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Protein-Polymer Conjugation—Moving Beyond PEGylation

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Abstract

In this review, we summarize —from a materials science perspective— the current state of the field of polymer conjugates of peptide and protein drugs, with a focus on polymers that have been developed as alternatives to the current gold standard, poly(ethylene glycol) (PEG). PEGylation, or the covalent conjugation of PEG to biological therapeutics to improve their therapeutic efficacy by increasing their circulation half-lives and stability, has been the gold standard in the pharmaceutical industry for several decades. After years of research and development, the limitations of PEG, specifically its non-degradability and immunogenicity have become increasingly apparent. While PEG is still currently the best polymer available with the longest clinical track record, extensive research is underway to develop alternative materials in an effort to address these limitations of PEG. Many of these alternative materials have shown promise, though most of them are still in an early stage of development and their *in vivo* distribution, mechanism of degradation, route of elimination and immunogenicity have not been investigated to a similar extent as for PEG. Thus, further in-depth *in vivo* testing is essential to validate whether any of the alternative materials discussed in this review qualify as a replacement for PEG.

Introduction

The seminal papers on protein PEGylation by Davis and coworkers in the late 1970's marked the beginning of an era of polymer conjugation to peptides and proteins to improve their delivery as therapeutics. These early studies established PEG as a "stealth" polymer, as defined by its ability to effectively prolong the *in vivo* circulation half-life and mask the immunogenicity of biomolecules [1,2]. Since then, the unique benefits of PEG have been profitably exploited by the pharmaceutical industry. With numerous PEGylated drugs approved by the Food and Drug Administration (FDA) to treat a variety of diseases and many more in clinical and pre-clinical development [3], PEGylation has become the most widely used approach to effectively improve the stability, solubility and pharmacokinetics and to reduce the immunogenicity of protein/peptide drugs. Over the years, PEG has been touted as a biocompatible and non-toxic material. However, after close to forty years of

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research and over two decades of clinical use, the limitations of PEG in clinical use have begun to emerge, which include its non-degradability and immunogenicity [4,5].

In applications where the molecular weight (MW) of the conjugated PEG is chosen to be greater than renal clearance so as to confer a long circulation half-life of the drug, the lack of degradation of PEG in systemic circulation may cause the polymer or the PEGylated conjugate to linger in circulation and eventually either clear through the liver or deposit in various tissues [6]. Several studies have shown that PEGylated proteins induced vacuole formation in organs including liver, kidneys and spleen [7-11]. One should keep in mind however, that in the majority of these studies, the PEGylated protein is bovine hemoglobin (bHb), which is known to be absorbed by the liver and spleen [11]. In addition, the various studies investigating this issue have reported some contradictory findings. In the case of bHb, while tests in a rat model showed that PEG-bHb but not unmodified bHb induced formation of vacuoles in the renal proximal convoluted tubules and splenic macrophages [10], another study conducted in a rabbit model showed the opposite result in the hepatocyte cytoplasm [9]. The same agents when tested in dogs all displayed renal tubular vacuole formation [7]. A separate study comparing chronic administration of a dimeric PEGylated TNF binding protein (PEG-TNFbp) and its non-PEGylated counterpart in rats showed that the PEGylated protein showed vacuolation in the renal cortical tubular epithelium while the non-PEGylated version did not. Vacuolation caused by high dosages of PEG-TNFbp was only partially reversible. Interestingly, when PEG was tested alone in two of these studies, it did not show any sign of vacuolation [8,10]. Thus, the currently available information is not sufficient to conclude whether there is indeed a cause-and-effect relationship between PEGylation and vacuolation. However, vacuolation observed with PEGylated proteins and its irreversibility associated with high dosages in some of these studies are sufficient cause for concern to warrant further investigation.

The immunogenicity of PEGylated drugs is another major—and somewhat controversial—concern that is currently under vigorous discussion. Studies have reported detection of anti-PEG antibodies in animal models and in patients treated with various PEGylated agents. Richter and Akerblom reported in 1983 that antibodies to PEG and ovalbumin were raised in rabbits immunized with PEGylated ovalbumin, though PEG alone of several different molecular weights (MWs) showed no or poor immunogenicity in rabbits and mice [12]. It is, however, also important to keep in mind that many therapeutic peptides and proteins of non-human origin are themselves immunogenic when administered in humans, and PEGylation can help mask the immunogenic epitopes on these molecules to reduce any immune response toward these therapeutics [6]. A case study in 2005 reported that a 45-year-old male patient with no known allergy developed severe hypersensitivity reaction within 2 min after receiving an intra-articular injection of a corticoid preparation containing 4 kDa PEG as excipient. Symptoms included nasal pruritus, conjunctivis and dizziness. Systematic testing revealed that the response was mediated by an IgE antibody that was specific to PEG [13]. Such a severe response upon the first injection suggests the possible presence of pre-existing anti-PEG antibodies in the patient. In fact, an early study conducted in 1984 reported that anti-PEG antibodies were detected in 0.2% of healthy blood donors and that the antibodies were predominantly of the Immunoglobulin M (IgM) isotype [14]. A more recent study by Garratty and coworkers reported a markedly higher, ~ 25% occurrence of

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anti-PEG antibodies, of both IgG and IgM isotypes, in 250 healthy blood donors [15]. The observed increase in the presence of PEG antibodies is likely due to the growing exposure of the general population to PEG in consumer and pharmaceutical products in recent years. Garratty reported that 60% normal donor sera agglutinated red blood cells modified with linear mPEG, which did not occur when cord sera from newborns were tested [16], further supporting this hypothesis. A direct consequence of PEG immunogenicity that adversely affects the therapeutic efficacy of PEGylated drugs is the accelerated blood clearance phenomenon. Rapid drug clearance has been reported in patients treated with PEG-asparaginase (up to a third of patients) [15] and PEG-uricase [17], which was strongly correlated with the presence of anti-PEG antibodies in both cases. In summary, while a definitive conclusion cannot be drawn about the immunogenicity of PEG in PEGylated therapeutics, its associated consequences raise serious concerns and stimulate rigorous investigations.

Thus, with the limitations of PEG becoming increasingly clear, the field is now witnessing a growing effort to search for alternative materials that either address the limitations of PEG or bring additional functional benefits to peptide/protein-polymer conjugates. This review aims to summarize the latest trends in the development of peptide/protein-polymer conjugates from a materials science perspective, by covering recent studies that have explored other polymers as alternatives to PEG (Figure 1). This review emphasizes studies that show promising *in vitro* and *in vivo* findings. We note that while some variability exists in the literature in terms of nomenclature of polymers, we will follow the naming convention of each individual article when discussing that work.

Non-Degradable PEG Alternatives

Poly(N-vinylpyrrolidone) (PVP)

As early as 1980, Shaltiel and coworkers had demonstrated that conjugating PVP to a group of protein isoallergens suppressed the level of allergen-specific IgE antibodies in treated mice [18]. However, the use of PVP for biomedical applications was limited by difficulty in synthesizing end-functional polymers with low dispersity, as the polymer was synthesized by conventional free radical polymerization. Recent reports by the Caruso [19] and Klumperman[20] groups on the successful synthesis of end-functionalized PVP's with low polydispersity by Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization and conjugation of the polymer to model biomolecules will likely stimulate increased interest in this polymer. Mayumi and coworkers compared various nonionic water-soluble polymers including PEG, PVP, polyacrylamide (PAAm), polydimethylacrylamide (PDAAm), polyvinyl alcohol (PVA) and dextran at the same MW for their potential as polymer modifiers of protein-based therapeutics. PVP proved to be the most promising candidate, as it showed the longest blood residence time and the lowest volume of tissue distribution. PVP conjugated to tumor necrosis factor-α (TNF-α) circulated longer than PEG-TNF-α at the same MW [21].

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Polyglycerol (PG)

The structural similarity of PG to PEG makes the polymer a logical candidate for biomedical applications. PG has demonstrated similar resistance to non-specific protein binding than PEG and a superior biocompatibility and toxicity profile than PEG [22,23]. Kizhakkedathu and coworkers showed that linear PG has a longer circulation half-life than several other water-soluble linear polymers of similar MWs tested previously, including PEG, while hyperbranched PG (HPG) has an even longer half-life, presumably due to its more compact structure [22]. The group also assessed the biocompatibility of high molecular weight (M_w ~ 120 kDa) LPG, hyperbranched (HPG) and linear PEG (M_w ~ 100 kDa) and found that LPG and HPG demonstrated excellent blood and cell compatibility while PEG showed dose-dependent activation of blood coagulation, platelets and the complement system, red blood cell aggregation and hemolysis, and cell toxicity [22]. Unfortunately, like PEG, PG is non-degradable. High MW HPG's (54,000 and 106,000 Da) have been shown to remain in the liver and spleen for at least 30 days post intravenous injection in mice [24]. A unique benefit of PG is that it carries multiple side-chain hydroxyls, which readily enables further functionalization or architectural variation [25]. A variety of architectures have been demonstrated with PG, including linear, hyperbranched [24], midfunctional, linear-block-hyperbranched [26] and side-chain functional [27]. Klok and coworkers conjugated PG with various architectures to lysozyme through squaric acid diethyl ester mediated coupling to reactive amine groups in the lysine side chains of the protein. A conjugate with midfunctional PG demonstrated significantly higher activity than its linear counterpart at a similar MW [26]. Harth and coworkers were the first to demonstrate *in situ* grafting of branched PG from Bovine Serum Albumin (BSA) using Ring Opening Polymerization (ROP) with the ability to control the degree of branching [28].

Poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA)

PHPMA is a neutral polymer generally accepted as nontoxic, biocompatible and nonimmunogenic [29,30]. The conjugation of PHPMA to proteins and peptides was limited until recently, because PHPMA was previously synthesized by conventional free radical polymerization that produced polymers with broad molecular weight distributions. Following the first report of synthesizing well-defined PHPMA with low polydispersity index (PDI< 1.1) using RAFT by the McCormick group [31], Davis and coworkers subsequently reported RAFT synthesis of PHPMA terminated with thiazolidine-2-thione that was conjugated to the amine groups of lysine residues in lysozyme [32]. In another study by the same group, RAFT was used to synthesize PHPMA polymer that was functionalized mid-chain with a thio-reactive pyridyldisulfide group. The polymer was subsequently conjugated to BSA and the mid-chain functionality was theorized to have an "umbrella-like" masking effect on the protein [33].

Polyoxazolines (POZs)

Early studies investigating the properties and *in vivo* fate of POZs have established this class of polymers as biocompatible materials with "stealth" properties [34,35]. Viegas and coworkers conjugated poly(2-ethyl 2-oxazoline) (PEOZ) of various MWs to granulocyte colony stimulating factor (G-CSF), a hematopoietic cytokine that regulates the growth and

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differentiation of hematopoietic progenitor cells to functionally activate the formation of mature neutrophils. An *in vivo* study in rats showed that an intravenously injected POZ conjugate with a 20 kDa MW polymer exhibited a 3-fold greater area under the curve (AUC) for neutrophil counts and up to 2 times higher AUC for white blood cell counts than the unmodified protein [36].

Veronese and coworkers synthesized POZs with methyl (PMOZ), ethyl (PEOZ) and propyl (PPOZ) side chains by living cationic polymerization and conjugated these polymers to BSA, catalase, ribonuclease, uricase and insulin. A 10 kDa MW PEOZ conjugated to insulin showed a 4-fold longer blood glucose control than unmodified insulin in naïve Sprague-Dawley rats. Furthermore, PEOZ conjugation to BSA more effectively masked the immunogenicity of the protein than conjugation of PEG of similar MWs [37]. Kabanov and coworkers synthesized amphiphilic block copolymers of POZ with methyl and butyl side-chains (PMOZ-b-PBOZ), which were then conjugated to horseradish peroxidase (HRP). The amphiphilic copolymers were shown to facilitate intracellular delivery of the protein in two different cell lines, a feature that PEG does not offer [38].

Degradable Synthetic PEG Alternatives

PEG-Based Degradable Polymers

The PEG-based, comb-like polymer, poly[oligo(ethylene glycol) methyl methacrylate] (POEGMA) has recently received much attention. Chilkoti and coworkers were the first to demonstrate grafting of a single chain of POEGMA from a defined site–the N or C terminus– of a protein using ATRP under aqueous conditions to yield site-specific and stoichiometric (1:1) conjugates with relatively low polydispersity and high yield [39–41●●]. These protein-POEGMA conjugates showed a 15–50 fold increase in blood exposure [39●●] and 50 fold increase in tumor accumulation [40●●] compared to the unmodified proteins upon intravenous administration to mice, thereby demonstrating that comb polymers that present short oligo(ethylene glycol) side-chains are a new class of PEG-like polymers that can significantly improve the pharmacological properties of proteins. In addition, POEGMA should, in principle, be biodegradable, as the OEG side-chains are connected to the methacrylate backbone by an ester linkage, which is susceptible to hydrolysis and enzymatic breakdown, and the degradation products are expected to be small enough to clear through renal filtration. Future studies are needed to prove these hypotheses.

A common approach to impart backbone degradability to PEG is by step-growth polymerization of telechelic, or di-end-functionalized PEG-based macromonomers. Several groups have demonstrated incorporation of reducible disulfide [42-44] and hydrolysable ester linkages[45,46] into the PEG backbone using this approach. However, polymers synthesized by this approach typically have a very broad MW distribution [47]. Anther approach is to polymerize PEG with comonomers that contain degradable linkages. The extent of degradation and the size of degradation products can be tuned by control of the fraction of comonomer in the resulting copolymer [48]. Lynd and coworkers demonstrated synthesis of poly[(ethylene oxide)-*co*-(methylene ethylene oxide)][P(EO-*co*-MEO)] by first copolymerizing EO with epichlorohydrin (ECH), followed by elimination of chloride to generate MEO, which contains hydrolysable vinyl ether bonds [48]. Cyclic ketene acetals,

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including 5,6-benzo-2-methylene-1,3-dioxepane (BMDO), 2-methylene-1,3- dioxepane (MDO), and 2-methylene-4-phenyl-1,3-dioxolane (MPDL), have been copolymerized with OEGMA by Nitroxide-Mediated Radical Ring-Opening Polymerization (NMP), to obtain PEG-based polymers with an adjustable amount of ester groups in the main chain. The synthesized polymers showed nearly complete hydrolytic degradation in 5% KOH aqueous solution, and were nontoxic to4, endothelial cells and macrophages [49].

Wooley and coworkers have synthesized amphiphilic graft copolymers of poly-(ε-caprolactone)-graft-poly(ethylene oxide) (PCL-g-PEO) by grafting 3 kDa PEO grafts and p-methoxybenzyl side chains onto the degradable polyester backbone of the statistical copolymer PCL-*co*-(2-oxepane-1,5-dione) via stable ketoxime ether linkages. Various compositions of the graft copolymer self-assembled into spherical and cylindrical micelles [50].

Another interesting approach was reported by Elisseeff and coworkers, where the Fenton reaction of hydrogen peroxide and ferric chloride at a neutral pH was utilized to partially degrade PEG to introduce hemiacetals randomly into its backbone, which is then hydrolyzable into aldehydes and alcohols under acidic conditions [51].

Santi and coworkers have developed a tunable PEG-based degradable depot for controlled release of peptide or protein drugs. The system consists of a hydrogel synthesized from multi-arm PEG macromonomers that are connected by β-eliminative linkers. Four arms of each macromonomer are cross-linked with neighboring macromonomers to form PEG hydrogels. Drugs are tethered to the remaining arms with similar linkers that cleave at slightly faster rates. The rate of drug release and depot degradation can be tuned by varying the chemistry of the linkers (Figure 2). When implemented with the peptide drug exendin-4 (exendin), extrapolation from in solution degradation results suggested that an optimized version of the system should provide an exendin depot requiring only once-a-month s.c. administration, which competes favorably with the currently longest-acting commercialized formulation that requires once-weekly s.c. injection [52●]. While the *in vitro* results are promising, more in-depth *in vivo* studies are necessary to further investigate the utility of this design.

Non-PEG-Based Degradable Synthetic Polymers

Poly(zwitterions) are polyelectrolytes that carry both positively and negatively charged groups with an overall neutral charge. They have recently emerged as a promising class of protein-resistant materials. Whitesides and coworkers showed that self-assembled monolayers of alkane thiols on gold surfaces that present zwitterionic —betaine and taurine — head groups were protein-resistant [53]. Building on these earlier results, Jiang and coworkers synthesized the zwitterionic brush polymers poly(sulfobetaine methacrylate) (PSBMA) and poly(carboxybetaine methacrylate) (PCBMA) on surfaces and demonstrated their excellent protein resistant properties [54-56]. A surface coating of PCBMA displayed superior resistance to non-specific protein adsorption from blood serum and plasma as compared to PEG [56]. The group subsequently conjugated PCBMA and PEG of similar MW and hydrodynamic radius (R_h) to the model enzyme α-chymotrypsin [57]. R_h-equivalent polymers are typically compared in these studies because poly(zwitterions) are

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known to have strong intramolecular ionic interactions, and thus adapt a more compact conformation compared to uncharged polymers. While PEGylation decreased the binding affinity of the enzyme to a peptide substrate, conjugation to PCBMA of similar R_h interestingly increased the binding affinity. This was suggested to be due to the ability of poly(zwitterions) to mediate ionic structuring of water, which draws water molecules away from hydrophobic regions of the protein, thus facilitating the interaction between the catalytic site in the enzyme and the substrate [57●]. Godwin and coworkers conjugated the zwitterionic polymer, poly(2-methyacryloyloxyethyl phosphorylcholine) (PMPC) and PEG with a similar R_h to interferon-α2a (IFN). The conjugates showed comparable *in vitro* anti-viral and anti-proliferative activity. In an *in vivo* pharmacokinetic study, however, PMPC-INF was found to have a longer half-life than PEG-INF [58]. Similar to POEGMA, the zwitterionic brush polymers that have been investigated are consisted of zwitterionic side-chains connected to (meth)acrylate backbones by ester linkages, which are susceptible to hydrolysis and enzymatic breakdown, rendering the polymers potentially side-chain degradable.

Polycarbonates have recently gained much interest for protein/peptide conjugation because of their biodegradability and synthetic ease [59]. The Hedrick group demonstrated that the cyclic carbonate monomer, pentafluorophenyl 5-methyl-2-oxo-1,3-dioxane-5-carboxylate (MTC-OC_6F_5), is a versatile building block for synthesis of functional polycarbonates by organocatalytic Ring Opening Polymerization (Figure 3). The active pentafluorophenyl ester on MTC-OC_6F_5 is amenable to substitution by suitable nucleophiles such as alcohols and amines to enable further functionalization with a large variety of moieties [60]. Side-chain functionalization can be done either pre- or post-polymerization, with the latter option offering more tolerance to different substituents [61]. The versatility of this highly modular approach was demonstrated by synthesis of a range of functional polycarbonates, which are of particular relevance to peptide/protein-polymer conjugates, such as polycarbonates with PEG [59], hydroxylcontaining [62●] and zwitterionic side-chains [63], all of which demonstrated promising protein-resistant properties and toxicity profiles.

Guan and coworkers approached the problem from a different perspective. Inspired by earlier structure-property studies conducted by Whitesides and Mrksich, which shed light on common properties found in protein-resistant materials including 1) hydrophilicity, 2) ability to accept hydrogen bonds, 3) inability to donate hydrogen bonds, and 4) net neutral charge [64,65], Guan and coworkers did some interesting work on rational design of degradable protein-resistant materials. They reported the development of a new carbohydrate-derived side-chain ether polymer with degradable ester bonds in the backbone, which was confirmed to have excellent protein resistance. While the polymer was synthesized by condensation polymerization in their initial report, which gave poor control over MW and high PDI, they later demonstrated that the polymer can be synthesized with good control of MW and PDI by living ROP of a permethoxylated ε-caprolactone [66].

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Naturally Degradable PEG Alternatives

Polysaccharides

In nature, glycosylation —the modification of proteins with carbohydrates— serves a number of functions, one of which is to stabilize and protect the proteins [67]. Inspired by nature, various groups have explored conjugation of polysaccharides to peptides and proteins. Duncan and coworkers conjugated dextrin to recombinant human epidermal growth factor (rhEGF) for wound healing applications. When testing the conjugates on epidermoid carcinoma (HEp2) cells, exposure to physiological concentration of α-amylase triggered dextrin degradation, which “unmasked” the bioactivity of rhEGF and effectively prolonged HEp2 proliferation for over 8 days [68].

Gregoriadis and coworkers conjugated the highly hydrophilic polysialic acid (PA), colominic acid (CA), to L-asparaginase by reductive amination. While there are concerns that conjugating the highly anionic PAs to proteins will alter the net surface charge, thus increasing the overall immunogenicity, pharmacokinetic studies of CA-asparaginase conjugates in mice with pre-existing anti-asparaginase antibodies showed that polysialylation in fact reduced the antigenicity of asparaginase and increased the circulation half-life of the enzyme by 3–4 fold [69]. In a later study by the same group, end-functionalized CA’s were conjugated to the amine groups on the N terminus and lysine residues in insulin. Compared to unmodified insulin, 39 and 22 kDa MW conjugates increased the duration of glucose control by 2- and 3-fold in normal female outbred T/O mice, respectively [70].

Hyaluronic acid (HA) has also been extensively used for peptide and protein conjugation. In addition to its biodegradability and an excellent safety profile, HA is highly amenable to functionalization, as it possesses four types of reactive functional groups: acetamide, carboxylic acid, hydroxyl and a terminal aldehyde [71]. Hahn and coworkers used different chemistries to conjugate various therapeutics to HA, including an agonistic peptide for the formyl peptide receptor like 1 (FPRL1) receptor for treating inflammatory diseases [72], exendin-4 for treatment of type 2 diabetes [73], and the angiogenesis inhibitor anti-fms-like tyrosine kinase-1 (anti-Flt1) peptide [74]. Because the conjugation chemistries used were not specific, a variable number of drug molecules per HA chain were obtained. Conjugation significantly extended the glucose-lowering effect of exendin in db/db mice for up to 3 days [73]. During a two-week treatment period in Sprague Dawley (SD) rats with corneas cauterized by silver nitrate, the HA conjugate of anti-Flt1 significantly enhanced inhibition of neovascularization compared to Avastin®, an FDA approved antibody that is an angiogenesis inhibitor, and the unmodified peptide [74].

Poly(amino acid)-Based Hybrid Materials

The polypeptide backbone of amino acid-based biomolecules is naturally enzymatically degradable. The concept of combining the “stealth” property of PEG with the intrinsic degradability of polypeptides was demonstrated in an early study by Schacht and coworkers. Poly[N-(2-hydroxyethyl)-L-glutamine] (PHEG) was synthesized and its hydroxyl groups were partially converted to reactive carbonate esters for subsequent conjugation with α-

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amino-ω-methoxy-PEG. The biodegradation of the polymer was confirmed in exposure to isolated rat liver lysosomal enzymes [75]. Li and coworkers later demonstrated that this class of hybrid polymers also displays a soluble-insoluble lower critical solution temperature (LCST) phase transition, and that the LCST was tunable by length of the PEG side-chain [76].

Recombinant Polypeptides

While synthetic polymers have long been the focus of peptide/protein-polymer conjugation, several recent studies have demonstrated that recombinant polypeptides are also excellent delivery platforms for peptide and protein therapeutics. The use of polypeptides instead of synthetic polymers for peptide/protein conjugation is advantageous in several ways, including: 1) the entire conjugate is genetically encoded and can thus be produced by standard recombinant expression without need for further chemical modification; 2) the end-product is monodisperse; 3) the length of the polypeptide can be precisely specified and tuned at the gene level; and 4) the conjugates are completely degradable into short peptides or amino acids.

Stemmer and coworkers demonstrated a simple approach to extend the circulation half-life of therapeutic peptides and proteins, by genetically fusing them to an unstructured recombinant polypeptide. Through rational design and repetitive screening, the group identified a polypeptide of choice, a single 864 amino acid long polypeptide that they named XTEN. Utility of the approach was demonstrated with exendin-4. Pharmacokinetics (PK) study of exendin-XTEN (E-XTEN) in cynomolgus monkeys showed a terminal half-life of 60 h. Glucose challenge in normal mice showed significantly longer glucose tolerance compared to unmodified exendin. Allometric scaling of PK results of E-XTEN in four different animal species predicted a half-life of 139 h in a 75 kg human. XTEN generated no substantial immune response, toxicity or kidney vacuoles in several animal models [77●●]. This technology is being commercialized by Amunix Operating Inc. and has been applied to a variety of therapeutic biomolecules [78-80] and an imaging agent [81]. Exendin-XTEN (VRS-859) is currently in a Phase I clinical trial for biweekly s.c. administration for treatment of type 2 diabetes and an XTEN fusion of human growth hormone (hGH-XTEN, VRS-317) is undergoing a Phase II clinical trial as monthly administration for treament of hGH deficiency [80].

More recently, Skerra and coworkers introduced the PASylation technology, in which a class of polypeptides consisted of randomized sequences of proline, alanine and serine (PAS) were developed as PEG mimetics for fusion to therapeutic biomolecules. Optimized PAS polypeptides were shown to be hydrophilic, uncharged and adapt disordered conformations and expanded hydrodynamic volumes. In the initial study, proof of concept was established with three model proteins: a recombinant Fab fragment of the humanized anti-HER2 antibody 4D5, human interferon α2b and hGH [82]. PAS polypeptides with various lengths were fused to the N or C terminus of the proteins at the genetic level, followed by recombinant expression of the fusion proteins in *E. coli* via periplasmic secretion. Interestingly, unlike PEGylated proteins, PASylated proteins showed almost quantitative retention of receptor-binding activities regardless of PAS polypeptide length.

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Pharmacokinetics of PASylated proteins assessed in BALB/c mice by i.v. injection showed up to 94-fold longer terminal half-life in the case of PASylated hGH in comparison to native hGH. The extent of half-life increase was found to be tunable by PAS polypeptide length. *In vivo* activity of PASylated hGH assessed in C57BL/6J-*Ghrhr*^{lit}/J growth retarded mice showed more than 6-fold increase in efficacy compared to hGH as assessed by body weight gain. Kidney, liver and spleen tissues harvested from mice treated with PAS-hGH daily for 10 d showed no histological abnormality. No immune reactivity toward the PAS moiety was detected in both immunization and repeated dose experiments [82]. XL-protein GmbH was founded based on PASylation and is currently applying the technology to a range of proteins and peptides for therapeutic and imaging applications [83–85].

Chilkoti and coworkers have exploited the unique LCST phase transition behavior of Elastin-Like Polypeptides (ELPs) to develop injectable depots for the sustained delivery of peptide therapeutics. ELPs are artificial polypeptides based on a pentapeptide repeat —Val-Pro-Gly-Xaa-Gly (VPGXG)— found in elastin, where X is any amino acid besides proline. Below their inverse transition temperature (T_t), ELPs are soluble in aqueous solution, but when the temperature is raised above their T_t, they undergo phase separation into an insoluble coacervate [86].

In addition to the common advantages of polypeptides mentioned above, ELPs offer many unique advantages that make them a particularly attractive platform for delivering therapeutic biomolecules. 1) The T_t of ELPs can be precisely tuned within a few degrees Celsius at the gene level by control of two orthogonal molecular parameters: the choice of the guest residue and ELP chain length [87,88]. 2) ELPs can be conveniently expressed in bacterial expression systems with high yield that in some cases exceeds 1 g/L in shaker flask [89]. 3) ELPs and their fusions can be purified to very high purity without the need for chromatography by inverse transition cycling (ITC), a batch purification process that the Chilkoti group had previously developed [90]. 4) *In vivo*, ELPs are biodegradable and non-toxic [91], and specific ELPs that have gone into clinical trials appear to be non-immunogenic. 5) Importantly, ELPs have a long circulation half-life ranging from 8-16 h depending on their composition and MW, which makes them attractive for the delivery of the many peptide and protein drugs that are rapidly cleared from circulation [91,92].

The unique LCST behavior of ELPs can be exploited by fusing a peptide or protein drug to an ELP that is soluble at room temperature but is designed to undergo phase transition when injected *in vivo*, driven by the change in temperature, resulting in the formation of an insoluble coacervate below the skin. The phase transition of ELP is reversible upon dilution such that in response to the concentration decay at the boundary layer of the coacervate's margins, the transitioned ELP slowly dissolves from its margins to the core, releasing ELP-drug fusions at a steady rate. The ability to form an injectable depot, a feature unique to ELPs among polypeptides, enables peptide and protein fusions to be released from a day to a week from a single s.c. injection. Chilkoti and coworkers demonstrated proof-of-concept of this approach with glucagon-like peptide-1 (GLP1), a peptide drug for treatment of type 2 diabetes [93●]. GLP1 has very short half-life of a few minutes [95], so that when injected subcutaneously, its *in vivo* efficacy, as judged by control of glucose levels in blood lasts only for 1-2 h. In contrast, a GLP1-ELP fusion that forms a depot in the s.c. space provided

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5 days of glucose control in mice after only a single s.c. injection. The importance of depot formation for long-term glucose control was underscored by a control, a GLP1-ELP fusion, where the ELP was designed with a T_t above body temperature, so that upon injection the fusion remained soluble in the s.c. space; this fusion only provided up to 24 h of glucose control.

Chilkoti and coworkers have also developed another system using ELPs that allows sustained release of the active peptide drug from an injectable depot, as opposed to the release of an ELP fusion. Proof-of-concept of this technology, that they named protease-operated depot (POD), was demonstrated by sustained release of GLP1 from a s.c. depot. The POD consisted of six consecutive repeats of GLP1 fused to an ELP (Figure 4a). The sequence of the ELP was optimized such that when injected s.c., the peptide oligomer-ELP fusion undergoes its LCST transition and forms a viscous, insoluble coacervate under the skin that serves as a drug depot. The linkages between GLP1 molecules were engineered to be cleavable by a protease to release intact GLP1 monomers over time (Figure 4b). The best performing POD system showed up to 120 h of glucose control in mice (Figure 4c), in stark contrast to unmodified GLP1, which only provides glucose reduction for 1 h (Figure 4d) [94●●].

The ELP fusion technology has been licensed to a start-up company, PhaseBio Pharmaceuticals, which is developing ELP fusions of peptide drugs for sustained delivery. PhaseBio has successfully taken this technology through a Phase II clinical trial for the delivery of GLP1 as a fusion with a depot-forming ELP, and a Phase I clinical trial of an insulin-ELP fusion. A vasoactive intestinal peptide -ELP fusion is in preclinical development, and will soon enter clinical trials.

Functions Beyond PEG-Like Properties

In an effort to further expand the repertoire of peptide/protein-polymer conjugation, recent studies have introduced new materials that offer functions beyond those offered by PEG. For example, oral delivery of peptides and proteins to the GI tract is particularly challenging, as it requires the protection of these therapeutics in an environment that was naturally evolved to denature them. PEG is ineffective in stabilizing orally delivered peptide and protein therapeutics in the upper gastrointestinal (GI) tract, particularly in the stomach, due to its charge neutrality and simple linear architecture. To address this challenge, Leroux and coworkers synthesized a polycationic dendronized polymer poly(3,5-bis(3-aminopropoxy)benzyl)-methacrylate (PG1) and conjugated it to a bacterial proline-specific endopeptidase. It was shown that while the unmodified enzyme is readily deactivated in the stomach, polymer conjugation stabilized it for over 3 hours and promoted mucosal adhesion of the enzyme in the stomach of female Sprague-Dawley rats. The observed benefits were attributed to the high positive charge density and dendronized architecture of PG1, which the authors speculated protect the enzyme from inactivation and promote interactions with mucin on the stomach wall [96].

Combining polymers with carbohydrates gives rise to a powerful class of hybrid materials that offers new and advanced functions. Complex carbohydrate structures of glycoproteins

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on host cell surfaces provide a binding site for many pathogens, including bacteria. Multivalent carbohydrate ligands can act as effective antagonists to carbohydrate-binding proteins, or lectins, and can thereby inhibit pathogenicity. Davis and coworkers conjugated a proteinase, subtilisin, to branched structures displaying one to four carbohydrate-tipped antennae. The resulting conjugates, capable of both binding lectins and degrading adhesion proteins, demonstrated nanomolar potency in inhibiting the co-aggregation of *A. naeslundii* with co-pathogen *Streptococcus oralis* [97]. In a similar study, Haddleton and coworkers synthesized a library of well-defined glycopolymers containing different ratios and densities of mannopyranoside and galactopyranoside using a combination of living radical polymerization and Huisgen [2+3] cycloaddition. A BSA conjugate of this polymer showed dose-dependent lectin-binding and significantly enhanced capacity to activate complement via the lectin pathway compared to unmodified BSA [98]. Finn and coworkers demonstrated conjugation of glycopolymers to the surface of viruses for multivalent presentation of lectin-binding carbohydrates. The glycopolymer-decorated viruses showed extraordinarily high binding affinities for lectins, and have potential application for delivery of drugs to cancer tissues that overexpress carbohydrate receptors[99].

Conclusion

PEGylation has been under the spotlight of pharmaceutical research and clinical development for long enough that some of its limitations are now beginning to emerge, which include lack of degradation, immunogenicity and limited functionality. Alternative polymers are hence being pursued in an effort to address these limitations and advance beyond the properties conferred by PEG-conjugates. Polymers that have been investigated include PVP, PG, PHMA and POZ's, and some of them appear to display superior "stealth" properties and safety profiles than PEG, though we note that these findings are still early and in many cases need further substantiation. The lack of *in vivo* routes of degradation of most of these materials still remains a concern. Thus, various approaches are under investigation to enable side-chain or backbone degradation of these polymers. While earlier approaches typically introduced backbone degradable linkages via polycondensation of telechelic monomers, recent efforts focused on intrinsically degradable polymers such as polycarbonates. Natural polymers such as polysaccharides and polypeptides that degrade into products naturally found in the body have also been explored. Polypeptide fusions of therapeutic proteins and peptides have the unique advantage that they can be entirely genetically encoded, which enables the facile synthesis of monodisperse conjugates with precisely tunable MW and easy scale-up by leveraging the capabilities of the biotechnology industry in manufacturing biologic drugs. Moving forward, several groups have started to expand the arsenal of peptide/protein-conjugation, by developing materials that have functions beyond those offered by PEG, such as combining synthetic polymers with functional carbohydrate motifs that can mediate active receptor binding.

In conclusion, the studies covered in this review give an overview of research on developing alternative materials for peptide/protein-polymer conjugation that either attempt to address some of the limitations of linear PEG or advance beyond its function. We note that despite its emerging limitations, PEG continues to be the most widely accepted polymer in clinical use for conjugation to therapeutic peptides and proteins to date. While some of the materials

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discussed here show promise, many have only been tested *in vitro* or primarily for their *in vivo* half-life extension properties. Other important features such as the immunogenicity and biological fate of these alternative materials have not been investigated to a similar extent as for PEG. Hence, extensive *in vivo* testing of these polymers is essential to validate whether any of them can replace the current gold standard—PEG— in pharmaceutical applications.

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● of special interest

●● of outstanding interest

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Highlights

- Limitations of conjugating poly(ethylene glycol) to proteins and peptides have become increasingly apparent.
- Various polymers have shown promise as PEG alternatives.
- Extensive future *in vivo* testing is essential to validate their suitability to replace PEG.

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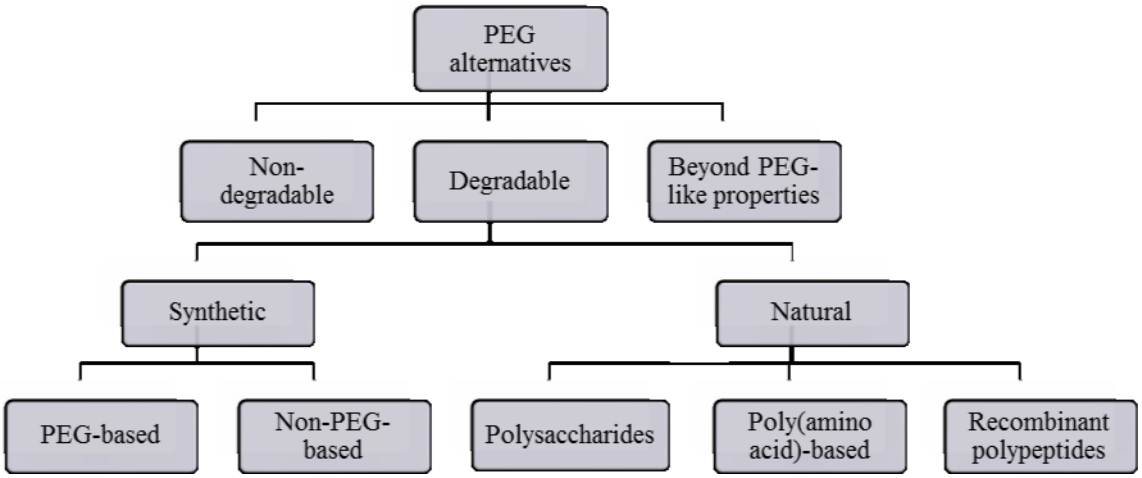

Figure 1.

Different types of polymers that have been investigated as alternatives to PEG. This schema is followed in the article.

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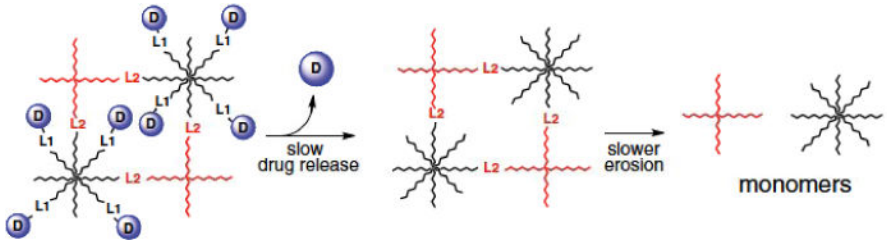

Figure 2.

Hydrogels formed by tethering drugs to and cross-linking multi-arms of PEG macromonomers with cleavable β-eliminative linkers. Varying chemistry of the linker enables tunable drug release and depot degradation [52●]. Copyright 2013 Santi and coworkers.

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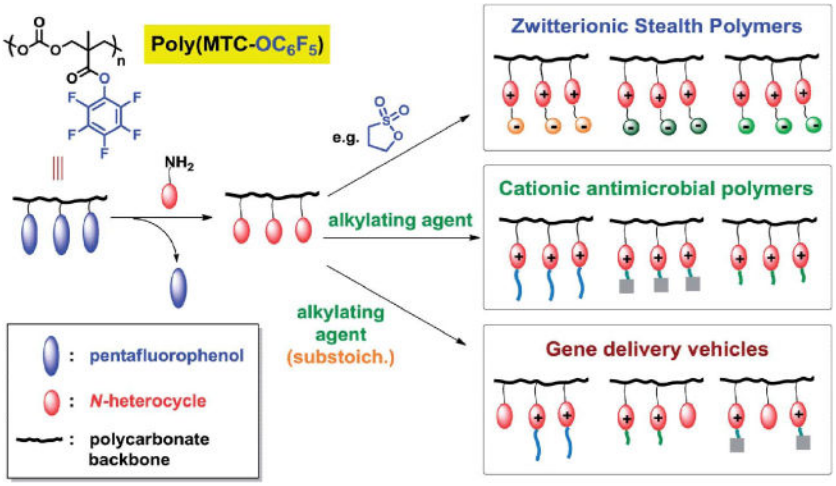

Figure 3.

Modular synthesis of a variety of functional polycarbonates using MTC-OC_6F_5 as a precursor monomer. As illustrated, MTC-OC_6F_5 is first polymerized via ROP. The pentafluorophenol side groups can undergo nucleophilic substitution by a primary amine connected by an aliphatic linker to a weakly nucleophilic N-heterocycle (eg. imidazole or pyridine). Selective chemical modification of the heterocycles can then be carried out using desired alkylating agents to generate polycarbonates with a diverse range of side-chain chemistries. Reprinted with permission from Hedrick and coworkers [62●]. Copyright 2014 American Chemical Society.

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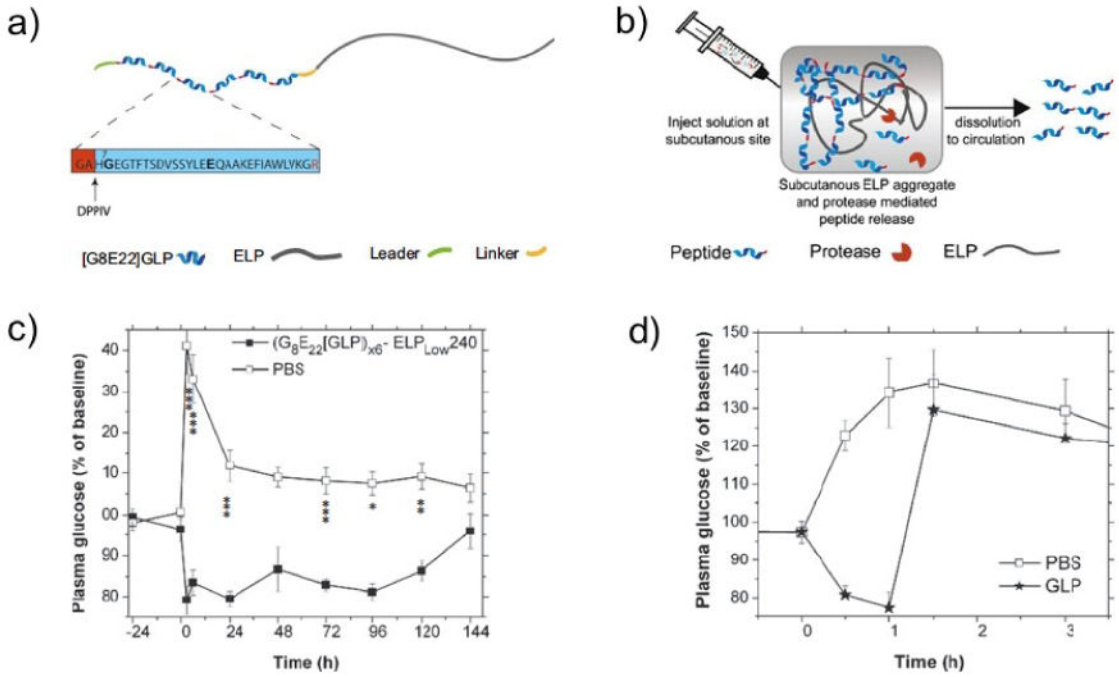

Figure 4.

Design and Characterization of GLP1 POD: a) Fusion protein in GLP1 POD design in which six consecutive repeats of GLP1 connected by protease-cleavable linkages are genetically fused to a thermally responsive ELP. b) Subcutaneous injection of the GLP-ELP POD triggers coacervation of the construct to form a depot. Protease cleavage releases monomeric GLP1 from depot over time. Daily fed blood glucose levels before and after a single s.c. injection of c) an optimized POD construct and d) GLP1 in C57BL6/J mice [94●●]. Copyright 2013 Chilkoti and coworkers.