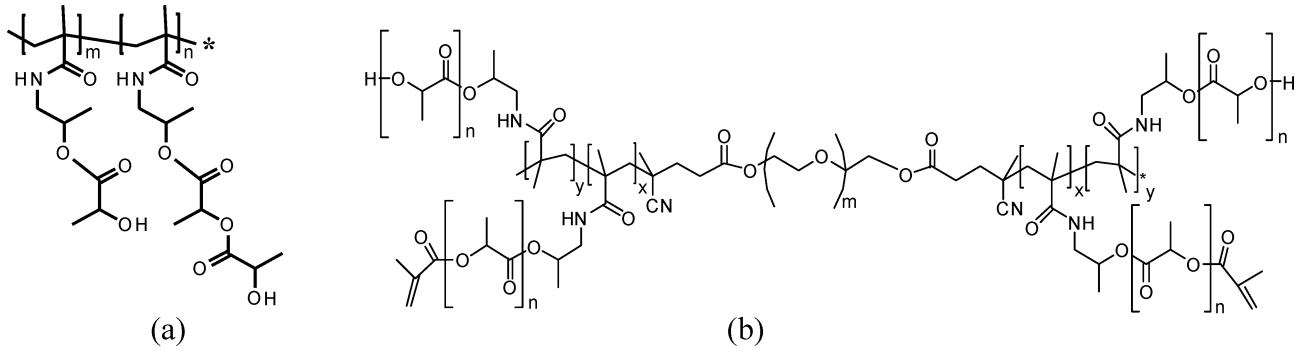

Figure 13. Chemical structures of (a) p(HPMAm-lac) and (b) methacrylated p(HPMAm-lac)−PEG−p(HPMAm-lac).

for controlled drug release aimed for wound healing purposes.[300] Indeed, significant high levels of re-epithelialization, cell proliferation, and collagen organization were observed. The sustained release of synthetic drugs like ketoprofen and spirolactone was also studied from PEG−PLGA−PEG hydrogels,[301] as well as the release of insulin, porcine growth hormone, and glycosylated granulocyte colony stimulating factor (in vitro and in vivo).[302,303] Taken together, all these studies confirmed an improved stability and capability to release drugs over an extended period of time (weeks) as compared to Pluronics formulations. More detailed overviews of the characteristics and efficacy of this type of copolymers can be found in other reviews.[277,279]

Several other thermosensitive copolymers of PEG with aliphatic polyesters were synthesized and applied for drug delivery. Some examples are AB, ABA, and BAB copolymers of PEG with caprolactone (PCL) and δ-valerolactone (PVL).[304−307] The in vitro and in vivo release of fluorescein isothiocyanate-labeled bovine serum albumin (BSA−FITC) was studied from PEG−PCL diblock copolymers gels and compared to that of Pluronic gels, and the studies demonstrated longer in vivo stability of PEG−PCL hydrogels and enhanced capability to provide sustained protein release over 10 days, as compared to Pluronic gels, where destabilization and drug release within 3 days was observed.[308,309]

Mikos et al. synthesized PEG-based triblock copolymers consisting of poly(propylene fumarate) (PPF) as middle block.[310] Compared to other PEG copolymers, PPF has the advantage of having unsaturated double bonds, suitable for stabilization of the hydrogels by chemical cross-linking. Biodegradable multiblock amphiphilic and thermosensitive poly(ether ester urethane)s consisting of poly-[(*R*)-3-hydroxybutyrate] (PHB), poly(ethylene glycol) (PEG), and poly(propylene glycol) (PPG) blocks were synthesized by Loh et al.[311] Aqueous solutions of these polymers were found to undergo a reversible sol−gel transition by micellar packing upon temperature changes at very low copolymer concentrations (2−5 wt %), and the authors showed that these systems are suitable for protein delivery.[312] Recently, Pluronic analogs containing middle blocks of poly(hexamethylene adipate) (PHA), poly(ethylene adipate) (PEA), and poly(ethylene succinate) (PESc) instead of PPO were synthesized. Because of the hydrophobic nature of PHA and PEA, strong hydrophobic interactions and micellization occurred, leading to formation of hydrogels only at relatively high concentrations, while the more hydrophilic PESs showed gelation at low concentrations.[313] A general drawback of some of these polyester-based copolymers is their very long degradation time (i.e., PCL degrades in vivo in 2−4 years[314]), which can lead to polymer accumulation in the body for long periods, limiting the use of these materials for chronic diseases, where controlled delivery systems need to be administered repeatedly.

In 2004, our department introduced a new class of thermosensitive and biodegradable polymers based on pHPMAm-lac (Figure 13a), which displays tunable LCST behavior from ∼10 to 60 °C by simply changing the length of the lactate side chains.[315] The polymer biodegradability is ensured by the presence of hydrolytically sensitive ester bonds in the lactate side chains. When the terminal lactate groups are cleaved by hydrolysis, the resulting polymer becomes water-soluble and can be eliminated by renal filtration, when its molecular weight is lower than the renal cutoff.[316] These thermosensitive polymers have been coupled to PEG by free radical polymerization using a PEG macroinitiator, yielding a copolymer with ABA triblock architecture, consisting of inner PEG B-block flanked by outer p(HPMAm-lac) A-blocks (Figure 13b). These polymers are suitable for the preparation of in situ gelling systems, whose mechanical properties and degradation behavior were improved by combining thermal self-assembly with photopolymerization upon polymer derivatization with methacrylate moieties.[259] The chemically stabilized hydrogels were suitable as controlled protein delivery systems, where model proteins were released according to diffusion governed kinetics, easily tailorable from 1 week to 2 months by changing polymer molecular weight, concentration, and degree of derivatization with methacrylate groups.[247,317,318] The potential of this thermosensitive hydrogel for tissue engineering was assessed by demonstrating good viability and differentiation of human mesenchymal stem cells (hMSCs).[259] It was further demonstrated that by randomly copolymerizing HPMAm-dilactate and NIPAm, a biodegradable thermosensitive polymer was obtained.[319]

Emerging thermosensitive hydrogels for protein delivery are also biodegradable polyphosphazenes, consisting of a hydrophilic PEG block and hydrophobic amino acids or a peptide block [L-isoleucine ethyl ester (IleOEt), D,L-leucine ethyl ester (LeuOEt), L-valine ethyl ester (ValOEt)] or di-, tri, and oligopeptides in the side groups.[320−322] Hydrogels were formed by intermolecular association of hydrophobic peptide chains, and when PEG was coupled to di-, tri-, and oligopeptides as side groups, hydrogels of higher mechanical strength were obtained, as compared to PEG−IleOEt polymer gels. Polymers containing depsipeptide (GlyGlycOEt) showed faster hydrolytical degradation because of the generation of carboxylic acid groups that made the polymers more hydrophilic, resulting in

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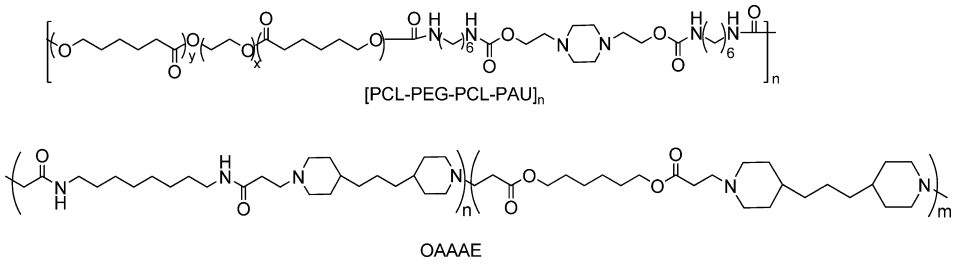

Figure 14. Chemical structures of multiblock copolymer ([PCL−PEG−PCL−PAU]$_n$) (top) and oligo(amidoamine/amino ester) (OAAAE) (bottom).[330,331]

sustained release of FITC−dextran and human serum albumin in vitro for about 2 weeks. The authors also studied strategies to decrease the burst release from polyphosphazene hydrogels by addition of chitosan, that, due to its positive charge, retained negatively charged proteins like BSA, gelatin type B (GB20), and FITC−BSA within the hydrogel network.[323] Application of these hydrogels as extracellular matrix for an artificial pancreas was investigated.[324]

4.1.2. pH Sensitive Hydrogels. Due to the specific pH range occurring at physiological, pathological, or subcellular sites such as stomach, intestine, endosome/lysosome, and tumor sites, pH is another stimulus used for the design of in situ gelling hydrogels.

Suitable polymers for this purpose are those bearing weak polyelectrolyte (polyacid, polybase) or polyampholyte sequences. pH-sensitive polymers rely on the protonation/deprotonation equilibrium, which depends on the pK_a of the acidic and/or basic moieties present in the polymer. Therefore, a pH-sensitive polymer can be charged (yielding a swollen state) or uncharged (yielding a hydrophobic/collapsed state) depending on the environmental pH.

Hydrogels comprising poly(methacrylic acid) (PMAA) grafted to PEG [P(MAA-*g*-EG)] showed pH sensitivity due to the interactions between the ether oxygen from the graft chain and the acidic groups. These complexes dissociate at higher pH and P(MAA-*g*-EG) hydrogels have been used as drug-delivery carriers for salmon calcitonin.[326,327] Recently, hydrogels that assembled at physiological pH were synthesized by grafting hydrophobic palmitoyl sequences to a biodegradable chitosan backbone. Hydrogelation was controlled by the degree of *N*-palmitoyl substitution and pH. A 15% derivatized chitosan of 1 wt % concentration aqueous system showed gelation upon increasing pH from 6 to 7.5, due to a transition from micelles to interconnected nanodomains. An in vivo study showed the formation of a gel at the site of injection, demonstrating its potential for biomedical applications.[328]

4.1.3. pH/Temperature-Sensitive Hydrogels. pH/temperature-sensitive copolymer hydrogels are prepared by introducing pH-sensitive moieties in a temperature-sensitive polymer. A pH/thermo-sensitive ABA copolymer was obtained by introducing carboxylic acid groups end groups into PLGA−PEG−PLGA triblock copolymers. Although the nonmodified triblock copolymer did not exhibit gelation upon increase of temperature, the carboxyl-capped PLGA−PEG−PLGA led to four states (sol, gel, precipitate, and turbid sol) depending on pH and temperature.[329]

pH- and temperature-sensitive multiblock poly(ester amino urethane)s were synthesized by coupling poly(amino urethane) (PAU) through a condensation reaction to PCL−PEG−PCL triblock copolymers to yield multiblock copolymers (PCL−PEG−PCL−PAU)$_n$. The incorporation of the ionizable PAU segments with tertiary amine groups in the macromolecule induced pH sensitivity (Figure 14). Below pH 6.9, the polymer is in a sol state in aqueous solution up to 60 °C due to the electrostatic repulsion of the piperazine groups. In contrast, at physiological pH of 7.4 the solution displays a sol−gel transition upon increasing temperature to 37 °C. The formation of the gel depended on the formation of interconnected micelles. The formation of a gel was assessed in vivo[330] and injectable poly(amidoamine)−poly(ethylene glycol)−poly(amidoamine) triblock copolymer hydrogels exhibiting pH and temperature sensitivity were designed for bioadhesive applications. The dual responsiveness depended upon the poly(amidoamine) outer blocks, which turned from a hydrophilic into hydrophobic state upon increasing pH and/or temperature. At low pH, a sol was observed up to 60 °C, while above pH 7.0 the micelles bridged, leading to the formation of a gel. In vivo experiments showed that upon subcutaneous injection of 12.5 wt % copolymer solution a white gel was obtained after 1 min.[332] The same group developed recently a thermosensitive and pH-sensitive hydrogel based on oligo(amidoamine/β-amino esters). The polymer solution of pH 6.6 could be injected subcutaneously in the back of a rat and gel formation occurred at physiological pH and temperature. This system showed prolonged insulin release in vivo (Figure 14).[331]

A thermosensitive triblock copolymer composed of poly(ε-caprolactone-*co*-lactic acid)−PEG−poly(ε-caprolactone-*co*-lactic acid) (PCLA−PEG−PCLA) was synthesized by ring-opening polymerization using ε-caprolactone (CL), lactide (LA), and PEG as macroinitiator. Separately, carboxylic acid terminated sulfamethazine oligomers (OSMs) were polymerized by chain transfer polymerization and coupled to terminal hydroxyl groups of the triblock copolymer, yielding a pentablock copolymer (OSMs−PCLA−PEG−PCLA−OSMs). An aqueous solution of this polymer showed a reversible sol−gel transition by a small pH change in the range of pH 7.4−8.0 and also by a temperature change in the region of body temperature, forming a gel at 37 °C, pH 7.4 (Figure 15). The block copolymers OSM−PCLA−PEG−PCLA did not form a gel at pH 8.0 in the tested temperature range (from 4 to 60 °C) because the hydrophobic interactions between PCLA−OSM blocks is perturbed by the ionized sulfonamide group of the OSM block. As the pH is decreased, OSM gets deionized, restoring the hydrophobic interaction between PCLA−OSM blocks and forming a gel. By exploiting both pH and thermosensitive functionalities of the polymer, it was possible to broaden the gel window and obtain a sol between 10 and 70 °C at pH 8.0. A solution of this polymer can be injected without concerns for premature gelation in the needle, and once in the body, the physiological pH triggers the gel formation.[333,334] The group of Lee et al. also synthesized diblock copolymer hydrogels based on a basic poly(β-

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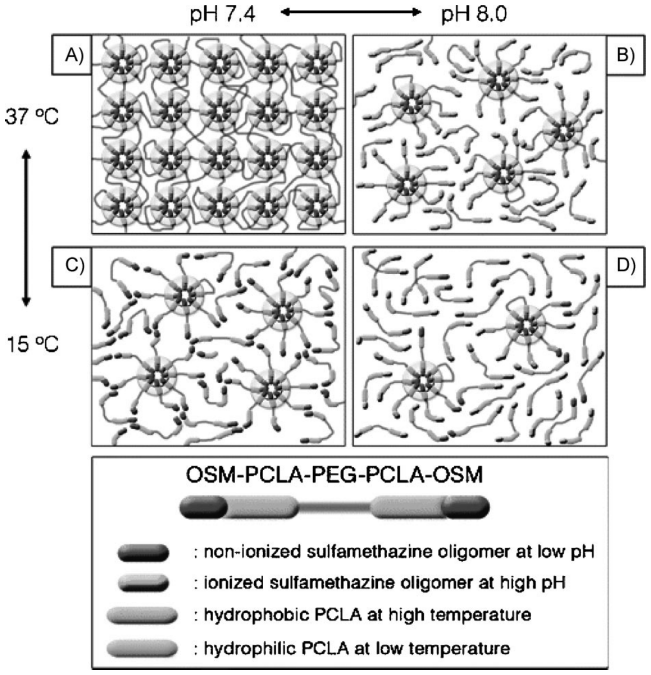

Figure 15. Schematic representation of the sol–gel mechanism of the pH/temperature-sensitive block OSMs–PCLA–PEG–PCLA–OSMs copolymer solution: (A) pH 7.4, 37 °C; (B) pH 8.0, 37 °C; (C) pH 7.4, 15 °C; (D) pH 8.0, 15 °C. Reprinted with permission from ref 333. Copyright 2005 American Chemical Society.

aminoester) (PAE) coupled to MPEG. A gel-to-sol transition at pH >6.0 was observed, when the temperature was increased as a result of micelle packing.[335]

Another dual responsive polymer is PAE–PCL–PEG–PCL–PAE pentablock copolymer, prepared by Michael addition polymerization of 4,4-trimethylenedipiperidine (TMDP), PCL–PEG–PCL diacrylate, and butane-1,4-diol diacrylate (BDA). Insulin, loaded into hydrogels based on the pentablock copolymer mentioned above, formed complexes with this polymer, lowering its LCST, and acted as physical cross-linker, which was confirmed by the longer stability of the protein loaded hydrogels as compared with the placebo gels.[336] The release of insulin depended on the degradation kinetics of the copolymers, and complete release of insulin was obtained in about 30 days.

4.2. Other Stimuli Responsive Polymers

4.2.1. Biomolecule Sensitive Hydrogels. On-demand release of drugs is particularly relevant for drugs that necessitate a more complex release profile able to mimic varying physiological concentrations over time (e.g., insulin or hormones). Glucose-sensitive hydrogels are insulin reservoirs of polymeric networks that ideally release the drug on demand when the glucose concentration exceeds a certain level. One strategy to achieve this goal relies on the use of pH-responsive hydrogels entrapping glucose oxidase, catalase, and insulin. *N*,*N*-Dimethylaminoethyl methacrylate (DMAEMA, pK_a 8.4)[337] has often been introduced into copolymer hydrogels to render them pH-sensitive. When glucose diffuses into the hydrogels, it is converted to gluconic acid due to the action of glucose oxidase. The formed gluconic acid causes a pH drop, responsible for the protonation of DMAEMA groups and swelling of the hydrogel due to increased electrostatic chain repulsions, resulting in larger pores in the gels and release of insulin.[241] A similar approach was reported using a sulfonamide-based glucose-responsive hydrogel.[338] Kitano et al. proposed poly(*N*-vinyl-2-pyrrolidone-*co*-phenylboronic acid) [p(NVP–PBA)] as a chemically regulated delivery system for insulin. The diol moiety on PBA allows the binding of glucose followed by pulsatile insulin release.[242,339] Brownlee et al. and Kim et al. pioneered the field of glucose-sensitive hydrogels using lecitin, known for its ability to bind carbohydrates. They loaded the hydrogel composed of cross-linked concanavalin A (a lectin having four binding sites) complexed to a glycosylated insulin in a pouch of Durapore membrane. In presence of glucose, its competitive binding with concanavalin A triggered the release of insulin.[340,341] Hydrogel systems sensing other biomolecules like antigens and proteins have been published as well; the reader is referred to more specialized reviews in which these systems are described and discussed.[342,343]

4.2.2. Drug-Sensitive Hydrogels. Recently, Weber et al. proposed a drug-sensing hydrogel based on gyrase subunit B (GyrB), reversibly cross-linked by coumermycin and able to release VEGF upon addition of novobiocin. The polymer forming the hydrogel is based on polyacrylamide functionalized with nitrilotriacetic acid chelating a Ni^{2+} ion to which GyrB can bind through a hexahistidine sequence. Through a mechanism of drug displacement, the addition of novobicin causes the cross-links to be partly broken, resulting in opening of the network structure and release of VEGF.[344] Antigen-sensing polymers have also been developed for the preparation of hydrogels. Miyata et al. reported on a semi-IPN hydrogel composed of two polymeric chains, each of them bearing either an antigen (rabbit IgG) or its specific antibody (goat anti-rabbit IgG). The presence of free target antigen induces a change in hydrogel volume followed by the release of encapsulated protein. They demonstrated that stepwise changes in antigen concentration can induce pulsatile permeation of a model protein (hemoglobin) through the network.[345]

4.2.3. Light-Sensitive, Electrosensitive, and Magnetic Field Sensitive Hydrogels. UV light has been used as a trigger for the (dis)assembly of hydrogels as well as for the release of encapsulated drugs. In order to design photosensitive hydrogels, a photochrome unit [e.g., azobenzene (AZOB)] has to be incorporated in the polymer structure. Azobenzene-modified polyacrylate with different spacers between the photochrome and the backbone was synthesized and BSA was combined with the polymer in aqueous medium. In the dilute regime, BSA/AZOB complexes were formed in equilibrium with unbound BSA and the affinity of the protein for the polymer depended on the length of the hydrophobic spacer and the presence of additional *n*-alkyl side groups. In the semidilute regime, physical cross-linking involving BSA greatly enhanced the strength of the hydrogel. In the two regimes, light was shown to modify the binding properties due to cis–trans isomerization of the azobenzene. Reversible release of BSA (up to 80% of the loaded protein) was obtained by exposure to UV.[346] When a ternary gel mixture of p(AA/C_{12}), αCD, and 4,4′-azodibenzoic acid (ADA) was irradiated with UV light, ADA isomerized from its trans to cis form, and the mixture underwent a gel-to-sol transition because αCD formed inclusion complexes more favorably with C_{12} side chains than with *cis*-ADA. When the ternary sol mixture was subsequently irradiated with visible light, ADA isomerized back from its cis to trans isomer and the mixture underwent a sol-to-gel transition. Furthermore, these gel-to-sol and sol-to-gel transitions

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occurred repeatedly by repetitive irradiations of UV and visible light.[347]

Similarly, mixtures of polyacrylamide bearing pendant AZOB moieties and βCD-derivatized poly(allylamine) lead to photo-responsive hydrogels.[348] Kwon et al. introduced a polymeric system, which rapidly changed from a solid into a liquid in response to small electric currents, by solubilization of the solid polymer complex upon decomposition into two water-soluble polymers. The system is based on poly(ethyloxazoline) that forms complexes with poly(acrylic acid) or poly(methacrylic acid), and modulated release of insulin was achieved with this polymeric system.[349]

4.3. Nanogels

Nanogels have, as the name implies, nanosize dimensions, and because of their size dependent applications, they might be regarded as smart hydrogels as well. Besides, many reported nanogels possess stimuli responsive behavior as described above for macrogels such as thermo- and pH-sensitivity.[350] Because many recent review papers describe the interest in nanogels for biomedical applications, we do not aim to give a complete overview here; we only shortly highlight the advantages of nanogels with a few appealing examples related to protein delivery.[351−354]

Many nanoparticulate systems with sizes between 10 and 1000 nm (but ideally <200 nm) such as nanocapsules, polymeric micelles, liposomes, and dendrimers have been developed for drug delivery applications.[355−357] Because of their small size, nanoparticles are able to circulate in the bloodstream (depending on their size and surface properties) for a couple of hours and overcome certain anatomical barriers. Besides, they can also reach tumor tissues, due to the enhanced permeation and retention (EPR) effect and coupling of targeting ligands on their surface aims for cellular recognition/internalization to increase efficacy of, for example, anticancer drugs loaded in such particles.[358,359] Nanogels are a relatively new class of nanoparticulate carriers that have been shown to deliver drugs intracellularly by different cellular uptake mechanisms (clathrin- and caveoli-mediated endocytosis, pinocytosis, and phagocytosis). Because of aimed intracellular drug delivery, especially pH-sensitive nanogels are of interest due to lower pH values in lysosomes, which might trigger the release of entrapped drugs.[360] So far, nanogels have been mainly exploited for the (targeted) delivery of low molecular weight drugs[351,361,362] but are also under investigation for the release of nucleic acid based drugs[363] and pharmaceutical proteins.

The group of Akiyoshi started working on nanogels in the 1990s and has shown the potential of their cholesteryl group-bearing pullulan (CHP) nanogels (Figure 16) for the delivery of several proteins, such as insulin, interleukin 12 (IL-12), and HER2 protein.[364]

In general, protein delivery from nanogels suffers from a limited release time due to the large surface area of nanogels compared to that of macrogels. For sustained release over longer periods of time, nanogels can be incorporated into macrogels. This technique was shown to be effective for the sustained release of erythropoietin (EPO) both in vitro and in vivo using the above-mentioned CHP nanogels encapsulated inside a hyaluronan hydrogel.[364]

Nanogels also have potential as delivery vehicles for protein-based vaccines. Fréchet et al. showed that acid-sensitive gels with a size of 200−500 nm could be used to activate cytotoxic T lymphocytes (CTLs).[366] The gels containing acid-labile cross-linkers could be loaded with antigens that remained encapsulated at physiological pH and were released upon phagocytosis by APCs.[367] Whether the particle size of these hydrophilic gels is important for triggering a CTL response was shown to be questionable, suggesting that hydrophilic nanogels behave differently than hydrophobic nanoparticles.[368]

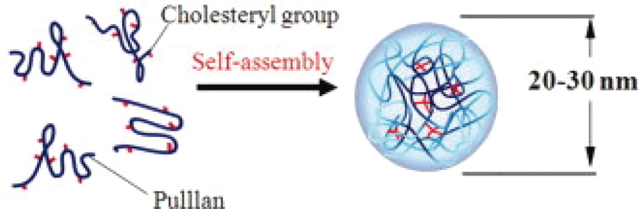

Figure 16. Formation of nanogel by self-assembly of CHP. Reprinted with permission from ref 365. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA.

5. GEL CHARACTERISTICS AND PROTEIN RELEASE MECHANISMS

Hydrogels are characterized in general by their mechanical strength, mesh size, and swelling properties. These properties all influence the performance of the materials in vivo and their drug release profiles.

Ideally, hydrogels have mechanical properties that match the mechanical properties of the tissue where they are placed in the body. The mechanical strength of hydrogels is usually measured using rheological analyses, giving information about (the frequency-dependent) viscosity (η) (if flow is possible) and shear (G) and Young's (E) moduli of the systems. Mechanical properties of natural tissues cover a wide range, e.g., bone tissues can have a Young's modulus of up to a few GPa.[369,370] Therefore, it is impossible to define ideal mechanical properties for hydrogels, as they depend on the site of application.

5.1. Mechanical Properties

Whether a polymer network can be regarded as a "gel" has been defined in different ways in literature. Many authors state the observation of gel formation upon a simple tube-inversion method. If the material does not flow upon inversion, it is called a gel.[371] Nevertheless, there are more quantitative methods to assess gel formation. Anseth et al. wrote a clear review about mechanical properties of hydrogels and ways how to control them by changing polymer composition and cross-link densities.[372] In this review, only a short summary is given about how to interpret mechanical data with respect to hydrogel network properties; for details about this subject, the reader is referred to Anseth's paper.[372]

For three-dimensional network systems, the shear modulus, G, can be used to calculate the number-average molecular weight between cross-links, M_c. Stronger materials are obtained when polymer networks have a higher cross-link density, reflected in a lower M_c. The relationship between M_c and G is given by the following equation:

$$G = \frac{\rho RT}{M_c}\frac{{r_0}^2}{{r_f}^2}\left(1 - \frac{2M_c}{M_n}\right)$$

Where ρ is the polymer concentration in g/m^3, R is the molar gas constant, and T is temperature. The front factor ${r_0}^2/{r_f}^2$ represents the ratio of the end-to-end distance in the network versus the end-to-end distance of isolated chains for which a

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value of 1 is often used, as the actual value is mostly unknown. When the number-average molecular weight of the linear chains before cross-linking, M_n, is much larger than M_c, the equation can be simplified to[373] $G = \rho RT/M_c$.

For hydrogels, which can be considered as swollen networks, the relationship between the shear modulus and the cross-link density, ν_e (mol/m^3), is often expressed as $G = \nu_e \Phi^{1/3} RT$, where Φ is the polymer volume fraction. Consequently, the relation between M_c and ν_e can be given by $\rho/M_c(1 - 2M_c/M_n) = \nu_e \Phi^{1/3}$.[374]

5.2. Mesh Size

Because the mesh size controls the maximum solute size that can diffuse through the gel, it is an important parameter to understand the release mechanism of entrapped proteins. The mesh size or correlation length (ξ) is polymer concentration dependent and is defined as the distance between two adjacent cross-links. The mesh size can be calculated using rubber elasticity theory from the number average molecular weight between cross-links, M_c as $\xi = (6M_c/\pi\rho N_A)^{1/3}$ with N_A Avogadro's number.[373] For cross-linked networks swollen in aqueous solution, also the following equation for the mesh size is often used: $\xi = \nu_{2,s}^{-1/3}(r_0^2)^{1/2}$ where $\nu_{2,s}$ is the polymer volume fraction in the swollen gel.[257] This equation is mostly used for hydrogel networks based on relatively simple linear polymers chains such as photopolymerized PEG-diacrylates for which the end-to-end-distance of the polymer chains, r_0^2, can be calculated easily from their chemical structure.[375,376]

Both rheological and swelling properties give information about the gel characteristics, but only average values for the mesh size can be obtained. However, heterogeneities in pore sizes can play an important role in the protein release kinetics. Other techniques have been used to explore the average mesh size and their size distribution over hydrogels. Scanning electron microscopy (SEM) is often used to investigate the morphology of hydrogels, even though the fast drying process is known to influence the morphology.[377,378] In the hydrated state, different techniques have been used to indirectly measure the mesh size by studying the diffusion of molecules within the gel such as confocal laser scanning microscopy (CLSM),[379] nuclear magnetic resonance (NMR),[380,381] fluorescence,[382,383] or scanning electrochemical microscopy (SECM), as reported recently by Jeerage et al.[378]

5.3. Protein Release Mechanisms: Background

As described in section 1, traditionally, protein therapeutics are administered parenterally upon reconstitution. The drug pharmacokinetics depend on the site of administration (intravenous, subcutaneous, intramuscular, etc.), the physicochemical properties (solubility, molecular weight, isoelectric point, etc.), and the elimination rate (via hydrolytic or enzymatic degradation or simply by kidney excretion). Advanced controlled delivery systems aim to improve the unfavorable protein pharmacokinetics, enhancing their therapeutic effect. The fluctuating plasma drug concentrations observed with traditional repeated bolus injections are avoided by the use of controlled releasing hydrogels that are potentially able to maintain drug levels within the therapeutic window, overcoming risks associated with potentially toxic or ineffective drug concentrations.

Generally speaking, the main mechanisms governing the protein release from hydrogels are diffusion and surface erosion. The Ritger–Peppas equation is often used to fit release data and determine which mechanism is responsible for release: $M^t/M^\infty = kt^n$, with M^t/M^∞ being the fractional drug release at time t. The constant k is a kinetic constant incorporating structural and geometric characteristics of the device.[384,385] If $n = 0.5$, the release is governed by Fickian diffusion. If $n = 1$, molecules are released by surface erosion, while both mechanisms play a role if n has a value between 0.5 and 1. This semiempirical power law equation was compared with different mathematical drug release models by Siepmann and Peppas.[386,387] Amsden also evaluated different models to describe solute transport in hydrogels taking different gel characteristics, such as polymer flexibility, into account.[388] To decide which mathematical model for drug release is the most suitable depends on the specific type of drug delivery device, its geometry, drug properties, and excipient type.[387]

The release mechanism depends on both the characteristics of the polymeric network and the protein. When the hydrogel pores are bigger than the hydrodynamic radius of the protein, diffusion is the driving mechanism for release, with a diffusion rate depending on the protein size. When instead the hydrogels pores are smaller than the protein radius, swelling or erosion/degradation (bulk or surface) are needed for release. Deviation from this behavior is observed when the hydrogel is triggered to swell/shrink by a specific stimulus, as described in other sections of this paper or when the polymer and protein interact via noncovalent interactions (i.e., electrostatic, hydrophobic interaction). The release of such hydrogels depends on the dissociation rate of the protein from the polymer matrix.

5.4. Diffusion-Controlled Release

Many of the gel matrices reported to date exhibit diffusion-controlled release, following Higuchi's kinetics, implying that the release is proportional to the square root of time.[389]

The protein release profiles can be generally fine-tuned in order to meet their specific medical needs. One of the most commonly used methods to modulate release is tailoring the hydrogel cross-link density. In this respect, synthetic polymers offer several advantages as compared to their natural counterparts, as both the polymer architecture and its chemical structure can be easily modulated.

Hiemstra et al. demonstrated that eight-arm poly(ethylene glycol)–poly(L-lactide) [PEG–(PLLA)$_8$] and poly(ethylene glycol)–poly(D-lactide) [PEG–(PDLA)$_8$] formed in situ gelling stereocomplex hydrogels suitable for the delivery of proteins. Lysozyme was released in vitro by diffusion in 16 days.[390] Subsequently, the same research group developed a hydrogel system based on dextran vinyl sulfone conjugates (dex–VS) cross-linked by Michael addition with tetrafunctional mercaptopoly(ethylene glycol) (PEG-4-SH). The release of several proteins [lysozyme, BSA, IgG, and basic fibroblast growth factor (bFGF)] was studied from hydrogels of different polymer concentrations. While a diffusional release was observed for lysozyme, BSA, and bFGF with release rates dependent on polymer concentration and protein size, IgG followed biphasic release, which was ascribed to diffusion during the initial phase (10 days) and to degradation during the following phase.[391]

BSA release was investigated from a thermogelling tricomponent multiblock poly(ether ester urethane)s consisting of poly[(*R*)-3-hydroxybutyrate] (PHB), poly(propylene glycol) (PPG), and poly(ethylene glycol) (PEG) based hydrogel by Li et al. It was demonstrated that BSA was released in a sustained manner for over 70 days; the first stage of release was diffusion-

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controlled, whereas the later stages were governed by erosion of the matrix.[392]

In situ gelling thermosensitive hydrogels formed by mixing chitosan solutions and glycerol-2-phosphate (β-GP) have been described for the delivery of insulin. The protein was released in vitro in 2 weeks by a diffusion-governed mechanism.[393]

Diffusion-controlled protein delivery was also observed by Van Tomme et al. from a self-assembled macroscopic hydrogel based on oppositely charged dextran microspheres. Encapsulated lysozyme, BSA, and IgG were released according to their molecular weight on a time scale from 20 to 60 days. The release was primarily diffusion-controlled during the entire release period for the studied proteins.[394]

A diffusion release behavior from photopolymerized thermosensitive methacrylated p(HPMAm-lac)−PEG−p-(HPMAm-lac) hydrogels was reported by Censi et al.[317] Model proteins [lysozyme, BSA, and immunoglobin G (IgG)] were quantitatively released in vitro, according to first-order kinetics, in 2−16 days, depending on protein size and polymer concentration (Figure 17). Diffusion was not governed by

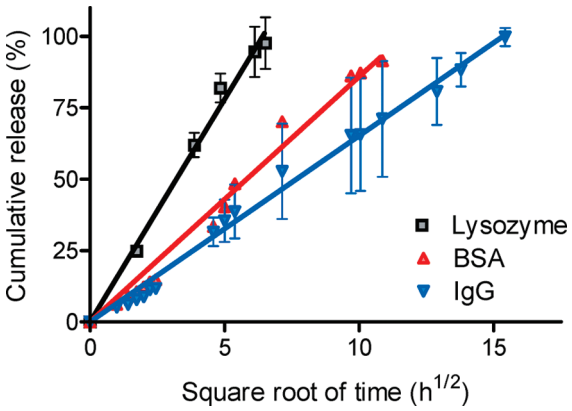

Figure 17. Diffusion-controlled release of model proteins (lysozyme, BSA, and IgG) ranging in molecular weight between 14 and 150 kDa from photopolymerized thermosensitive p(HPMAm-lac)−PEG−p-(HPMAm-lac) of 20 wt % polymer concentration. Reprinted with permission from ref 317. Copyright 2009 Elsevier.

swelling and degradation, which occurred on a longer time scale than the release. The diffusivity of the protein, calculated from release profiles according to Fick's second law, decreased due to the hydrogel matrix 9−20 times as compared to protein's diffusion in water.

In a subsequent study, the same group studied how to fine-tune the protein release by changing the molecular structure of the polymer. The influence of PEG's molecular weight and the extent of methacrylation on the cross-linking density of the hydrogels and BSA release was explored. The release of BSA could be extended up to 2 months by increasing the methacrylation extent and PEG molecular weight. Again, the release mechanism was diffusional, however, the hydrogels, having a relatively hydrophobic character, showed biphasic protein release, likely from the gel hydrophilic and hydrophobic phases, respectively.[247] Surprisingly, the protein release rate decreased with increasing PEG molecular weight. This finding is in contrast with previously published studies. Hubbell et al. in their pioneering work on photopolymerized hydrogels based on PEG and α-hydroxy acid showed that the diffusivity of proteins in the hydrogels decreased with PEG molecular weight, and protein release was governed by a combination of diffusion and degradation. It was reasoned that the PEG molecular weight determined the hydrogel mesh size.[128,136] In the hydrogel studied by Censi et al.,[247,317] a different polymer assembly was observed, as the hydrogel was cross-linked by a tandem method: hydrophobic interactions of the thermosensitive p(HPMAm-lac) chains, followed by subsequent photopolymerization. The PEG molecular weight was varied whereas the thermosensitive chain length was kept constant, implying that at body temperature the hydrogels of shorter PEG blocks had a greater hydrophobicity, as compared to analogues of longer PEG molecular weight. As a result, a more extensive phase separation with formation of bigger hydrophilic pores was observed for shorter polymers. The diffusivity of BSA was higher in hydrogels with higher porosity, which was confirmed by confocal laser microscopy studies (CLSM) that revealed the existence of bigger hydrophilic micropores in hydrogels of shorter PEGs. Differences in hydrogel inner structure were clearly visible, as shown in Figure 18. CLSM emerged in this work, as well as in a paper by Vermonden et al.,[318] as a powerful technique to investigate the hydrogel's internal structure, especially in systems where phase separation into hydrophilic and hydrophobic domains was observed.

5.5. Degradation-Controlled Release

Stereocomplex PEG−$(PLLA)_8$/PEG−$(PDLA)_8$ based hydrogels that showed diffusion-controlled release when loaded with lysozyme, released IgG (MW = 150 kDa) in 20 days with nearly zero-order kinetics, meaning that the initial mesh size of the hydrogel was bigger than the hydrodynamic radius of lysozyme and smaller than that of IgG, which needed matrix degradation to be released. Similarly, the release of interleukin-2 (IL-2), investigated both in vitro and in vivo, showed degradation mediated kinetics for 10 days.[390]

Pluronics gels have been used to encapsulate and release proteins, such as insulin[288] and IL-2.[395] In both studies, zero-order kinetics were observed. However, the major shortcomings of these gels, as for many other physical hydrogels, are their weak mechanical strength, rapid erosion, and fast release of the therapeutics from the gel networks.[282]

A constant release rate of human insulin over 2 weeks was observed in vitro from injectable PLGA−PEG−PLGA (ReGel) systems, but incomplete release was obtained.[303] This drawback was overcome by addition of 0.2% (w/v) zinc, resulting in a release of 90% of the loaded insulin. A similar study was conducted with the incretin hormone glucagon-like peptide-1 (GLP-1), which was released in vitro from ReGel over 5 days. Extended zero-order release to over 2 weeks without burst effect was observed using zinc-complexed GLP-1.[396]

Another example of an insulin-releasing hydrogel is based on PAE−PCL−PEG−PCL−PAE.[336] Insulin was loaded into the matrix, forming an ionically linked insulin−PAE complex. An in vitro study showed an almost zero-order release for up to 20 days. The in vivo efficacy of insulin-loaded gels was also assessed by implanting them subcutaneous in both healthy and STZ-induced diabetic rats. It was shown that insulin was maintained at a constant steady-state level for 15 days in healthy rats and further that insulin levels were controlled by the amount of insulin loaded into the copolymer and the copolymer concentration in the hydrogel. Blood glucose and plasma insulin levels of diabetic rats showed an efficacy of the delivery system for more than 1 week with a single injection.[397]

Surface-eroding self-assembled hydrogels based on PEG/chol and PEG/βCD, showing nearly zero release of lysozyme, BSA,

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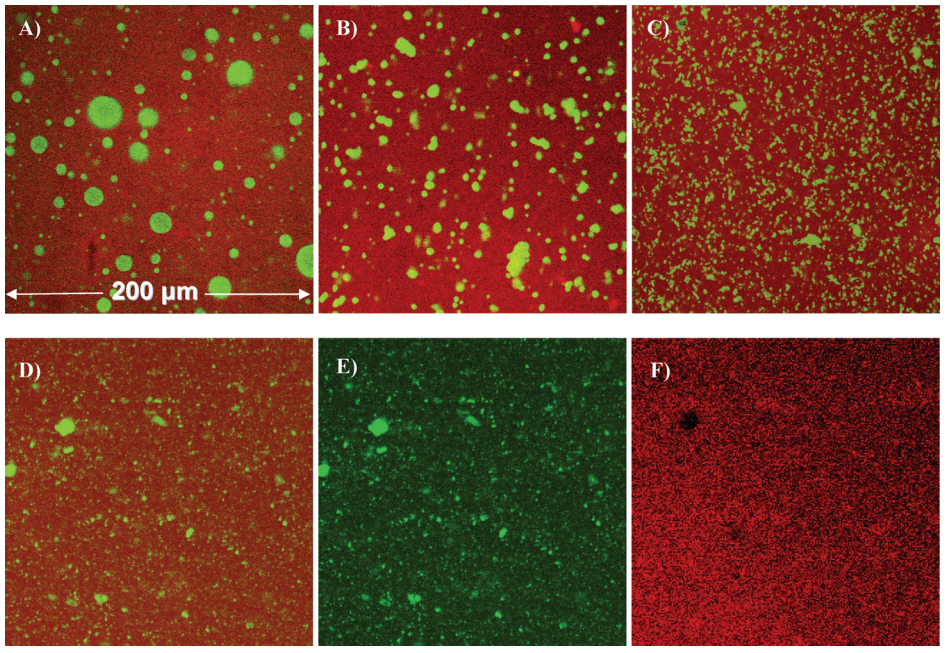

Figure 18. Confocal laser scanning microscopy pictures of photopolymerized p(HPMAm-lac)−PEG−p(HPMAm-lac) triblock copolymer hydrogels of different PEG molecular weight and constant p(HPMAm-lac) length. Hydrogels were double stained with hydrophobic Nile Red (in red) and hydrophilic fluorescein isothiocyanate FITC−BSA (in green) and microscopic pictures of hydrogels (35 wt % solid content) composed of (A) PEG 4 kDa, (B) PEG 10 kDa, (C) PEG 20 kDa, and (D) PEG 40 kDa were taken. Pictures E and F show hydrogels of PEG 40 kDa stained with only FITC−BSA and NR, respectively. Reprinted with permission from ref 247. Copyright 2010 American Chemical Society.

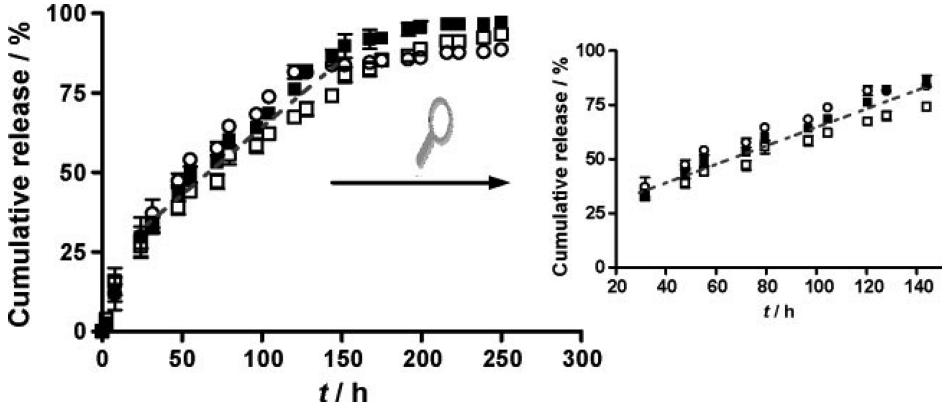

Figure 19. Surface-eroding hydrogels composed of multiarm PEG−cyclodextrin/PEG−cholesterol inclusion complexes. Almost zero-order release kinetics of model proteins (lysozyme, BSA, and IgG). Reprinted with permission from ref 398. Copyright 2009 Wiley-VHC.

and IgG, were investigated by van de Manakker et al. Regardless of the polymer molecular weight the proteins were released in vitro in approximately 200 h (Figure 19). The release, however, could be tailored by the polymer content of the hydrogels.[398]

5.6. Modulating Protein Release

Although significant progress has been achieved in the development of injectable biodegradable polymeric hydrogels, some challenges still remain. Initial burst or very fast release, which is observed in many protein releasing hydrogels both in vitro and in vivo, are limiting factors for many applications. In vivo burst release may be ascribed to the rate of gelation of injectable in situ gelling systems. When the sol−gel transition is not immediate, premature leakage of the protein in the surrounding tissue prior to complete gelation might occur. Burst or fast release can be also ascribed to hydrogel network defects or inhomogeneities as well as high diffusivity.

Some approaches to improve hydrogel performances and overcoming burst and fast release have been proposed. Synthetic flexible polymers like the photopolymerizable thermosensitive p(HPMAm-lac)−PEG−p(HPMAm-lac) triblock, investigated by Censi et al. and described in section 4.2, showed the capability to tailor the protein release profiles by designing modular polymer structures and extend the release time according to the aimed pharmacokinetics of the drug.[247]

Another successful strategy is the introduction of functionalities in the polymer structure like charged groups or binding sites, which can prevent fast protein diffusion by polymer interaction. One example of such system has been described in section 4.3. The release mechanism of proteins from PAE−PCL−PEG−PCL−PAE hydrogels depends on two concomitant factors, the ionic interactions between partial positive charges in PAE blocks and negative charges in insulin and degradation of the PAE blocks.[397]

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The RADA16 peptide, self-assembled by formation of β-sheet structures, first reported by Zhang[399] has been used to encapsulate and deliver several proteins for intramyocardial delivery. This peptide has also been used to deliver platelet-derived growth factor BB (PDGF-BB),[400] stromal cell-derived factor-1 (SDF-1),[401] and insulin-like growth factor I (IGF-I)[402] to decrease myocardial infarct. Also in this case, the slow and controlled release of the active proteins is due to the amphiphilic nature of the self-assembling peptide that interacts with loaded proteins, which slows down diffusion and subsequent release kinetics.

Similarly, the release of FITC−albumin from polyphosphazene hydrogels was controlled using chitosan[393] and was sustained over 2 months without a burst in the presence of chitosan, in contrast to the observed release over 1 month from gels without chitosan. The prolongation of release time was ascribed to the formation of an ionic complex between chitosan and FITC−albumin. The polyphosphazene hydrogel from Park et al. has also been used to entrap pancreatic islets,[324] which is an alternative way to overcome burst and uncontrolled release of insulin, as the protein release rate depends on the capability of cells to stay viable and produce the protein. In comparison with both rat islets entrapped in other hydrogels and free islets, rat islets in the polyphosphazene hydrogel retained higher cell viability and showed insulin production and consequently release of this protein from the gel for over a 28-day culture period. In a subsequent study, polyphosphazene hydrogels were used to encapsulate hepatocytes as spheroids or single cells.[403] Over a 28-day culture period, the spheroid hepatocytes maintained a higher viability and produced albumin, whereas single hepatocytes showed lower levels of albumin secretion from the hydrogel.

An alternative approach to prevent burst release is the combination of two delivery systems in one composite matrix. For example, Leach et al. developed a photopolymerizable (PEG−)glycidyl methacrylate−hyaluronic acid [(PEG−)GM−HA] that showed remarkable diffusivity, leading to fast release of BSA (approximately 60% within 6 h). The duration of the release could be prolonged to a certain extent by increasing the polymer concentration, but the longest duration of release (up to several weeks) was achieved by incorporating BSA−PLGA microparticles within the hydrogel matrix (Figure 20).[142]

5.7. Methods To Measure Release Kinetics

The method generally applied to evaluate the release from gel-based drug delivery systems relies on release studies. When release curves show a square root dependency on time and the hydrogel matrix remains constant in size and geometry during the experiments, the diffusion coefficient of encapsulated drugs can be calculated.[384] However, these experiments are time-consuming and sometimes poorly predictable because high variation among results can be observed depending on the method used for release studies in vitro (geometry of the dosage form, sampling method, volume of acceptor medium, swelling/degradation/erosion of the hydrogel matrix, etc.). An emerging technique to investigate the mobility of molecules embedded in hydrogel matrices is an analytical method named fluorescence recovery after photobleaching (FRAP).

FRAP experiments are performed on hydrogels loaded with fluorescently labeled protein, which is photobleached in small regions (typically 10−50 μm^2) within the gel using an optical microscope equipped with a light source. After bleaching the probe molecules, the fluorescence intensity within the bleached

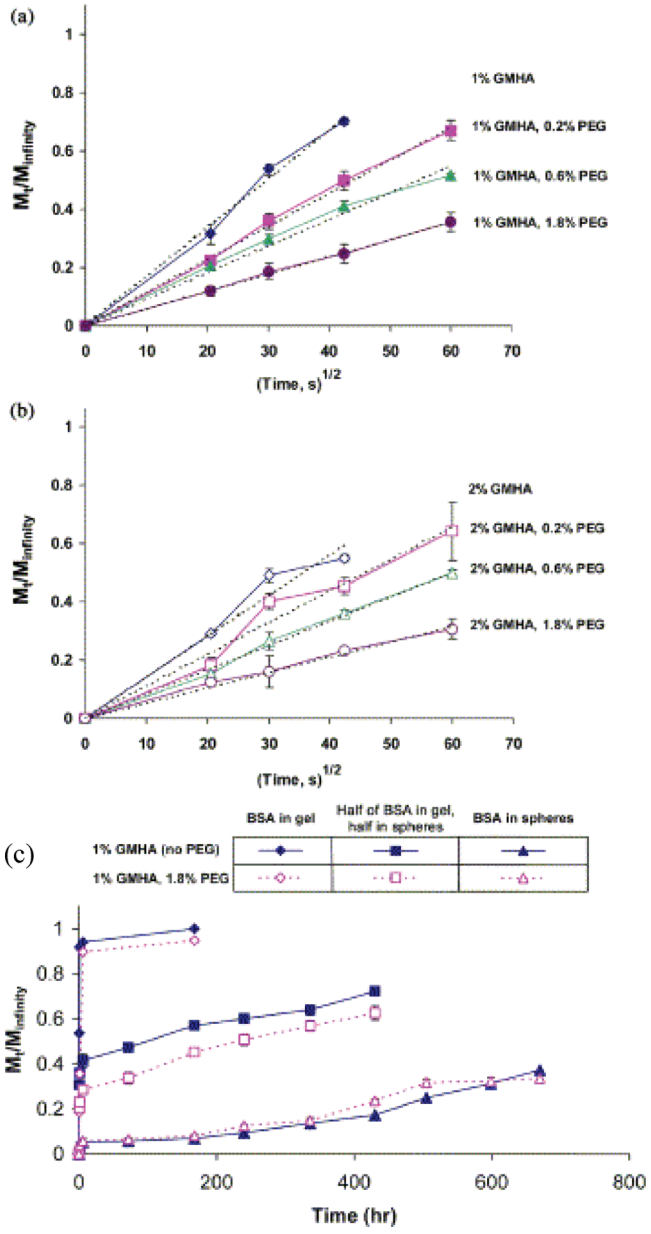

Figure 20. BSA release profiles from photo-cross-linked (PEG−)-glycidyl methacrylate−hyaluronic acid hydrogels. The fast diffusive release of the protein from the hydrogels could be retarded by increasing PEG and GMHA concentration (a, b), while the incorporation of BSA-encapsulating PLGA microparticles allowed extension of the release time up to several weeks (c). Reprinted with permission from ref 142. Copyright 2005 Elsevier.

region recovers due to diffusion of unbleached molecules from the surroundings and the diffusion coefficient of the protein in the matrix can then be calculated from the resulting recovery profiles.[404] FRAP experiments have also been used to evaluate the mobility of molecules in cells and biological tissues; for example, Braeckmans et al. explored the mobility of macromolecules in bulk three-dimensional biological materials, such as vitreous body isolated from bovine eyes and lung sputum expectorated by cystic fibrosis patients.[405] Recently, several research groups characterized hydrogel-based drug delivery systems by FRAP. De Smedt et al. were pioneers in combining confocal laser scanning microscopy (CLSM) and FRAP to study localized diffusion coefficients in hydrogels and relate

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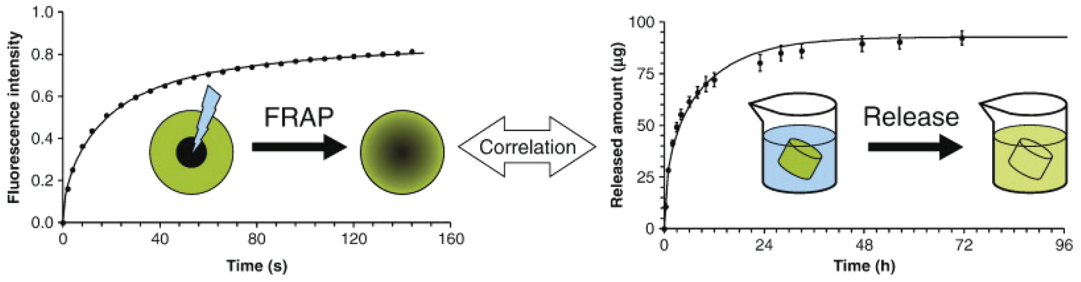

Figure 21. Schematic comparison between the classical approach of release study method and the FRAP technique. Reprinted with permission from ref 412. Copyright 2010 Elsevier.

mobility of FITC−dextrans to the rheological properties of the gels.[406] Vermonden et al. synthesized a series of thermogelling p(HPMAm-lac)−PEG−p(HPMAm-lac) triblock copolymer hydrogels containing PEG's middle blocks of different molecular weight and used FRAP analysis to investigate how macromolecular diffusion can be controlled according to polymer design, concentration, and temperature. FITC−dextrans were used as fluorescent probe and the results revealed that its diffusivity decreased with increasing polymer concentration, temperature, and PEG's molecular weight.[407] In another study, Branco et al. incorporated FITC−dextrans of different molecular weight in peptide-based hydrogels, formed by peptide self-assembling in response to pH and ionic strength by formation of amphiphilic β-hairpins. Dextran mobility within and release from hydrogels of varying solid content was studied and it was found that the release was influenced by diffusion and charge interaction between dextran and peptide. Moreover, the results observed in bulk release experiments correlated very well those obtained by FRAP.[408]

Similarly, the possibility to tailor the molecular architecture of galactomanna hydrogels as well as guar−drug conjugates to entrap and limit the diffusion of model drugs was researched by Burke et al. Also, in this study FRAP experiments were a valuable tool to study to which extent the hydrogel matrix was able to restrict the drug mobility and a fast screening method to design formulations with extended release profiles.[409]

Kuijpers et al. performed parallel FRAP and bulk release studies to investigate the mobility of lysozyme in gelatin−chondroitin sulfate hydrogels containing 5, 10, and 20% of chondroitin sulfate. The results of lysozyme release experiments, which revealed that release was governed by diffusion and electrostatic interactions between the protein and the hydrogel matrix, were confirmed by FRAP analysis. These studies showed that the combination of chondroitin sulfate with cross-linked gelatin gels led to a significant increase in the lysozyme loading capacity of the gel and a prolonged release time (by charge interaction).[410] A study by Norde et al. using oxidized potato starch polymer microgels showed that FRAP experiments are indeed very suitable to reveal the correlation between electrostatic interactions and the mobility of lysozyme in hydrogels.[411]

A good correlation between FRAP and release data was found by Van Tomme et al. and Censi et al., whose hydrogel systems[247,317,394] are described in others sections of this paper. Surface-eroding hydrogels, releasing the drug with zero-order kinetics, showed immobile proteins by FRAP.[398]

A more systematic comparative study between the measured diffusion coefficients by FRAP and the release kinetics was carried out recently by Brandl et al. (Figure 21).[412] They used gels prepared by step-growth polymerization of PEG, loaded with FITC-labeled dextrans. The translational diffusion coefficients of the incorporated FITC−dextrans were measured by FRAP and pulsed field gradient NMR spectroscopy. Because the determined diffusion coefficients agreed well with those obtained from release studies and predicted mobility from mechanical testing, FRAP and pulsed field gradient NMR spectroscopy were proposed as alternatives to release experiments.

However, although FRAP always reflects very well the relative differences in macromolecular diffusivity between hydrogels of different composition and cross-linking degree, the technique has some limitations. Quantitative correlation between release experiments and FRAP is not always possible, as they rely on remarkably different setups. The time scales of FRAP and release experiments are totally different (FRAP monitors protein diffusivity for minutes while release experiments are performed over days to weeks); moreover, FRAP experiments measure diffusion coefficients on a microscale level, unlike release experiments that provide data on the macrodiffusivity. Furthermore, swelling processes, often influencing the diffusion of entrapped molecules, can occur only when the gel is exposed to aqueous medium in a release experiment setting; FRAP does not take swelling/matrix degradation/erosion into account. Thus, FRAP can be used as a complementary technique to release experiments to rapidly and qualitatively evaluate the potential of newly developed drug delivery systems for controlled release purposes.

5.8. Protein Stability

The potential of drug delivery systems to enter the clinic and make an impact on patient's life strictly depends on their ability to release *active* proteins, besides providing their sustained release. Therefore, the assessment of protein stability needs to be implemented in the evaluation of hydrogel-based delivery systems, in order to certify the pharmacological drug activity and the lack of immunogenicity. The stability of the protein has to be maintained during hydrogel preparation, storage, and release.

The maintenance of protein's native structure still represents an issue for many hydrogel formulations, as very often incomplete release due to aggregation, chemical binding between protein and polymer, oxidation, deamidation, etc. might occur.[413]

As already mentioned, it was reported that BSA loaded in chemically cross-linked hydrogels by radical polymerization covalently coupled to the polymer, due to the role of BSA as chain transfer agent during the polymerization.[135] Cadee et al. showed that oxidation of recombinant human interleukin-2 (rh-IL2) plays an important role when dextran-based hydrogels were cross-linked using potassium peroxodisulfate (KPS). The

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extent of oxidation, however, could be reduced significantly upon addition of an antioxidant.[414] In a recent study, it was shown that postloading of gels with proteins exploiting reversible charge interactions is a suitable method to avoid their unwanted chemical modification.[415]

Protein loading into polymer matrices can result in loss of the 3D structure (protein denaturation). Denatured protein molecules can form larges aggregates, the structures of which can finally induce an immunological response, e.g., the formation of antibodies that bind to and sometimes neutralize the activity of the native therapeutic protein. It was shown that aggregation of recombinant human interferon β (rhIFN-β) plays a major role in the immunogenicity during the treatment of multiple sclerosis.[416] Although protein denaturation and aggregation have been shown to occur in protein/PLGA formulations,[417] hydrogel matrices, due to their high water content, have a better compatibility with proteins compared to the relatively hydrophobic polymer matrices like PLGA. Furthermore, the mobility of proteins in hydrogel matrices is limited, which contributes to the stability of the entrapped protein pharmaceutical. Indeed, in many papers it has been shown that the structure and bioactivity of proteins released from hydrogels were retained. Therefore, generally speaking, proteins entrapped in hydrogel matrices are less susceptible for denaturation/aggregation than in other types of matrices.

A number of complementary techniques to evaluate the structural changes of proteins are available, and several of those need to be combined in order to obtain a full characterization of the protein stability. Liquid chromatography (HPLC, SEC) is one of the fundamental methods to investigate possible changes in the primary structure of the protein, including oxidation, deamidation, or the presence of (ir)reversible aggregates. Also mass spectrometry gives information on changes in the primary structure.[418]

Other techniques of interest, aimed at characterizing structural changes in protein pharmaceuticals, are Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), fluorescence spectroscopy, far and near circular dichroism (CD), etc. and they are reviewed in more specialized papers.[419]

6. HYDROGEL/PROTEIN FORMULATIONS UNDER PRECLINICAL AND CLINICAL DEVELOPMENT

In this section, we provide an overview of the hydrogel systems that are taken into development by industries and that are in advanced preclinical or clinical studies or on the verge of commercialization. Although significant efforts were made to compile a comprehensive overview, it cannot be ruled out that some technologies currently in the translational step from academia to industry have been missed.

Endo Pharmaceuticals is present on the market with a soft and flexible 12-month hydrogel implant named Vantas that delivers histrelin, a luteinizing hormone-releasing hormone (LHRH) agonist, for the palliative treatment of advanced prostate cancer. The implant consists of a histrelin acetate drug core inside a nonbiodegradable cylindrically shaped hydrogel reservoir. The hydrogel reservoir is a hydrophilic polymer cartridge composed of 2-hydroxyethyl methacrylate, 2-hydroxypropyl methacrylate, trimethylolpropane trimethacrylate, benzoin methyl ether, Perkadox-16, and Triton X-100.[420] The same technology is used for the product Suprelin LA, a histrelin-releasing hydrogel implant used for the treatment of children with central precocious puberty. Currently, Endo Pharmaceuticals is conducting implant phase III clinical trials on octreotide implants (9 months release) for the treatment of acromegaly[421,422] and phase II clinical trials on octreotide implants for carcinoid syndrome.[423] Although well-tolerated and biocompatible, Endo's implants are nonbiodegradable and the removal of the exhausted device is consequently needed. Surgical explantation of the empty device can be circumvented using more advanced systems based on biodegradable polymers.

The first example of an injectable in situ forming hydrogel is a thermosensitive hydrogel by Protherics Salt Lake City, Inc., named ReGel. The company was formerly known as MacroMed, Inc. and changed its name to Protherics Salt Lake City, Inc. after its acquisition by Protherics plc (January 2007). Currently, Protherics Salt Lake City, Inc. operates as a subsidiary of BTG plc. ReGel is based on a triblock copolymer of PLGA−PEG−PLGA and is administered as a free-flowing liquid upon reconstitution. It was reported that the phase transition at body temperature led to some degree of volume shrinkage, contributing to an initial burst release effect. The most advanced application of ReGel is the delivery of paclitaxel for the local management of breast and esophageal cancer. A joint effort with Diatos SA made it possible to bring ReGel-based formulation of paclitaxel (Oncogel) up to phase II/b clinical trials. Recently, ReGel extended its use to protein delivery with two registered products, Cytoryn and hGHD-1, that have yet to undergo clinical trials. Cytoryn is a depot formulation that combines ReGel technology with commercially available IL-2, proleukin (Chiron) in a dual syringe administration device and is indicated for the treatment of renal carcinoma and melanoma (peri/intratumoral administration). The fully bioactive protein was released in a 3−4-day period and weekly administration of Cytoryn demonstrated in animal models to improve tumor stasis and survival and to have negligible side effects as compared to conventional IL-2 administration.[424] hGHD-1 is a novel injectable depot formulation of human growth hormone (hGH) utilizing the ReGel drug delivery system for the treatment of patients with hGH deficiency.[425] Other protein-based compounds formulated in ReGel and described in literature are Zn−insulin[303] and glucagon-like-peptide (GLP-1).[396]

H.P. Acthar Gel by Questcor is a depot formulation of the adrenocorticotropic hormone (ACTH) in 16% porcine gelatin to provide a prolonged release after intramuscular or subcutaneous injection. The formulation is currently approved in the United States for the treatment of acute exacerbations of multiple sclerosis, nephrotic syndrome, infantile spasms, and 15 other diseases and disorders. Acthar is administered every 24 or 72 h and provides a longer circulation time for ACTH, which is normally eliminated in man in approximately 15 min.[426]

MedinCell, a start-up company founded in 2002 and based on the drug delivery research of Michael Vert, is developing MedinGel, a proprietary technology for the subcutaneous and intramuscular controlled release of peptides, proteins, and small molecules. The hydrolytically degradable hydrogel matrices are constituted of a poly(lactic acid) (PLA) segments attached to a central poly(ethylene oxide) (PEO) block of various lengths. These triblock copolymers were prepared by ring-opening polymerization of lactide in the presence of PEG, using a nontoxic Zn metal or CaH_2 as co-initiator.[427] Hydrogels are prepared by introduction of water into organic solutions of copolymers with appropriate hydrophilic/hydrophobic and soft/hard segment ratios of PLA and PEO. Typically, the

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drug is added to a polymer solution in tetraglycol and water is subsequently added. The presence of water leads to phase separation and formation of reversible hydrogels, where the PLA segments are assembled into hydrophobic microdomains and PEO is responsible for the swelling of the three-dimensional networks. The inventors of this injectable technology claim that MedinGel is suitable for the controlled delivery of hydrophilic and/or hydrophobic drugs. Although some results on protein delivery in vitro have been published,[428] demonstrating release and retention of structural activity of BSA and fibrinogen, MedinGel did not reach clinical trials for any specific application.

The same research team that reported on a bioerodible thermogelling material (ReGel) synthesized a polymer displaying thermosensitive properties by aliphatic modification of a biodegradable triblock copolymer based on PEG as middle block and poly(L-lactide-*co*-caprolactone) as side chains. The thermosensitivity of the polymer is ascribed to the aliphatic modification.[429] Preliminary studies in vivo demonstrated that the thermogel is formed after subcutaneous administration in mice and that the depot was stable for 2 weeks. Upon acquisition of the license by InGell, a recently established company located in The Netherlands, this technology was named InGell Gamma and is being developed as a controlled delivery system for small molecule drugs and peptides. Other hydrogel delivery platforms, initially developed by Hennink et al. and later taken into product development by Octoplus (OctoDex) and InGell (InGell Delta 1 and 2), are based on dextran. InGell Delta 1 is composed of a dextran backbone grafted with either D- or L-oligolactate chains containing 5−15 lactic acid units. The depot formation is driven by stereocomplexation and preclinical studies performed on tumor-bearing mice demonstrated the suitability of this hydrogel for the controlled delivery of IL-2. The density of oligolactates and the grafting density allowed tailoring of the release and degradation rate. The release of the encapsulated protein was dependent on diffusion.[430]

In a hydrogel system first described by Hennink et al., dextran (dex) backbone was derivatized with hydroxyethyl methacrylate (HEMA) moieties for the preparation of hydrogel-based and chemically cross-linked microspheres.[431,432] This gel system was named OctoDex by OctoPlus.

In comparison to PLGA microspheres, this delivery system was found more advantageous for labile molecules like proteins for a number of reasons. First, the preparation method avoids the use of organic solvents, as it relies on water in a water emulsion technique, where an aqueous solution of modified dextran polymer, polymerization agents, and protein drug is emulsified in a continuous PEG phase. The chemical cross-linking of the particles by radical polymerization using potassium peroxydisulfate (KPS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) leads to the formation of a three-dimensional network that entraps the protein. Second, the hydrophilicity of the microspheres allows overcoming stability issues associated with the use of hydrophobic polymers like PLGA. Finally, it has been shown that OctoDex formulations are burst-free, and no acidification of the particle microenvironment and autocatalyzed degradation of the polymer were observed. Several proteins were encapsulated in OctoDex microspheres, and their release was studied in preclinical studies. Specifically, IgG was used as a model protein, and tailorable release profiles in vitro as a function of cross-link density were reported. IL-2 in OctoDex microspheres was administered intratumorally in mice and compared to five intratumoral injections of free IL-2. For both treatments, having the same total IL-2 dose, similar survival rates were observed.[433] The feasibility of the formulation of larger structures like liposomes in OctoDex was also demonstrated.[434] Typically, swelling and degradation of the particles control the release mechanism. OctoDex microspheres loaded with human growth hormone were studied in mice and man. Single subcutaneous administration of the microspheres in mice resulted in a good correlation between hGH released in vitro and in vivo effects. Administered to healthy volunteers, it produced an increase (over 7−8 days) in hGH serum concentrations. A good in vitro/in vivo correlation of release and increased serum concentration of biomarkers [insulin-like growth factor-I (IGF-I), IGF binding protein-3 (IGFBP-3)] was found, indicating that bioactive hGH was released from the microspheres.[435]

An advanced hydrogel delivery platform is PolyActive, which is under development by OctoPlus. This biodegradable polymeric system is based on a series of poly(ether ester) multiblock copolymers composed of PEG and poly(butylene terephtalate) (PBT). PolyActive is another example of a flexible polymer system of which the amount and length of the building blocks offer the possibility to customize the matrix characteristics to a specific delivery need. Similar to other polymeric systems, PolyActive can be processed into several formulations, including microspheres and gels. The PEG soft segment gives the material elastic properties, while the PBT hard segment acts as a cross-linker via physical interactions. The cross-links are reversible and form when the polymer is below the melting or glass transition temperature. The first biomedical application of PolyActive was in the field of tissue engineering. When it was found that the polymer supported calcium phosphate formation, PolyActive was commercialized as bone cement restrictor (SynPlug) by IsoTis in 2001. PolyActive attracted also interest as a drug delivery material. A number of model and therapeutically relevant proteins were encapsulated and released from PolyActive both in vitro and in vivo with excellent correlation. Research also showed evidence for maintenance of protein stability and activity during formulation and release. Some factors have been identified as release modulators. For instance, lysozyme forms reversible aggregates when encapsulated in PolyActive and its release is affected by deaggregation dynamics. It has also been reported that protein−polymer interaction and polymer degradation influenced the release.[436−438] The degradation of the matrix occurs mainly by hydrolysis of the ester bonds, and higher weight fractions of hydrophobic blocks result in slower degradation rates. As for other described technologies, due to the hydrogel-like nature of this system, the acidic degradation products are more rapidly extracted from the degrading matrices than, for example, from PLGA microparticles.

PolyActive in the form of microspheres emerged as an interesting candidate for the formulation of cytokines. The protein is encapsulated and the microspheres are formed using the W/O/W double emulsion method. The amphiphilic nature of the polymer stabilizes the emulsion during the preparation process, and it was claimed that the presence of PEG in the aqueous phase stabilizes the protein.

IFN-α2b-loaded PolyActive microspheres, named Locteron, were designed by OctoPlus as a 2-week controlled delivery system for the treatment of hepatitis C. A phase I clinical trial was reported in 2008[439] and this formulation recently

completed the dose-finding phase IIb clinical trial by Biolex Therapeutics. Currently, hepatitis C is commonly treated with IFN-α2b, a cytokine having a half-life of 3 h. As a result of its short circulation time, high dosing and frequent administrations are required, which are associated with severe side effects such as fatigue and influenza-like-illness that necessitate discontinuation of the therapy.[440] A common approach to overcome such drawbacks is to use pegylated IFN-α2b (i.e., PEG-Intron or Pegasys), which assures longer half-life and a once weekly administration regime. Locteron promises to further improve therapeutic outcomes as compared to pegylated IFN, as its controlled release mechanism results in the gradual delivery of interferon-α2b to patients over the duration of 2 weeks, avoiding the early peak plasma levels of the active interferon that characterize the pegylated interferons. By reducing dosing and plasma peaks, Locteron has the potential to reduce the frequency and severity of flu-like symptoms and depression commonly experienced by patients treated with pegylated interferons. A press release from Biolex, highlighting the major results of Locteron's clinical trials, claimed that clinical data supported the expected product profile. The viral response rate to Locteron was comparable to that of the pegylated protein, while there was a statistically and clinically significant reduction in flu-like adverse events and depression. As a result of that, substantially reduced use of concomitant medications (analgesics and antipyretics) and lower therapy discontinuations due to adverse events were achieved.[441]

The French company Flamel developed a nanogel formulation named Medusa, which is a self-assembled poly(amino acid) nanoparticulate system particularly suitable for the extended release of a broad range of biologics (including proteins, antibodies, peptides, and vaccines) and of small molecules (injectable drugs). However, the release time-scale for Medusa technology can hardly go beyond 1 week. This delivery system is composed of an amphiphilic molecule consisting of hydrophilic poly(glutamic acid) and hydrophobic vitamin E. In water, self-assembly occurs, resulting in the formation of stable nanoparticles that can be stored as aqueous suspension or lyophilized. Medusa can be administered, upon reconstitution, using small needles and its preparation method avoids the use of W/O/W emulsion, allowing higher scalability of the product and a protein friendlier formulation procedure, as compared to microparticles. The nanogel has been proven to be safe and biodegradable and a Drug Master File for Medusa was filed by Flamel with the FDA in February 2011. Medusa protein-based products have been tested already in a number of clinical trials:[442]

- IFN-α XL, a long acting human interferon-α-2b (α2b) for the treatment of hepatitis C virus infection (HCV), has successfully completed two phase 1 trials and is currently in phase 2 study by Flamel (in comparison to Pegintron);
- IFN-β XL, a sustained release formulation of human interferon-β-1a (β1a) for the treatment of multiple sclerosis currently being tested in a multicenter phase 1 study by Merck-Serono;
- FT-105, a long-acting basal human insulin for the treatment of type I and II diabetes, has successfully completed a phase 1 study by Flamel (in comparison to Lantus);
- IL-2 XL, a long-acting human interleukin-2 for the treatment of renal cell carcinoma, for which proof-of-concept has been obtained in a phase 1/2 study by Flamel (in comparison to Proleukin).

Other Medusa-based products that are at preclinical stage and obtained proof-of-concept in animal model include

- hGH XL, a long-acting human growth hormone (hGH) for the treatment of growth disorders;
- GLP-1 XL, a long-acting human glucagon-like peptide-1 analog for the treatment of type II diabetes.

A concern related to the subcutaneous administration of protein-loaded nanogels is the possibility for these nanoparticles to penetrate the dermis, eliciting an immune response via dendritic cells. To our knowledge, no immunogenic events were reported for Medusa formulations, but especially when dealing with nanoparticulate systems, immunogenicity needs to be assessed for each formulated protein.

7. CONCLUSIONS

Protein-loaded hydrogels are studied with the aim to increase the therapeutic outcome and to improve patient compliance. Although many innovative and attractive hydrogel concepts have been published over the last 20−30 years, only a limited number of hydrogel/protein formulations has reached advanced preclinical and clinical evaluation. To bring these mainly academic concepts to industrial application and exploration, involvement of industry is essential. First, the very high costs associated with clinical trials cannot be carried by academic research groups. Second, for important issues like scaling up, sterilization (for protein/hydrogel formulations this means aseptic production), and reproducibility of production, industrial input and knowledge are required.

As a whole, the field of hydrogels for protein delivery is making important steps toward clinical application and is demonstrating to surpass other well-established technologies, like PLGA microparticles, which only product for the delivery of proteins (Nutropin Depot) was withdrawn shortly after release. Hydrogel's soft and hydrophilic nature and mild preparation methods seem to be particularly well-suited to enhance efficacy, reduce dosing interval, and provide a more convenient dosage route of large and labile proteins. To date, the proteins formulated in hydrogels that are paving their way to market are mostly cytokines and growth hormones.

The successful use of protein-loaded hydrogels for in vivo applications faces several technical challenges. On one hand, the high water content and soft nature of hydrogels is a key-feature for the successful formulation of proteins, but on the other hand, this characteristic typically results in relatively rapid release of proteins from the gel matrix over the period of hours or days, burst release, low mechanical strength, and short durability. The release profiles are generally much shorter than those that can be achieved using microspheres or macroscopic devices based on more hydrophobic polymers (for example, PLGA). Extensive research established that fast diffusion can be tackled by increasing polymer concentration or cross-linking density or applying chemical cross-linking methods. However, these strategies pose significant challenges for hydrogel application in vivo. The concentration of polymers is indeed often limited by the aqueous solubility of the gel precursors or the resulting high viscosity and poor injectability of the solutions. Furthermore, for some (chemical) cross-linking methods the reactive gel precursors have to be injected using double-barreled syringes or some other inconvenient devices.

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From the point of view of the living body, issues such as biocompatibility, biointegration, and medical safety are key considerations. Biocompatibility depends critically on the interactions that occur at the tissue/material interface. Such interactions depend on surface characteristics, chemical composition, physical nature, and degradation products of the material.[443] Optimizing and modulating these interactions represent major scientific challenges with clinical issues such as (avoiding) inflammation and foreign body response presenting particular problems.

Even when biocompatibility issues are solved, commercialization of a hydrogel-based delivery system is still not an easy endeavor. In general, advanced delivery systems are often victim of difficult scale-up processes, high manufacturing costs, and limited sales potential. Insurance companies are less likely to reimburse for novel and often costly formulations if a less expensive variation exists (i.e., pegylated proteins). Finally, a better understanding of the healthcare requirements and communication with investors, the medical profession, and the public will help expediting the commercial exploitation of hydrogels.

To conclude, although hydrogels hold potential in overcoming the unique formulation challenges of biotherapeutics, there is only a limited number of formulated protein and peptide drugs available. Nevertheless, the continuous progress in this field is likely to expand hydrogels' role in the biopharmaceutical market. Furthermore, with the advent of biosimilars (a biopharmaceutical or other biological product whose patent protection expired and can be therefore manufactured by a party other than the original developer using either identical or different manufacturing processes), the need for advanced delivery systems, including hydrogels, will become critical for the future branded products.

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Author Contributions

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Biographies

Tina Vermonden obtained her M.Sc. degree in molecular sciences at Wageningen University (The Netherlands). She obtained her Ph.D. degree in 2005 in the group of Prof. E. J. R. Sudhölter at the Laboratory of Organic Chemistry at Wageningen University with a thesis on supramolecular coordination polymers. In 2005, she joined the Department of Pharmaceutics at Utrecht University (The Netherlands) as a postdoc working on thermosensitive hydrogels as biomaterials. During an exchange project, she worked on the diffusion of macromolecules in hydrogels at the Department of Pharmaceutics at the University of Minnesota (Minneapolis, USA) in the group of Prof. R. A. Siegel in 2008. In 2009, she was appointed as Assistant Professor at the Department of Pharmaceutics in Utrecht. Her primary research interests are biomaterials for tissue engineering and drug delivery.

Roberta Censi was born in Fermo, Italy, in 1980. She studied Industrial Pharmacy at the University of Camerino, Italy, where she graduated with honors in 2006, and subsequently worked as a formulation scientist under the supervision of Dr. P. Di Martino. In 2007, she moved to The Netherlands to join Prof. W. E. Hennink's group (Utrecht University), where she completed her Ph.D. in 2010, working on the development of thermosensitive hydrogels for protein delivery and tissue engineering. She is currently working as a postdoctoral fellow on a joint project between the Universities of Utrecht and Camerino. Her main research interests include novel polymeric systems for pharmaceutical and biomedical applications.

Prof. Wim Hennink obtained his Ph.D. degree in 1985 at the Twente University of Technology (The Netherlands) with a thesis on biomaterials research. From 1985 until 1992 he had different positions in industry. In 1992, he was appointed as Professor at the Faculty of Pharmacy of the University of Utrecht. Currently, he is Head of the Department of Pharmaceutics, a position he took in 1996. From 1997 until 2004 he was European Editor of the *Journal of Controlled Release*. His main research interests are in the field of polymeric drug delivery systems. He has published ~400 papers and book chapters and holds 20 patents.

LIST OF ABBREVIATIONS

4EDMAB	4-*N*,*N*-dimethylaminobenzoate

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α/βCD	α/β-cyclodextrin
AA	acrylic acid
ACTH	adrenocorticotropic hormone
ADA	4,4′-azodibenzoic acid
APCs	antigen presenting cells
AZOB	azobenzene
bFGF	basic fibroblast growth factor
β-GP	glycerol-2-phosphate
BIS	*N*′,*N*″-methylenebisacrylamide
BSA	bovine serum albumin
CD	circular dichroism
CHP	cholesteryl group bearing pullulan
CLSM	confocal laser scanning microscopy
CTLs	cytotoxic T lymphocytes
CQ	camphorquinone
DCC	dicyclohexyl carbodiimide
dex	dextran
dex−VS	dextran vinyl sulfone
dex−HEMA−DMAE	methacrylated dextran−HEMA−dimethylaminoethyl
DMAEMA	*N*,*N*-dimethylaminoethyl methacrylate
DNA	DNA
DS	degree of substitution
DSC	differential scanning calorimetry
DTT	dithiothreitol
ECM	extracellular matrix
EPO	erythropoietin
EPR	enhanced permeation and retention effect
FDA	US Food and Drug Administration
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
FTIR	Fourier transform infrared spectroscopy
G	α-L-guluronic acid
G	shear modulus
GLP	glucagon-like peptide-1
GMHA	glycidyl methacrylate−hyaluronic acid
GMIDA	glycidyl methacrylate−iminodiacetic acid
GyrB	gyrase subunit B
HA	hyaluronic acid
HA−MA	methacrylated HA
HAse SD	hyaluronidase from *Streptococcus dysgalactiae*
HA−SH	thiolated HA
HEMA	(2-hydroxyethyl) methacrylate
hGH	human growth hormone
hMSC	human mesenchymal stem cells
HPLC	high-performance liquid chromatography
I2959	Irgacure 2959
IFN	intraferon
IGF-I	insulin-like growth factor I
IgG	immunoglobulin G
IL-2	interleukin 2
IleOEt	L-isoleucine ethyl ester
im	intramuscular
iv	intravenous
KPS	potassium peroxodisulfate
LCST	lower critical solution temperature
LHRH	luteinizing hormone-releasing hormone
M	β-D-mannuronic acid
M_c	number-average molecular weight between cross-links
MMP	matrix metalloprotein
M_n	number-average molecular weight
MPEG	monomethoxyPEG
M_w	weight-average molecular weight
N_A	Avogadro's number
NASI	*N*-acryloxysuccinimide
NIPAm	*N*-isopropylacrylamide
NMR	nuclear magnetic resonance spectroscopy
NR	Nile red
OAAAE	oligo(amidoamine/amino ester
OSM	carboxylic acid terminated sulfamethazine oligomers
PA	poly(L/DL- alanine)
PAA	poly(acrylic acid)
PAE	poly(β-amino ester)
PAU	poly(aminourethane)
PBS	phosphate-buffered saline
PBT	poly(butylene terephtalate
PCL	poly(ε-caprolatone)
PCLA	poly(ε-caprolatone-*co*-lactic acid)
PDGF-BB	platelet-derived growth factor BB
PEA	poly(ethylene adipate)
PEG	poly(ethylene glycol)
PEG-4-SH	tetrafunctional mercapto-PEG
PEGDA	poly(ethylene glycol) diacrylate
PEG−VS	poly(ethylene glycol) vinyl sulfone
PESc	poly(ethylene succinate)
PEO	poly(ethylene oxide)
PDLA	poly(D-lactide)
PHA	poly(hexamethylene adipate)
PHB	poly[(*R*)-3-hydroxybutyrate]
PHPMAm	poly(*N*-(2-hydroxypropyl)-methacrylamide
PHPMAm-lac	poly(HPMAm esterified with (mono or di)lactoyl lactate
PHPMA-*co*-DAMA	poly(*N*-(2-hydroxypropyl)-methacrylamide)-*co*-*N*-(*N*′,*N*′-dicarboxymethylaminopropyl)-methacrylamide
PL	poly(D-lysine)
PLA	poly(lactic acid)
PLGA	poly(lactic-*co*-glycolic acid)
PLHMGA	poly(lactic-*co*-hydroxymethyl glycolic acid)
PLLA	poly(L-lactide)
PLX	poly(propylene glycol)−poly(ethylene glycol)−poly(propylene glycol)
PMAA	poly(methacrylic acid)
P(NVP−PBA)	poly(*N*-vinyl-2-pyrrolidone-*co*-phenylboronic acid)
PPF	poly(propylene fumarate)
PPG	poly(propylene glycol)
PPO	poly(propylene oxide)
PPODA	poly(propylene glycol) diacrylate
PVL	δ-valerolactone
PVP	poly(*N*-vinylpyrrolidone)
QT	pentaerythritol tetrakis 3′-mercaptopropionate
R	molar gas constant
rhIFN-β	recombinant human interferon beta

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rhIL-2	recombinant human interleukin 2
sc	subcutaneous
SDF-1	stromal cell-derived factor 1
SEC	size exclusion chromatography
STZ	streptozotocin
SECM	scanning electrochemical microscopy
T	absolute temperature
TA	pentaerythritol triacrylate
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TEOA	triethanolamine
TGF	transforming growth factor
UCST	upper critical solution temperature
UV	ultraviolet
ValOEt	L-valine ethyl ester
VEGF	vascular endothelial growth factor

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