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In situ-forming implants present an attractive parenteral delivery platform for proteins and peptides owing to their ease of application, sustained-release properties, tissue biocompatibility and simple manufacture.

Injectable implants for the sustained release of protein and peptide drugs

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Protein and peptide macromolecules have emerged as promising therapeutic agents in recent years. However, their delivery to the target site can be challenging owing to their susceptibility to denaturation and degradation, short half-life and, therefore, poor bioavailability. *In situ*-forming implants present an attractive parenteral delivery platform for proteins and peptides because of their ease of application, sustained-release properties, tissue biocompatibility and simple manufacture. In this review, we discuss the various mechanisms by which polymer systems assemble *in situ* to form implant devices for sustained release of therapeutic macromolecules, and highlight recent advances in polymer systems that gel in response to a combination of these mechanisms. Finally, we examine release mechanisms, marketed products and limitations of injectable implants.

Rapid advances in protein and peptide pharmacology, and the use of DNA recombinant technology for large-scale manufacture of such molecules, has given these molecules the reputation of being safe, effective and highly potent therapeutic agents [1]. Paradoxically, the advancement in the development and synthesis of therapeutic peptides and proteins has significantly outpaced the development of adequate delivery systems [2]. Proteins and peptides show poor oral bioavailability owing to extensive degradation by enzymes in the gastrointestinal tract, as well as limited permeability across the gastrointestinal mucosa. Their inherent physicochemical properties, such as large molecular size, short plasma half-life, immunogenicity, and their tendency to undergo aggregation, adsorption and denaturation, further limit their oral bioavailability to less than 1% [3]. Alternative routes, such as buccal, nasal, rectal, vaginal and transdermal, have been investigated for the administration of proteins and peptides, but studies have demonstrated that enzymatic degradation in these tissues is comparable to that in the gastrointestinal tract [4,5]. Hence, proteins and peptides are generally administered parenterally, but need to be given frequently owing to their short half-life [6], thus reducing patient compliance.

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Suspensions and emulsions have traditionally been used to formulate parenteral injections. However, these dosage forms do not allow for controlled release of the drug. Moreover, emulsions exhibit limited stability and a short shelf-life [7]. Various controlled release systems, such as microemulsions, micelles, liposomes, niosomes and nanoparticles, have been developed over the past few decades. Although these formulation techniques have been shown to be beneficial in the therapeutic delivery of macromolecules, they also have several drawbacks, including dosage form migration away from the injection site. Therefore, injectable implants, which are syringeable liquid formulations that are injected intramuscularly or subcutaneously and solidify *in situ* to form solid or semi-solid drug depots, have been developed as a versatile alternative for the controlled parenteral delivery of therapeutic agents [8].

Injectable implants can deliver proteins and peptides at a controlled rate over a prolonged period of time, thereby reducing the dose and frequency of administration [9,10]. Given that they also eliminate the need for repeated injections and surgery, both before implantation and after the release is complete, injectable implants significantly improve implant application and, thus, patient compliance [11]. Major advantages of injectable implants over other drug delivery systems include their composition of pharmaceutically acceptable ingredients, ease of formulation and drug loading, and, in most cases, biodegradability, biocompatibility and nontoxicity [12]. In addition, polymers used in injectable implants often stabilize proteins and peptides owing to surface activity, preferential exclusion, steric hindrance of protein–protein interactions or by increasing viscosity and limiting protein structural movement [10]. Protein conjugation with poly(ethylene glycol) (PEG), for example, is known to increase protein stability and reduce immunogenicity by steric hindrance [13]. Similarly, hydroxypropyl-β-cyclodextrin (HP-β-CD) has been shown to prevent precipitation of porcine growth hormone by thermal and interfacial denaturation [14]. Certain polymers can also inhibit the chemical denaturation of proteins, with dextran, for example, limiting the metal oxidation of human relaxin [15]. Therefore, it is apparent that incorporation of protein and peptide drugs into these polymer matrices might also limit their enzymatic degradation in the plasma.

The formulation of *in situ*-forming implants using biodegradable polymers to deliver drugs was first reported during the early 1990s [16,17]. Dunn *et al.* [17] devised a biodegradable injectable implant system for animals, using both thermoplastic and thermosetting polymers. Since then, several injectable implant formulations for the delivery of peptide hormones [18], proteins [19], contraceptive steroids [20], narcotic antagonists [16] and anti-inflammatory agents [21] have been developed. In this review, we discuss the various mechanisms by which polymeric injectable systems assemble *in vivo* to form semisolid or solid implants that release drug molecules in a sustained fashion. We also highlight recent advances in polymer systems that gel in response to a combination of these mechanisms, and examine drug release mechanisms, marketed products and limitations of injectable implants.

In situ gelling mechanisms

Typically, injectable implants are liquids or syringeable semisolids that solidify *in situ* to form viscous gels. The gelling mechanism can be classified into three broad categories (Fig. 1): (i) *in situ* precipitation, wherein the polymer is rendered insoluble under physiological conditions owing to a combination of physical forces, such as hydrogen bonding, hydrophobic interactions and ionic bonding between polymer chains. Precipitation of the polymer occurs owing to phase separation, sol–gel transition at physiological temperatures or in response to a change in pH; (ii) *in situ* crosslinking, wherein chemical crosslinking of polymer chains is initiated upon a change in temperature or ion concentration, as well as by photo-irradiation or in the presence of enzymes; and (iii) *in situ* solidifying systems, which are either hot melts that solidify on cooling to physiological temperatures or lyotropic liquid crystals, which self-assemble in aqueous solutions. Table 1 provides an overview of recent research in the area of *in situ* gelling polymeric systems.

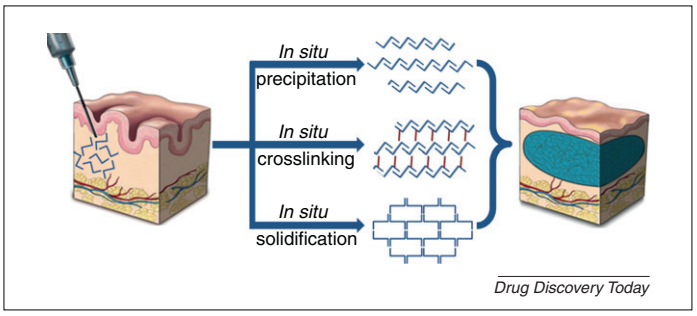

FIGURE 1

Overview of *in situ* implant-forming mechanisms. Modified, with permission, from [154].

In situ *precipitating systems*

Phase-sensitive systems

Dunn *et al.* [17] first proposed the formulation of an injectable *in situ*-forming implant system in 1991 by incorporating drugs into a solution of a biodegradable and biocompatible polymer in a water-miscible, biodegradable and biocompatible solvent. Upon injection of this system, the organic solvent diffused out, resulting in direct contact of the polymer with aqueous physiological fluids, leading to precipitation of the polymer and formation of an implant system. Commonly used biodegradable and biocompatible phase-sensitive polymers are poly-D,L-lactide (PLA), poly-D,L-lactide-co-glycolide (PLGA) and poly-ε-caprolactone-co-lactide (PCL) [8,22].

As carrier vehicles, several water-miscible organic solvents have been considered. *N*-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO) and tetrahydrofuran-2-pyrollidine have been most widely used. Although NMP and DMSO are the most preferred, their use is restricted owing to controversial reports regarding their toxicity [23]. Organic solvents used for the solubilization of polymers require careful consideration because they might also denature proteins and peptides, and induce inflammatory reactions [24]. Glycofurol has been used as a pharmaceutically acceptable, nontoxic solvent for PLGA implants without compromising its pharmacokinetic properties [25]. Moreover, PLGA dissolved in PEG500-dimethyl-ether showed exceptionally good local tolerability and biocompatibility [26]. A completely solvent-free *in situ* gelling system was obtained by simultaneously injecting aqueous solutions of naturally occurring β-glucan and a water-soluble

TABLE 1

Overview of recent research in the area of *in situ* gelling polymeric systems

Drug	Polymer	Gelling mechanism	Study outcomes	Ref
Rosiglitazone	PLGA in triacetin	Phase-sensitive precipitation	Sustained release of rosiglitazone over seven days	[11]
Insulin	PLGA in benzyl benzoate and benzyl alcohol	Phase-sensitive precipitation	Low burst release, good pharmacodynamic response over extended time period, good biocompatibility and stability	[31]
Soluble tumor necrosis factor	PLGA in glycofurol	Phase-sensitive precipitation	Increase in bioavailability and long-term action	[25]
Plasmid DNA	PLGA in glycofurol	Phase-sensitive precipitation	Sustained release over 70 days, ease of preparation and customizable drug release profile	[139]
Recombinant human growth hormone (rhGH)	Fluoroalkyl-ended PEG	Phase-sensitive precipitation	Improved *in situ* stability with minimal burst release and no protein aggregation	[140]
Lysozyme	PEG–PLLA and PEG–PDLA star blocks	Stereocomplexation	Sustained release over ten days	[107]
Bovine serum albumin	Oxidized CMC and *N*-succinyl chitosan	pH-dependent precipitation	Low burst release and negligible cytotoxicity	[141]
Repaglinide	Chitosan and/or PVP-glutaraldehyde interpenetrating network	pH-dependent precipitation	Controlled release over 12 h	[38]
Insulin	PEG–PAF	Temperature-dependent sol–gel transition	A single dose of insulin exhibited a hypoglycemic effect for up to 18 days	[51]
FITC-dextran	PEG–sebacic acid polyester	Temperature-dependent sol–gel transition	Reduced initial burst release	[142]
Horseradish peroxidase	PEG–PCL–PEG	Temperature-dependent sol–gel transition	Rapid *in situ* gelling and sustained release for at least 45 days	[143]
Cyclosporine A	PVA, chitosan and glycerophosphate	Temperature-dependent sol–gel transition	Controlled release and absorption with increased bioavailability	[41]
Recombinant human bone morphogenetic protein	Poloxamine	Temperature-dependent sol–gel transition	Sustained release for several days at body temperature with low cytotoxicity	[45]
Polyelectrolyte complex of rhGH	Poly(organo-phosphazene)	Temperature-dependent sol–gel transition	Reduced burst release and improved *in vivo* stability of rhGH	[134]
Tissue plasminogen activator	PEG-oligo α-hydroxy acid-methacrylates	Photo crosslinking	Retention of enzymatic activity, sustained release over five days	[61]
Growth factors	Azido-chitosan-lactose	Photo crosslinking	Sustained release for seven days with retention of protein activity	[144]
Horseradish peroxidase	Ca-Alginate and methacrylated dextran	Ion-mediated crosslinking	Mild preparatory conditions retained protein activity	[71]
rhGH	Cholestryl-substituted hyaluronic acid	Ion-mediated crosslinking	Easy protein loading, plasma levels maintained within a narrow range, no initial burst release	[74]
α-Amylase/lysozyme	Hyaluronic acid-tyramine	Enzyme-mediated crosslinking	Sustained release with no initial burst, protein activity was not affected	[145]
Ethinyl estradiol	Glyceryl palmitostearate/apricot oil	Lyotropic liquid crystal formation	Improved efficacy and bioavailability	[146]
Rivastigmine	*N*-stearoyl-L-alanine methyl ester	Lyotropic liquid crystal formation	Low burst release and sustained release for 11 days with low systemic exposure	[147]

polymer, such as PEG and dextran, resulting in a biodegradable and biocompatible implant. Thus, mechanical strength, gelling rate and drug release were controlled by optimizing the ratio of β-glucan to the other water-soluble polymers [27].

Extensive studies have already been performed to evaluate the potential of phase-sensitive systems for the delivery of proteins and peptides [1,20,28]. *In vitro* simulated studies have shown that the Atrigel™ system, based on PLGA, significantly improved the bioavailability of several model protein drugs by inhibiting enzymatic degradation [9]. Other injectable PLGA implants have also been examined as depot systems for long-term sustained delivery of drugs and have yielded promising results [11,25]. However, the major disadvantage of these systems is their high initial burst release, which is the result of the lag time between the injection of the system and the precipitation of the polymer. Various studies have shown that an increase in polymer molecular weight and/or polymer concentration, by virtue of forming more viscous solutions, can reduce the initial burst release of proteins and peptides. A corresponding decrease in the drug release rate from systems containing a higher molecular weight and/or a higher concentration of polymers is also common [25]. The use of a more hydrophobic solvent, such as ethyl benzoate or triacetin, can also reduce the burst effect and enhance sustained release [11,29–31]. This has been suggested to occur owing to slower phase inversion, resulting in less porous, more fluid, two-phase gel structures that release drug molecules more uniformly [32,33]. Reduction in burst release from PLGA-based systems has also been observed in the presence of some surfactants, such as Triton X-100, which have a plasticizing effect on PLGA in the presence of water, resulting in physical aging of the polymer and reduction in surface pore depth [34].

pH-sensitive systems

Polyelectrolytes are macromolecules that ionize in certain solvents to form polymer ions [10]. Similar to all weak acids and bases, the degree of ionization of polyelectrolyte chains is pH dependent. This property has been exploited to formulate smart polymers that gel *in situ* at physiological pH. To be used in the formulation of injectable implants, a polyelectrolyte should remain unionized at physiological pH.

Chitosan, a natural, biodegradable and biocompatible polysaccharide with low systemic and local toxicity [35], is soluble in acidic solutions owing to protonation of its amine groups. At physiological pH, it loses the protons and systematically precipitates to form a hydrated gel [36]. However, chitosan alone forms rapidly degrading loosely structured weak gels that are unsuitable for sustained delivery [37]. One approach to improve the structural strength of chitosan is by blending it with other polymers. Vaghani *et al.* [38] crosslinked a chitosan and polyvinylpyrrolidone (PVP) blend with glutaraldehyde to form a semi-interpenetrating polymer network that gelled *in situ* at physiological pH. Their investigations revealed that the porosity of chitosan gels is a function of the crystallinity of the polymer, which can be reduced by blending it with PVP. Hydrophobic modification of chitosan can also improve its structural strength. One example includes the modification to *N*-palmitoyl chitosan, which enabled rapid formation of a dense nontoxic pH-sensitive chitosan hydrogel at pH 6.5–7.0 [39].

A significant concern relating to pH-sensitive polymer systems is that the solutions are not neutral when constituted, which can have a negative impact on the stability of proteins and peptides as well as cause local irritation upon injection. Moreover, pH alone is a poor control for *in situ* gelling implants and, thus, several attempts have been made to modify polyelectrolytes to prepare thermosensitive gelling systems [40–42].

Thermosensitive systems

Certain polymers show abrupt changes in their solubility with changes in the environmental temperature. These polymers are characterized by a lower critical gelation temperature (LCGT) and an upper critical gelation temperature (UCGT) (Fig. 2). LCGT and UCGT are functions of the hydrophilic–hydrophobic balance on the polymer backbone as well as the free energy of mixing [24]. As the environmental temperature approaches LCGT, polymer–water interactions become unfavorable compared with water–water and polymer–polymer interactions. Therefore, these polymers show reduced viscosity below the LCGT, above which it increases sharply. At UCGT, the polymer chains achieve a high kinetic energy and become increasingly randomized, resulting in rupture of the gel structure and a decrease in viscosity. Therefore, polymer systems with a LCGT between room and physiological temperature are excellent candidates for injectable implant systems [10].

Triblock PEO–PPO–PEO copolymers, commonly known as Poloxamers® or Pluronics®, were the first polymers capable of temperature-dependent sol–gel transitions approved by the US Food and Drug Administration (FDA). They show gelation at body temperature when used at concentrations of 15% (w/w) and above [43]. However, some animal studies have demonstrated that Poloxamers might be toxic at this concentration [44]. Poloxamines, which are x-shaped PEO–PPO block copolymers with an ethylenediamine core (Tetronic®), have recently been evaluated as a controlled-release injectable platform for delivery of recombinant bone morphogenetic protein [45]. Although poloxamines are nonbiodegradable, it is believed that they are rapidly eliminated in urine, thus exhibiting minimal toxicity. However, unmodified Poloxamers, have been shown to elevate plasma cholesterol and triglyceride levels owing to possible stimulation of 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase activity in the liver [46]. Moreover, nonbiodegradable polymers are generally undesirable because they can accumulate in the body. Nevertheless, recent studies have shown that the introduction of hydrophilic groups, such as polylactide [23] and PEG [47] to such

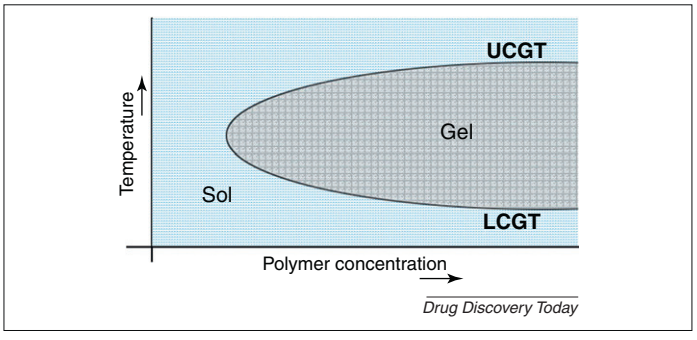

FIGURE 2

A typical sol–gel phase diagram. Abbreviations: LCGT, lower critical gelation temperature; UCGT, upper critical gelation temperature.

thermosensitive copolymers often imparts biodegradability to them.

Extensive studies have been performed to develop and characterize biodegradable thermosensitive polymer systems owing to their ease of formulation and administration, absence of organic solvents, good tolerability and wide application in the biomedical field [23]. Jeong *et al.* [48–50] introduced a series of polymer blocks, including a PEG-block-poly(alanine-co-phenyl alanine) (PEG–PAF) matrix, which undergoes sol–gel transition at physiological temperature at concentrations as low as 3.0–7.0% (w/w). These PEG–PAF polymer matrices undergo enzymatic degradation in the subcutaneous tissue and show no residual toxicity [51]. Kang *et al.* [52] synthesized copolymers of PEG and PCL with at least 5% poly (L-lactide) (PLLA) in the PCL segment, which undergo sol–gel transition at body temperature. The size and viscosity of this implant was modulated by changing the ratio of PEG, PCL and PLLA and, thus, the system could be tailored to release the drug over a period of a few weeks to a few months. As for pH-sensitive systems, a high initial burst release of the drug owing to slow *in vivo* sol–gel transition is also the major drawback of thermosensitive systems. However, by optimizing the chain-length ratio between the hydrophilic and hydrophobic segments of the copolymers, significant improvements in their release characteristics and stability can be achieved. Gels with larger hydrophilic PEG blocks, for example, have lower stability and show a higher initial burst release [23]. A novel approach to reduce burst release from PCL–PEG–PCL systems includes their coupling to a KRGDKK (Lys-Arg-Gly-Asp-Lys-Lys) peptide. *In vitro* drug release studies from these systems have shown excellent sustained release characteristics for over one month without an initial burst [53].

Many attempts have also been made to prepare temperature-responsive chitosan systems to improve release kinetics and enable administration of proteins and peptides at neutral pH. Chitosan and glycerophosphate (GP) mixtures, for example, remain free-flowing liquids at room temperature, even at neutral pH, as GP promotes protective hydration of chitosan chains and thus prevents their aggregation. At higher temperatures the hydrophobic interactions between the chitosan chains are strengthened and the polymer precipitates to form a hydrogel. However, this system has limited potential in drug delivery owing to a high burst release [40,54,55]. By contrast, polyvinyl alcohol (PVA)-blended chitosan-GP systems have shown great potential as *in situ*-forming implants exhibiting improved bioavailability, low burst release and controlled release over extended periods [36]. Besides GP, several other polyols, such as 1,3-propanediol, 1,2-propanediol, glycerol and mannitol or polyoses, such as trehalose, have been suggested as stabilizing agents for chitosan, with the added advantage of chitosan–mannitol and chitosan–trehalose systems allowing lyophilization to improve the formulation shelf-life [56]. Another interesting temperature-sensitive system includes a combination of chitosan and sodium bicarbonate. At physiological

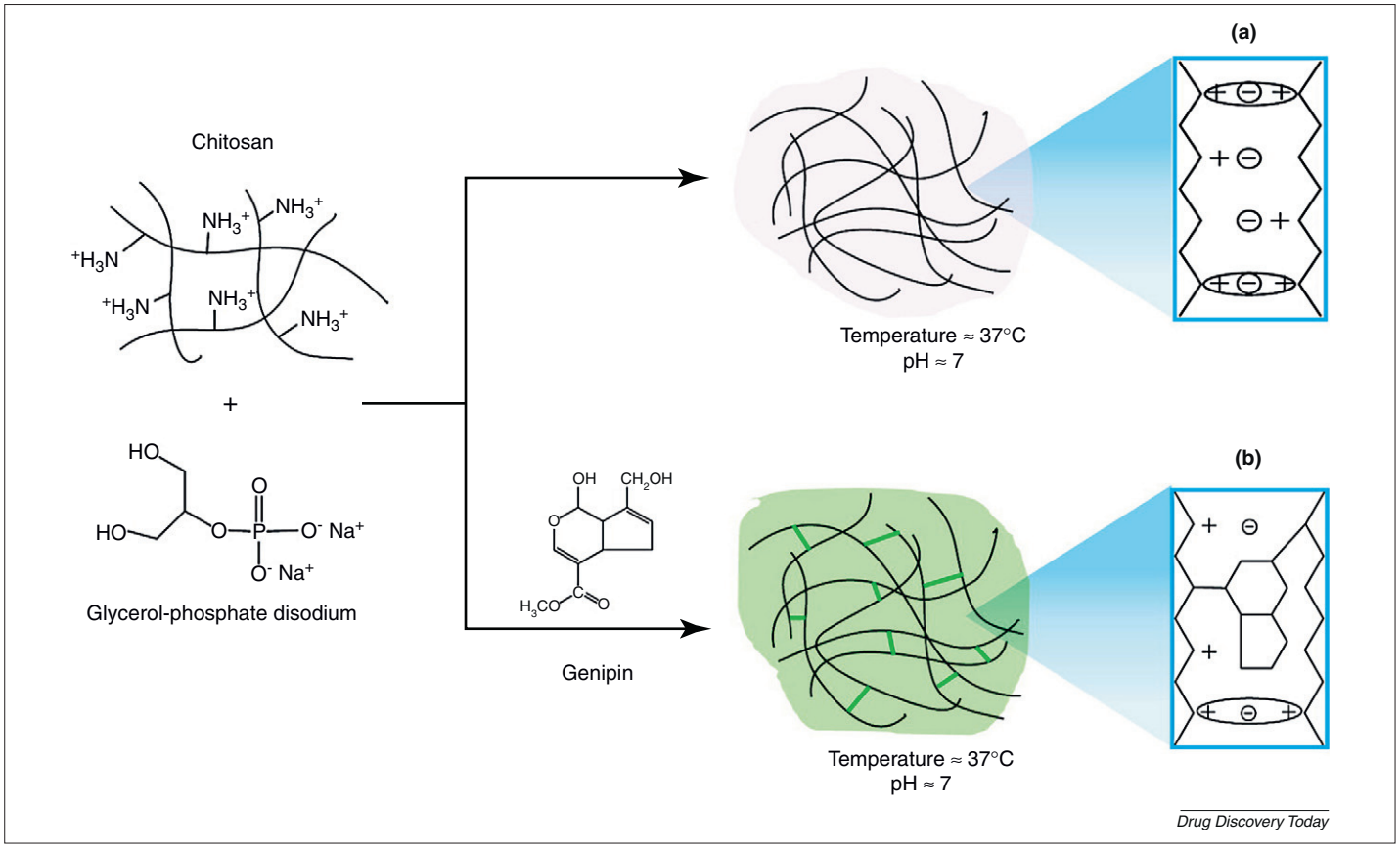

FIGURE 3

Schematic representation of chitosan-based hydrogel systems formed by **(a)** sol–gel transition owing to deprotonation of chitosan; and **(b)** chemical crosslinking in the presence of the crosslinking agent genipin.
Reproduced, with permission, from [58].

temperatures, the bicarbonate ion deprotonates chitosan to form water and carbon dioxide, and the polymer precipitates. Thus, by changing the bicarbonate concentration, the morphology and release characteristics of the implant can be controlled [57].

In situ crosslinking systems

Thermosetting systems

Thermosetting polymers are liquids having good flowability when initially constituted, but that crosslink at elevated temperatures to form viscous gels. Unlike thermosensitive polymers that show a transition to the gel state owing to a change in polymer–water interactions above the LCGT, gelation in thermosetting polymers is a consequence of the chemical reaction between polymeric chains, often in the presence of a catalyst or initiator. This approach was first introduced by Dunn *et al.* [17], who synthesized a copolymer of D,L-lactide or L-lactide with ε-caprolactone, using a polyol initiator and a catalyst (peroxide) to form biodegradable thermosetting systems for drug delivery in animals. The major advantage of this system was its excellent syringeability. However, it had several drawbacks, including a high burst release of drug during the first hour after injection, carcinogenicity of the polyol initiator and necrosis in the surrounding tissues owing to the exothermic crosslinking process [8].

Improved thermosetting crosslinking systems have since been synthesized by adding genipin, a naturally occurring chemical crosslinking agent, to neutralized chitosan–GP systems described in the previous section. The resulting hydrogel formed by chemical crosslinking in addition to deprotonation and precipitation of chitosan (Fig. 3) and, thus, showed improved mechanical and chemical strength. Other advantages of this system included localization at the injection site, rapid formation, nontoxicity and controlled surface porosity [58].

Photoresponsive polymers

Photosensitive polymers are biocompatible, biodegradable, polymerizable and at least partially water-soluble macromeres or prepolymers, which are photocured *in situ*, usually in the presence of photoinitiators, such as ethyl eosin, camphorquinone and derivatives of acetophenone [10]. Macromeres usually comprise at least one biodegradable region [usually poly-lactic acid, poly-glycolic acid, poly-(anhydride), poly-(amino acid) or poly-lactone], at least one water-soluble region (such as PEG, polyvinyl alcohol, polysaccharides, such as hyaluronic acid (HA), or proteins, such as albumin) and at least two regions that can be polymerized by free radicals (preferably acrylates, methacrylates, diacrylates and other biocompatible photopolymerizable groups) [8]. The intensity of light, the choice of the photoinitiator, and the number of reactive double bonds present significantly affect the rate of polymerization [59].

Hubbel *et al.* [60] patented a polymerizable hydrogel containing a central PEG core flanked by oligo α-hydroxy acids (PLA and/or PGA) and acrylate groups on either side. In this system, eosin dye was used as photoinitiator and polymerization was instigated by UV light. Further investigations on this system have revealed that the molecular weight of PEG and the type of α-hydroxy acid used significantly influenced the release rate of the drug [61]. Other polymers investigated include alginate and HA modified with methacrylate (MA) to form soft, flexible viscoelastic gels on photolysis. These gels swelled rapidly up to 14 times their dry weight in the presence of an optical trigger and exhibited excellent mechanical properties, which were further optimized by controlling the degree of methacrylate modification [62]. Swelling behavior and elasticity of methacrylated HA gels were further improved by incorporating PEG-diacrylate as co-macromonomer and mechanical properties were modulated by changing the HA molecular weight and the concentration of PEG-diacrylate [63].

Several attempts have been made to synthesize photopolymerizable hydrogels based on chitosan, owing to its excellent biocompatibility. Photo crosslinkable chitosan hydrogels, prepared by including lactose moieties (lactobionic acid) and photoreactive azide groups (*p*-azidobenzoic acid) on the chitosan backbone have found numerous biomedical applications [64–66]. UV polymerizable chitosan hydrogels have also been developed by derivatizing chitosan with vanillin or hydroxybenzaldehydes. Given that these chitosan hydrogels were prepared using naturally occurring, biodegradable and biocompatible substances, they were found to be nontoxic and showed excellent tolerability [56]. Overall, photo crosslinked polymers are advantageous because the polymerization reactions are rapid, thus reducing burst release. However, low tissue penetration of the initiating light and the formation of reactive oxygen species at high initiator concentrations limit their application in a clinical setting.

Ion-mediated crosslinking

Interaction of electrolytes with di- or trivalent polymers can also result in gel formation owing to crosslinking of polymer chains by ionic interactions. Alginate is a natural polysaccharide that reacts with divalent ions, such as Ca^{2+}, to form three-dimensional networks by creating ionic interchain bridges [67]. This property has been exploited in the preparation of temperature-sensitive microspheres containing Ca^{2+} in a sodium alginate suspension as an injectable implant that gels rapidly at physiological temperatures [68,69]. Soluble macromolecules, such as cytokine interleukin-2, were readily incorporated into these self-gelling alginate matrices by mixing them with the formulation before gelation, whereas immune-stimulatory cytosine-phosphate-guanine (CpG) oligonucleotides were attached to the surface using alginate microspheres as modular components [69]. In another study, Ca^{2+} was released *in situ* from hydroxyapatite (HAP) nanocrystals in response to a pH drop mediated by hydrolysis of D-glucono-δ-lactone, resulting in strong homogenous gel formation with an alginate dispersion [70]. More recently, injectable polysaccharide hydrogel systems based on calcium alginate and two dextran methacrylate derivatives have been suggested for protein delivery, because they can be prepared under mild conditions without compromising the activity of the macromolecules. Mechanical properties of the hydrogels were controlled by alteration of the methacrylation extent [71]. However, one major drawback of this system included the slow and uncontrolled degradation of the implant [72]. To counter this, hydrolytically sensitive groups were introduced on the calcium alginate-methacrylated dextran hydrogel by oxidation with periodate [73]. The oxidized alginates degraded at physiological conditions without compromising the inherent properties of native alginate.

Another polysaccharide used to form ion-mediated nanogels is HA [74], which is a biodegradable, biocompatible polysaccharide naturally present in vertebrates [75,76]. Nakai *et al.* [74] substituted the cholesteryl groups on HA to form nanogel dispersions in water, which self-assembled *in situ* in the presence of physiological salts

(Na^+ and Cl^-), thus forming hydrogels. Given that these gels spontaneously entrap large quantities of proteins and protect them from enzymatic denaturation, they have become particularly attractive for macromolecule delivery.

Enzyme-mediated crosslinking

A recent development in chemically crosslinked hydrogels is the use of enzymes to initiate the crosslinking reaction. Enzymatic crosslinking usually occurs under mild conditions, is nontoxic and the catalyzing enzymes often occur naturally within the body [77,78]. Another major advantage of these systems is that the crosslinking reaction can be modulated by controlling the activity of the enzyme [79]. A pioneering enzymatically crosslinked hydrogel was introduced by Sofia *et al.* [79] in 2002, demonstrating that poly(aspartic acid) polymers functionalized with aromatic groups could be crosslinked in the presence of peroxidases. Since then, tyramine conjugates of various water-soluble polymers have been chemically crosslinked in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2), and have been suggested as potential injectable controlled-release systems [57,80–82]. Lee *et al.* [83], for example, described HA–tyramine conjugates that gel *in situ* in the presence of HRP and H_2O_2. The mechanical strength and rate of gelation of this hydrogel was found to be directly proportional to the concentration of H_2O_2 and HRP, respectively. A similar crosslinking mechanism was also observed in dextran–tyramine conjugates, which have shown great versatility in biomedical applications [57,81,84]. Thus, enzymatically crosslinked polymers could provide a promising alternative for the sustained delivery of proteins and peptides.

In situ solidifying and/or swelling systems

Thermoplastic pastes

Polymers exhibiting a low molecular weight and glass transition temperature (T_g) and, consequently, a relatively low melting point (25–65 °C), are used to formulate thermoplastic pastes. These polymers are injected as melts and solidify at body temperature to form sustained-release drug delivery systems [85]. Such polymers have good syringeability at elevated temperatures and can be molded to the desired shape by application of heat packs [86]. Thermoplastic pastes can be synthesized from monomers of D,L-lactide, glycolide, ε-caprolactone, trimethylene carbonate, dioxanone, ortho-esters and anhydrides [87–89]. The major limitation of thermoplastic pastes is their injection at elevated temperatures (≥60 °C), which can result in the denaturation of proteins and peptides, as well as formation of scar tissue, which can encapsulate the formed implant, therefore inhibiting the release of the drug into the surrounding tissue [90].

Lyotropic liquid crystals

Lyotropic liquid crystalline systems are water-insoluble amphiphilic lipids that self-assemble in aqueous solutions to form lyotropic crystals [91]. Given that these systems can incorporate and control the release of drugs of varying sizes, they find extensive application in the delivery of protein and peptide drugs [92]. The use of phase transformation from a liquid to a liquid crystal allows administration of a low viscosity matrix by injection, which transforms into a viscous liquid crystalline phase upon dilution in body fluids. Glycerol monooleate and its derivatives have been studied intensively for this purpose owing to their biocompatibility and biodegradability [93], and have been used as drug delivery systems for several proteins and peptides, showing improved stability [94] and biological activity [95] of the incorporated macromolecules.

Recently, two lyotropic liquid crystalline systems based on phytantriol and glyceryl monooleate, respectively, have been investigated as stimuli-responsive controlled-release systems. The internal nanostructure of these liquid crystals exists predominantly in the cubic phase at lower temperatures, transforming into the hexagonal phase upon temperature increase. Drug release from these systems is diffusion controlled, with a faster release observed from the cubic phase, exhibiting larger aqueous channels than the hexagonal phase. Thus, by thermally inducing a reversible transition from the bicontinuous cubic phase to the hexagonal phase, a temperature-sensitive drug release system can be obtained [96] (Fig. 4). The transition temperature can be optimized by adding ethyl acetate [97] or oleic acid [98]. However, a limitation of such temperature-sensitive drug release systems includes the lack of specificity in the heat source. By incorporating hydrophobized gold nanorods into the liquid crystalline matrix, which resonate upon photoirradiation with a specific light source providing remote heat, nanostructure crystal phase transition and, consequently, a change in the drug release rate, can be obtained [99].

Another novel lyotropic system of poly(ester anhydride) based on sebacic acid and hydroxy oleic acid has recently been proposed

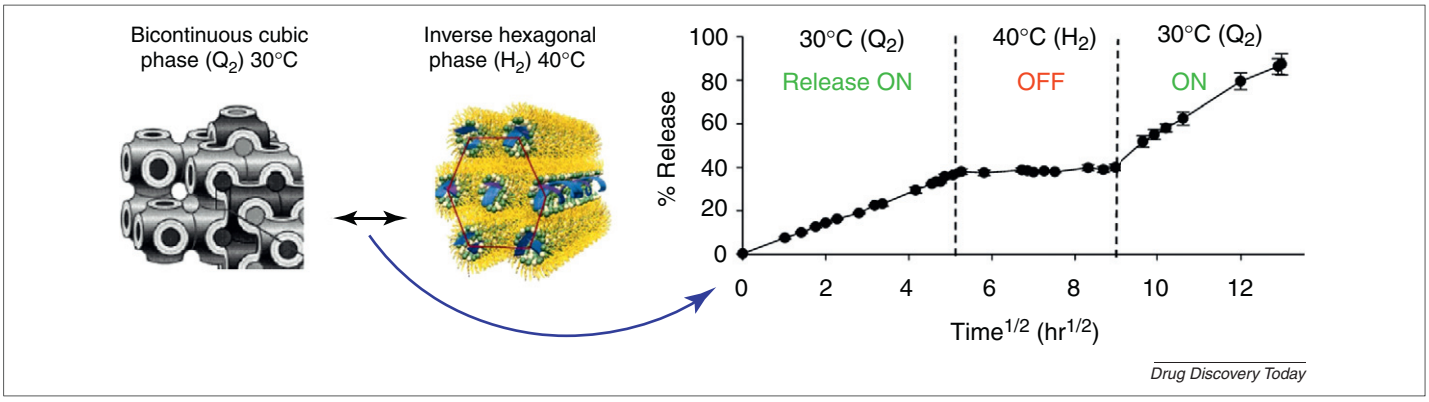

FIGURE 4

Drug diffusion is faster from the large aqueous channels in the cubic phase compared with the hexagonal phase. By controlling the temperature of the system, a reversible phase change in the crystal nanostructure can be induced, altering the drug release rate.
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for site-directed, injectable administration and controlled release of paclitaxel for tumor management in orthotropic prostate cancer [100]. Polyanhydride systems are relatively stable *in vivo*, exhibit a long shelf-life, and the mild preparatory conditions preserve protein and peptide drugs [101], highlighting them as promising injectable implants for macromolecule delivery. Overall, these systems prove advantageous owing to their low cost and lipase biodegradability. However, variability in liquid crystal formation upon injection because of inconsistency in surrounding body fluid or the high initial viscosity when injecting an already preformed cubic phase might limit their application *in vivo*.

Recent combination systems

Novel polymer systems that gel *in situ* by a combination of the above mechanisms have gained popularity during recent years because they have the potential to significantly improve mechanical strength [102], protein stability [103], encapsulation efficiency [104] and drug release profiles [40]. Numerous efforts have been directed toward the development of chitosan hydrogel systems that respond to both pH and temperature. Neutralized chitosan–GP solutions, for example, have been used extensively as described earlier. The pH sensitivity of chitosan–GP systems can be enhanced by increasing the amount of amino groups on the chitosan backbone. Thus, quarternized chitosan–GP mixtures have been developed as intelligent carrier systems that gel at physiological temperatures to form polymeric implants that release the drug in a pH-dependent fashion [40]. Thermosensitive and pH-sensitive synthetic polymer blocks have also been evaluated for potential use as injectable implants. Shim *et al.* [103] synthesized a pH- and temperature-sensitive polymer by coupling a sulfonamide derivative as the pH-sensitive moiety, with a thermosensitive PCL–PEG–PCL block copolymer. This system demonstrated a sol–gel transition between pH 7.4 and 8.0, with a simultaneous change in temperature, and proved exceptionally suitable for delivery of acid-labile and hydrophobic proteins.

Hydrogels formed by interaction of physical forces are often inferior in terms of mechanical strength to hydrogels formed by chemical crosslinking. Thus, considerable research has been dedicated to strengthen thermosensitive hydrogels by initiating crosslinking between the polymer chains. HA modified by Pluronic F127 copolymer shows sol–gel transition between 20 °C and 40 °C, but additional vinyl groups can further be photopolymerized by UV irradiation to improve the mechanical strength [105]. Another novel thermo- and photosensitive *in situ*-gelling ABA triblock copolymer with improved stability was introduced by Censi *et al.* [106]. The A block of the triblock copolymer comprised thermosensitive poly(N-(2-hydroxypropyl) methacrylamide lactate) [p(HPMAm-lac)], whereas the B block comprised hydrophilic PEG. At physiological temperature, the A blocks associated to form weak gels, in which chemical crosslinking was induced by UV irradiation to improve the mechanical strength.

Recently, Hiemstra *et al.* [107] suggested a system of PEG–PDLA (poly-D-lactide) and PEG–PLLA star-block copolymers that were mixed *in situ* to form hydrogels by stereocomplexation between the star-block enantiomers (Fig. 5). MA star block copolymers were further photopolymerized to improve the mechanical strength. Photopolymerization occurred rapidly (1–2 min) at low initiator concentrations [0.003% (w/w)]. Formed gels degraded within

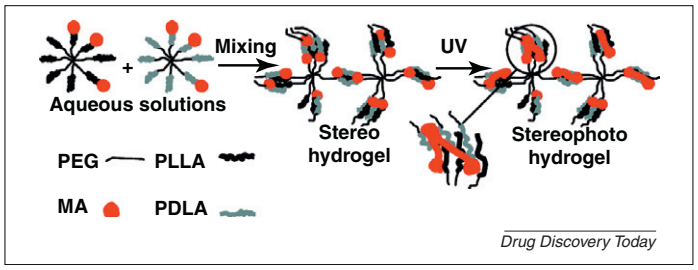

FIGURE 5

Schematic representation of *in situ* stereocomplexation and photopolymerization of star block copolymers.
Abbreviations: PEG, poly(ethylene glycol); MA, methacrylate; PLLA, poly (L-lactide); PDLA: poly (D-lactide).
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7–16 weeks and could be modulated by changing the degree of methacrylation [108]. These examples demonstrate the advantage of combination systems over a single gelling mechanism and, therefore, warrant further investigation as injectable implants for macromolecule delivery.

Drug release mechanisms

It is generally accepted that the drug release from a polymer matrix can be by: (i) diffusion through water filled pores; (ii) diffusion through the polymer; (iii) osmotic regulation of flow; and (iv) erosion or degradation of matrix components [109]. Drug release from hydrolytically degraded matrices uses a combination of diffusion and erosion. During the diffusion process, the drug located on or near the implant surface diffuses out rapidly, forming a drug-depleted layer adjacent to the polymer surface. The drug molecules in the interior of the implant then have to traverse through a progressively thickening layer of the polymer and, therefore, the rate of diffusion steadily decreases. This can be controlled by decreasing the distance traveled by the drug (i.e. by decreasing the size of the implant). However, most often, this phenomenon is compensated for by erosion of the polymer matrix so that it progressively loosens up, increasing the permeability of the drug through the polymer matrix [110].

Matrices can degrade either homogeneously (hydrolysis occurs throughout the polymer matrix), or heterogeneously (hydrolysis is restricted to the surface of the device). Perfectly heterogeneous degradation of polymer matrices is preferable owing to zero-order release of the drug. This can be achieved by increasing the hydrophobicity of the polymer to limit the entry of water into the matrix, making degradation only a surface phenomenon [110]. During homogeneous degradation, the matrix retains its original shape up to a critical point, after which it collapses and dissolves completely. PLA, PLGA and poly-(ε-caprolactone) systems are assumed to undergo homogeneous degradation and show a sigmoidal degradation curve. When placed in an aqueous environment, water is absorbed into the polymer matrix and PLGA begins to degrade. It has been demonstrated that the rate of hydrolytic degradation of PLGA ester bonds is slower than the rate at which water is absorbed into the matrix. Therefore, the entire device is wetted rapidly, resulting in homogenous degradation of the polymer throughout the matrix [111].

Drug release from such matrices can sometimes be biphasic; however, triphasic release profiles are more common (Fig. 6). Phase

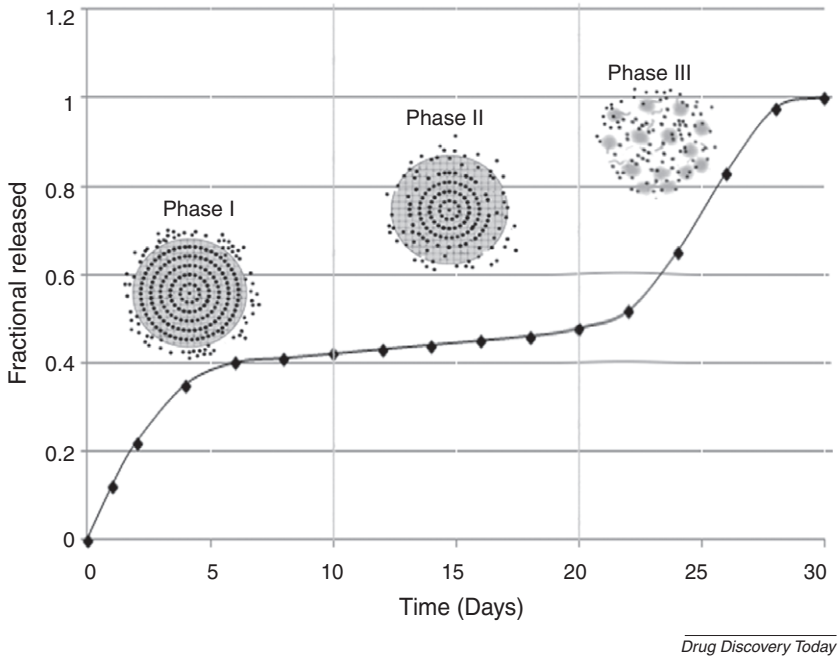

FIGURE 6

Typical triphasic release profile from poly-D,L-lactide-co-glycolide (PLGA) matrices. Phase I shows a high initial burst release, phase II is diffusion-controlled slow release and phase III is characterized by fast release owing to erosion of the polymer matrix.

I of a typical triphasic release profile is characterized by 'burst release' attributed to nonentrapped drug molecules. The second phase is usually a period of slow release during which the drug slowly diffuses out of the polymer system, whereas the third phase, also called the 'second burst', is fast drug release at the onset of erosion [109]. Besides this typical triphasic drug release profile from polymer matrices, several other release characteristics can be obtained, depending on the polymer used. By carefully controlling the polymer molecular weight and polymer concentration, the degradation of the polymer and, consequently, the drug release rate and profile can be adjusted. An increase in the molecular weight of the polymer generally results in lower porosity, reduced influx of water and, therefore, slower degradation. Consequently, the rate of drug release decreases and the duration of drug release is prolonged [18].

Marketed products

Several injectable implant systems have already made it to the market (Table 2). Atrigel® (PLGA in NMP) is a proprietary drug delivery system suitable for systemic and localized drug delivery. It was developed by Dunn *et al.* [17] and licensed to Atrix Laboratories for subgingival delivery of antimicrobials to treat periodontal disease. Several Atrigel-based systems have since been approved by the FDA and are currently on the market. Atridox™ (Atrix Laboratories, USA), indicated for the treatment of chronic adult periodontitis, and Doxirobe™ (Pfizer Animal Health, USA), used for veterinary periodontitis, are two-syringe systems that form drug depots on simultaneous injection of Atrigel and doxycycline hyclate [112–114]. Eligard® (Sanofi Aventis, USA) is another injectable system based on the Atrigel technology that releases leuprolide acetate over one, three, four or six months. Eligard comprises two separate sterile syringes, one containing the Atrigel system and the other containing leuprolide acetate, whose contents have to be mixed immediately before injection [115,116].

SABER™ (DURECT, USA) is a multicomponent sustained-release system based on sucrose acetate isobutyrate (SAIB), a pharmaceutically acceptable solvent, and one or more additives. The drug is dispersed or dissolved in SAIB and injected intramuscularly or subcutaneously [117]. *In situ*, it forms a viscous depot by phase dispersion. The potential of SABER to deliver various drug molecules has been investigate and it has been shown that sustained release of intact growth hormone could be achieved *in vivo* for at least seven days [118]. SABER has also been used for the sustained release of bupivacaine (POSIDUR™) over 72 h to treat local postsurgical pain. This system is currently in Phase III clinical trials [119].

ReGel® (BTG, UK) is a filter-sterilizable PLGA–PEG–PLGA triblock copolymer system that gels *in situ* by temperature-mediated sol–gel transition. Its inherent ability to stabilize poorly soluble and sensitive drugs is one of its major advantages [120]. Cytoryn™ is a ReGel/interleukin-2 intratumoral injection that has shown sustained release for 72–96 h and presents a promising platform for cancer immunotherapy [121]. hGHD-1 is a protein delivery system for sustained release of human growth hormone from ReGel [122], whereas OncoGel™ is an intralesion depot system used for sustained release of paclitaxel over six weeks [123]. Phase II clinical trials of the OncoGel system have revealed prolonged release of paclitaxel with good tolerability and low systemic exposure [124]. InGell® Gamma (InGell Labs, Netherlands) is another thermosensitive triblock PCL–PEG–PCL system being developed for commercial use to deliver a variety of proteins, peptides and other therapeutic agents. Owing to the presence of ε-caprolactone, InGell Gamma is reportedly more stable than ReGel and allows longer sustained-release profiles [125,126].

TABLE 2

Commercially available products or delivery platforms based on *in situ* gelling hydrogels

Product name and/or composition	Gelling mechanism	Drug delivered	Benefits and/or main study outcomes	Ref
Atrigel® (PLA and/or PLGA in biocompatible solvent)	Phase-sensitive precipitation	Doxycycline hyclate (Atridox® and/or Doxirobe®)	Sustained release for 1–4 weeks	[114]
		Leuprolide acetate (Eligard®)	Reliable sustained release and good tolerability	[116]
		Bovine serum albumin and/or insulin	Inhibition of enzymatic degradation of protein	[9]
SABER™ (sucrose acetate isobutyrate with additives and biocompatible solvent)	Phase-sensitive precipitation	Human growth hormone	Low burst release, increased persistence and sustained release for seven days	[118]
		Bupivacaine (POSIDUR™)	Sustained release over 72 h	[119]
		Pingyangmycin (Zein and/or Zein-SAIB)	Low burst release and sustained release over one week	[148]
		Risperidone [SAIB–PLA–ethanol (Relday™)]	Biocompatible, sustained release and low burst release	[149,150]
ReGel® (PLGA–PEG–PLGA)	Thermosensitive sol–gel transition	Interleukin-2 (Cytoryn™)	Significant reduction in tumor growth and systemic activation of tumor immunity	[121]
		Human growth hormone (hGHD-1)	Easy sterilization and drug loading	[122]
		Paclitaxel (Oncogel®)	Sustained release, good tolerability and low systemic exposure	[123]
		Insulin	Sustained release over one week, good tolerability and no inflammation	[151,152]
BST-Gel® (chitosan–GP)	Thermosensitive sol–gel transition	Paclitaxel (Paclitel®)	Low toxicity, low systemic exposure and no Cremophor or organic solvent	[54,127]
		Camptothecin	No toxicity and more effective than single intraperitoneal injection	[128]
		Antitumor necrosis factors	10–30% drug loading and slow release limits systemic exposure	[153]
		Bovine serum albumin	Versatile and sustained release depot system	[153]
Octodex™ (dextran grafted with PLLA and PDLA)	Stereocomplexation	Immunoglobin G and/or lysozyme	Quantitative sustained release with full preservation of enzymatic activity of lysozyme	[129]
InGell® Delta (dextran grafted with D- and L-lactide oligomers)	Stereocomplexation	Interleukin-2 (rhIL-2)	Excellent biocompatibility and therapeutic efficacy, and low initial burst release	[132]
InGell® Gamma (PCL–PEG–PCL)	Thermosensitive sol–gel transition	Various proteins, peptides and small molecules	Soft, robust gels with little burst release that are capable of solubilizing hydrophobic drugs	[125]

BioSyntech Inc. (Canada) developed a thermosensitive chitosan-based platform technology by combining chitosan with β-glycerophosphate (GP). This sustained release hydrogel is registered as BST-gel™ and has been suggested for site-specific prolonged delivery of several drugs. Paclitel® (Paclitaxel/BST-gel) has been suggested for treatment of breast cancer, and intratumoral injections provided sustained release for several days with minimal systemic exposure and, thus, reduced toxicity [54,127]. Attempts have also been made to incorporate other chemotherapeutic drugs, such as camptothecin, into the BST-gel system [128].

A hydrogel of dextran solutions grafted with L- or D-lactic acid, respectively, which assemble by stereocomplexation in an aqueous medium, was first developed by De Jong *et al.* [129] and has subsequently been commercialized by OctoPlus (Netherlands) as Octodex™. Once formed, the gel degrades rapidly into two harmless degradation products, lactic acid and dextran. Octodex hydrogels are particularly suitable for delivery of proteins and peptides because, unlike Octodex microspheres, they do not contain organic solvents, crosslink under mild conditions by stereocomplexation and only show a mild drop in pH during degradation [130]. InGell Delta (InGell Labs) is another dextran-backbone hydrogel, which assembles *in situ* by stereocomplexation of D- or L-oligolactate chains grafted separately to dextrans. As in the case of Octodex, InGell Delta is an excellent platform for protein delivery owing to mild preparation conditions, *in situ* gelation and subsequent degradation [131,132].

Limitations of injectable implants

The major limitation of most injectable implants is the high initial burst release. The amount of drug released owing to the burst effect can sometimes be higher than the recommended safety margin and, therefore, can cause tissue irritation, pain and toxicity. Several attempts have been made to minimize burst release by increasing polymer density and polymer concentration, altering the hydrophobicity of the injection medium and improving the response to environmental stimuli. Minimizing burst release by complexing or polymerizing the drug molecule has also been proposed [133]. Park *et al.* [134] have shown that, by complexing human growth hormone with poly-L-arginine or dimerizing it with zinc ions, the initial burst release of the drug can be sup-

pressed. Local toxicity might also be observed because of unreacted monomers as a result of incomplete implant formation. Moreover, unlike preformed implants, minimal control can be exercised over the shape and size of the *in situ*-formed systems [24], thus, variability in the rate of degradation of the polymer matrix and/or heterogeneous drug release might be observed, which again can contribute to over- and/or underdosing and/or toxicity. Although several novel imaging techniques have been used to evaluate implant formation *in vivo* [135], the complex and highly variable processes of implant formation, drug release and tissue response are difficult to predict.

Finally, although incorporation into polymer matrices often inhibits enzymatic denaturation of proteins, drug instability can also arise owing to polymer–protein interactions. Hydrophobic interactions between hydrophilic proteins and hydrophobic polymers can cause irreversible aggregation, resulting in reduced activity of the protein. Another cause for concern is the acidic microenvironment resulting from degradation of lactide and glycolide polymers, which again can initiate protein degradation [136–138]. Protein stability could be improved by using PEG-grafted poly-L-lactide copolymers, which are amphiphilic in nature [138]. The PEG side chain inhibits protein–polymer adhesion and, therefore, stabilizes the protein in aqueous media. *In situ*, this systems forms micro-sized gel implants with improved protein stability and release profile owing to shorter protein diffusion distance.

Concluding remarks

With the continuous advancement in biotechnology and DNA recombinant mechanisms for the development of protein and peptide drugs, the demand for efficient drug delivery methods is continuously increasing. *In situ*-forming injectable implant systems provide a suitable mechanism for delivery of proteins and peptides, not only because of their sustained release properties, but also their ability to protect macromolecules against degradation. With continuing efforts, injectable implants can further be optimized to respond to internal and/or external stimuli and, thus, modify their release characteristics. These systems promise a bright future for the formulation of protein and peptide drug delivery systems and research with various peptide hormones has already suggested that these systems are suitable as contraceptives or in hormone replacement therapy. Several researchers have investigated different polymer systems and their various gelling mechanisms to control implant shape and size and, therefore, the drug release kinetics. Combining two or more gelling approaches could further enhance the gelling efficacy of these systems and help to optimize drug release profiles.

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