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PERSPECTIVE

Peptide/Protein−Synthetic Polymer Conjugates: *Quo Vadis*

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ABSTRACT: Peptide/protein−synthetic polymer conjugates, which covalently combine one or more copies of a peptide sequence or protein with one or more synthetic polymer elements, offer unique possibilities to integrate the properties and functions of bio(macro)molecules and synthetic polymers in a single hybrid material. This article provides a status report of the field of peptide/protein−synthetic polymer conjugates. First, the main synthetic strategies for the preparation of peptide/protein−synthetic polymer conjugates will be discussed. In the last two sections, selected properties and applications of peptide−synthetic polymer conjugates and protein−synthetic polymer conjugates will be highlighted.

Introduction

Peptide/protein−synthetic polymer conjugates are hybrid materials which covalently combine one or more copies of a peptide sequence or protein with one or more synthetic polymer elements.[1,2] Combining peptides/proteins with synthetic polymers in a single hybrid material is of interest as it provides unique opportunities to combine the best of two worlds. This is illustrated in Table 1, which lists several characteristic properties of peptides/proteins and synthetic polymers. The properties in Table 1 are, rather arbitrarily, classified as strengths or weaknesses. It is, of course, obvious that the classification of a certain property as a strength or a weakness depends very much on the specific application. Nevertheless, the overview in Table 1 suggests that judicious combination of peptide/protein elements with synthetic polymers is an interesting strategy to synergistically combine the properties of these different classes of materials and to overcome some of their limitations.

Table 2 provides a brief overview of the early history of the field. The first reports of a peptide−synthetic polymer conjugate go back to the early 1950s.[3,4] These articles described the synthesis of a polyvinylpyrrolidone−glycyl-L-leucine−mescaline conjugate, which was designed as a depot to allow enzyme-mediated drug release. In the 1970s, Davis and Abuchowski found that covalent attachment of poly(ethylene glycol) (PEG) to bovine serum albumin (BSA) and bovine liver catalase was a useful strategy to reduce or eliminate the immunogenicity of these proteins and increase their blood circulation times.[5,6] Another therapeutic benefit of combining proteins and synthetic polymers was discovered in the mid-1980s by Maeda et al., who studied the pharmacokinetics of conjugates of the antitumor protein neocarzinostatin (NCS) and styrene−maleic anhydride copolymers, which were designated as smancs.[7−9] These conjugates were found to preferentially accumulate in tumor tissue due to a passive targeting mechanism, which was referred to as the enhanced permeability and retention (EPR) effect. Other early examples of peptide−synthetic polymer conjugates include a variety of hybrid di- and triblock copolymers prepared by ring-opening polymerization of α-amino acid *N*-carboxyanhydrides (NCA's) using appropriate synthetic polymer macroinitiators.[10−14] The interest in these polymers, which were regarded as model substances for biological systems, was primarily in their structure-forming properties.

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The aim of this Perspective is to provide a status report of the field: highlighting recent advances and identifying possible bottlenecks and areas for further research. The remainder of this paper consists of three sections. First, the main synthetic strategies for the preparation of peptide/protein−synthetic polymer conjugates

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Table 1. Characteristic Properties of Peptides/Proteins and Synthetic Polymers, Highlighting the Potential Benefits of Combining These Different Classes of Polymers in a Single Hybrid Material[a]

	peptides/proteins		synthetic polymers
+	hierarchical structure formation	−	"limited" control over nanoscale structure
+	recognition and binding (active targeting and adhesion)	−	biologically "inactive"
−	toxicity	+	biocompatibility
−	immunogenicity	+	resistance to enzymatic degradation
−	enzymatic degradation	+	increased blood circulation time ("stealth effect"; poly(ethylene glycol))
		+	passive targeting (EPR effect)

[a] Properties are, rather arbitrarily, classified as strength (+) or weakness (−). The specific attribution of a property as a strength or a weakness, of course, very much depends on the particular application of a material.

Table 2. Overview of Early Developments in the Field of Peptide/Protein−Synthetic Polymer Conjugates

year	author(s)	contribution	reference(s)
1950s	Jatzkewitz	first report of a peptide−synthetic polymer conjugate (polyvinylpyrrolidone−mescaline)	3, 4
1960s/1970s	Davis et al.	protein PEGylation	5, 6
1970s	Spach, Gallot, Yamashita et al.	peptide−synthetic hybrid block copolymers (via ring-opening polymerization of α-amino acid *N*-carboxyanhydrides)	10−14
1980s	Maeda et al.	enhanced permeability and retention effect (passive targeted delivery of therapeutic protein−synthetic polymer conjugates)	7−9

will be briefly reviewed and selected advances highlighted. Over the past years, significant progress has been made in the synthesis of peptide/protein−synthetic polymer conjugates. As an in-depth discussion of all these developments would go beyond the scope of this Perspective, only a selected number of examples that are believed to be of particular interest will be discussed. The final two sections will focus on properties and applications and successively discuss peptide−synthetic polymer conjugates and protein−synthetic polymer conjugates. The properties and applications that will be discussed in these last sections are not meant to be exhaustive, but rather represent a (personal) selection that will hopefully stimulate further work in the area.

Synthesis

Table 3 presents an overview of the main synthetic approaches that are available for the preparation of peptide/protein−synthetic polymer conjugates. The approaches listed in Table 3 are classified according to the method used for the preparation of the peptide/protein part as well as with respect to the conjugation strategy used. The three main techniques that are used for the synthesis of the peptide/protein part are (i) α-amino acid *N*-carboxyanhydride (NCA) ring-opening polymerization, (ii) solid phase peptide synthesis (SPPS), and (iii) protein biosynthesis. Each of these techniques has specific advantages and drawbacks, which will be discussed below. The synthesis of the conjugates can be performed in a convergent or divergent fashion. Convergent synthesis involves the coupling of presynthesized peptide/protein and synthetic polymer building blocks. Divergent synthesis can be carried out in two ways: (i) by polymerization of synthetic monomers using a peptide/protein macroinitiator and (ii) by synthesizing a peptide on a soluble or solid-supported synthetic polymer. The remainder of this section will briefly review the three main peptide/protein synthetic methods, in particular from the point of view of conjugate synthesis, and highlight recent advances.

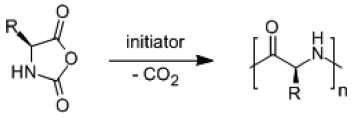
NCA Ring-Opening Polymerization. The NCA ring-opening polymerization (Scheme 1) is the most commonly applied technique for the large-scale preparation of synthetic polypeptides and polypeptide (hybrid) block copolymers. This method uses simple starting materials, allows the preparation of high molecular weight polypeptides, and does not affect the stereochemistry at the chiral center of the amino acid building blocks.[15−18] Like most other polymerization reactions, the NCA ring-opening polymerization yields neither perfectly monodisperse nor sequence specific polypeptides. This strategy has been successfully used, however, for the preparation of a broad variety of (hybrid) block copolypeptides and other complex polypeptide architectures.[18] NCA's are very sensitive monomers, and polymerizations carried out with traditional nucleophilic (primary amines) or basic (tertiary amines, alkoxides, hydroxides) initiators typically proceed via multiple competitive chain growth pathways, which obviously restricts control over polymer architecture as well as molecular weight and molecular weight distribution. Over the past decade, however, a number of techniques have been developed that allow to overcome these problems and enable a "living" NCA polymerization. Most of these techniques focus on controlling the reactivity of the growing polymer chain end. One approach to achieve a "living" NCA ring-opening polymerization involves the use of transition-metal-based initiators such as Ni(COD), $(PMe_3)_4Co$, or (dppe)Pt(MBS-H).[19−21] Other initiators that have been reported to promote "living" NCA polymerization include primary amine hydrochlorides[22] and *N*-trimethylsilylamines.[23,24] When high-vacuum conditions are used to purify solvents and monomers and to carry out the polymerization reaction, primary amines have also been demonstrated to allow polymerization of NCA's in a living fashion.[25]

Most of the peptide−synthetic polymer conjugates that have been prepared via NCA ring-opening polymerization are AB or ABA type hybrid block copolymers.[18] These hybrid block copolymers are usually obtained in a two-step divergent strategy that involves the use of primary amine end-functionalized synthetic polymeric macroinitiators to start the NCA polymerization.[18,26,27] Over the past few years, however, a number of alternative pathways have also been explored. Deming et al. have demonstrated that amido−amine nickelacycle end groups can be incorporated in synthetic polymers, which can subsequently be used as macroinitiators for the controlled NCA polymerization.[28−30] Well-defined peptide−synthetic hybrid block copolymers have also been prepared via NCA ring-opening polymerization using *N*-trimethylsilylamine[24] or primary amine hydrochloride[22,31] end-functionalized macroinitiators or by means of high-vacuum polymerization techniques and conventional primary amine initiators.[32] Most of the hybrid block copolymers reported so far have been obtained using presynthesized macroinitiators. An interesting strategy that obviates the need to separately prepare a synthetic polymeric initiator is the use of dual, or bifunctional, initiators. This approach has been pioneered by

Table 3. Overview of the Main Synthetic Strategies for the Preparation of Peptide/Protein−Synthetic Polymer Conjugates

	conjugate synthesis	
peptide/protein synthesis	convergent	divergent
α-amino acid *N*-carboxyanhydride (NCA) ring-opening polymerization	• click coupling of α-alkyne/α-azido polypeptides with alkyne/azide functionalized nonpeptidic polymers[38−40]	• NCA ring-opening polymerization using primary amine,[18,26,27,32] amido−amine nickelacycle,[28−30] *N*-trimethylsilylamine,[24] or primary amine hydrochloride[22,31] macroinitiators • one-pot synthesis using dual initiators for NCA ring-opening polymerization and NMP/ATRP[33−35]
solid phase peptide synthesis (SPPS)	• N-terminal PEGylation of solid supported peptides[44−47] • Solution click coupling of azide/alkyne chain end/side chain functionalized synthetic polymers with azide/alkyne functionalized peptides[48−51]	• SPPS of the peptide segment on a PEG modified solid support[45,52−54] • controlled radical polymerization of vinyl monomers using peptide-based initiators or chain transfer agents[57−67] • (controlled) polymerization of peptide-functionalized monomers[68−74]
protein biosynthesis	• protein PEGylation[82,84,85] • bioorthogonal (Staudinger ligation,[86] click chemistry,[49,87] and oxidative coupling[88]) conjugation of appropriate functional proteins and synthetic polymers	• ATRP or RAFT polymerization of vinyl monomers using protein-based macroinitiators or chain transfer agents[90−95]

Scheme 1



Heise, Menzel, and co-workers, who have developed various initiators that allow (controlled) NCA polymerization as well as the controlled polymerization of vinyl monomers such as styrene or methyl methacrylate via nitroxide-mediated polymerization (NMP) or atom transfer radical polymerization (ATRP) in one pot.[33−35] In addition to divergently growing a polypeptide block from a synthetic polymer initiator, peptide−synthetic hybrid block copolymers can also be prepared by convergent coupling of presynthesized polymer segments. A very attractive reaction for the convergent synthesis of peptide−synthetic hybrid block copolymers is the Huisgen 1,3-dipolar cycloaddition reaction ("click chemistry"). This reaction not only is high yielding but also is compatible with a broad range of functional groups,[36,37] which makes it particularly attractive for the synthesis of biopolymer conjugates. The use of click chemistry for the convergent synthesis of peptide−synthetic hybrid block copolymers was first described by Taton, Lecommandoux, and co-worker, who used this strategy to prepare poly(γ-benzyl-L-glutamate)-*b*-poly(2-(dimethylamino)ethyl methacrylate) (PBLG-*b*-PDMAEMA) diblock copolymers[38] and has been used to prepare various other hybrid block copolymers since then.[39,40]

Solid Phase Peptide Synthesis. Solid phase peptide synthesis nowadays is a routine technique that allows access to peptides containing up to 50 amino acid residues with perfect control over chain length and monomer sequence.[41,42] For the convergent synthesis of peptide−synthetic polymer conjugates, either via selective modification of a suitable end-reactive polymer or via functionalization of appropriate reactive side chains, a broad range of reactions is available. A detailed overview of these coupling conditions has been published in a recent overview.[43] Traditional peptide coupling conditions are frequently used for the synthesis of peptide−synthetic polymer conjugates. These reaction conditions are attractive as they generally provide high conversions and are compatible with standard solid phase peptide synthesis protocols. N-terminal modification of solid-supported peptides with carboxylic acid-modified poly(ethylene glycol) (PEG) derivatives, for example, has been widely used to prepare a variety of peptide−synthetic polymer conjugates.[44−47] A drawback is that these reactions are usually not bioorthogonal and cannot be used to synthesize conjugates in homogeneous (aqueous) solution from unprotected peptides. There is, however, a plethora of bioorthogonal coupling reactions that can be used for this purpose. Examples include various Pd^0-catalyzed coupling reactions, Staudinger ligation, cycloaddition reactions (Diels−Alder, 1,3-dipolar cycloaddition), reductive alkylation, oxime and hydrazone formation, thiol addition reactions, and oxidative coupling.[43] Although various of these strategies have been used to prepare protein−synthetic polymer conjugates (vide infra), only the Huisgen 1,3-dipolar cycloaddition reaction has been employed in several instances for the synthesis of peptide−synthetic polymer conjugates.[48−51] It is obvious that the range of other bioorthogonal coupling reactions that is available provides ample opportunities for the synthesis of novel, complex peptide−synthetic polymer conjugates without the need for possible complex protective group strategies.

The divergent synthesis of peptide−synthetic polymer conjugates can be carried out on the solid phase as well as in homogeneous solution. Divergent solid phase synthesis of peptide−synthetic polymer conjugates is frequently carried out using commercially available Tentagel resins in which poly(ethylene glycol) chains are attached to the solid support via a labile linker.[45,52−54] To avoid aggregation and facilitate the synthesis of difficult peptide sequences, so-called switch ester segments can be introduced in the peptide backbone. After completion of the synthesis, the peptide backbone can be re-established via a selective O → N acyl switch.[52] In addition to the use of switch ester segments, there are various other strategies that can be used to prepare difficult peptide sequences, including the use of backbone amide protecting groups[55] or microwave synthesis.[56] These latter two approaches, however, to the best of our knowledge have not yet been explored for the preparation of peptide−synthetic polymer conjugates. Instead of grafting the peptide segment step-by-step from a soluble or insoluble synthetic polymer support, the synthetic polymer segment can also be grown from appropriate initiator modified resin-bound peptides using controlled radical polymerization techniques.[57−59] These polymerization techniques have also been used to

synthesize peptide−synthetic polymer conjugates in homogeneous solution. Using peptide-functionalized chain transfer agents (CTA's), for example, peptide−synthetic polymer conjugates can be prepared via reversible addition−fragmentation chain transfer (RAFT) polymerization.[60,61] While the peptides may be attached to either the Z or the R group of the CTA, the latter approach is particularly attractive as it can be used to generate thiol end-functionalized peptide−synthetic polymer conjugates. The thiol end group represents a versatile handle for further bioorthogonal chain end functionalization and can also be used to coat gold substrates with a monolayer of the conjugates. Several reports have described the synthesis of peptide−synthetic polymer conjugates using cyclic or linear peptide ATRP initiators.[62−66] These ATRP initiators were typically obtained by modification of the N- or C-terminal amino acid or a suitable side-chain functional group with the appropriate ATRP initiator. Recently, Maynard et al. have taken this concept a step further and synthesized artificial amino acids with side-chain ATRP initiators that are compatible with standard Fmoc SPPS.[67] With these amino acids it is now possible to synthesize peptide initiators with exact control over the conjugation site without having to rely on the occurrence of a particular amino acid in that peptide or the use of elaborate protective group strategies.

A final, divergent approach for the synthesis of peptide−synthetic polymer conjugates is based on the polymerization of peptide-based monomers.[43] A variety of peptide-based monomers have been polymerized via conventional free radical polymerization,[68,69] ring-opening methathesis polymerization,[70−72] or controlled radical polymerization.[73,74]

Protein Biosynthesis. Some of the limitations of the NCA ring-opening polymerization (no precise control over chain length and monomer sequence) and SPPS (maximum chain length restricted to ~50 amino acids) can be overcome by using biosynthetic methods. By introducing an appropriately engineered recombinant plasmid into a bacterial host, this technique can be used to produce high molecular weight proteins with precisely controlled chain lengths and monomer sequences.[75] Over the past two decades, the scope of protein biosynthesis has significantly expanded with the development of techniques that allow the incorporation of a broad range of noncanonical α-amino acids.[76−81] A variety of these noncanonical α-amino acids contain functional groups that can act as reactive handles for bioorthogonal modification reactions. This is very attractive from the point of view of protein−synthetic polymer conjugate synthesis as it allows precise control over the conjugation site without having to rely on the frequency of occurrence or average surface accessibility of a certain amino acid residue.

The first protein−synthetic polymer conjugates were prepared by Davis et al. and were obtained following a convergent strategy that involved reaction of a PEG-dichlorotriazine derivative with bovine liver catalase or bovine serum albumin.[5,6] Over the past three decades PEGylation has developed into a well-established technology to modify therapeutic proteins and improve their stability and solubility, reduce renal clearance, enhance circulation half-life, reduce immunogenicity and antigenicity, and prevent proteolytic degradation. Nowadays, a variety of PEGylated protein based drugs are available on the market, including blockbuster drugs such as Pegintron, Pegasys, Neulasta, and Mircera which are used to treat diseases such as Hepatitis C, cancer, and others.[82] It is worth mentioning that the conjugation chemistries that have been developed to attach PEG to proteins have also found use to prepare hybrids of proteins with various other synthetic polymers.[83] The first-generation PEGylation agents, which in addition to PEG-dichlorotriazine included PEG-tresylate, PEG-succinimidyl carbonate, PEG-benzotriazole carbonate, PEG-*p*-nitrophenyl carbonate, PEG-trichlorophenyl carbonate, PEG-carbonylimidazole, and PEG-succinimidyl succinate, were effective in generating the desired conjugates but also suffered from several drawbacks. These were due to PEG impurities, the restriction to low molecular weight PEG derivatives, the formation of unstable linkages, and lack of selectivity in modification.[84] The latter is due to the fact that most PEGylating agents are reactive toward multiple amino acid side-chain functional groups. Since most proteins contain multiple copies of a certain amino acid residue, this makes it difficult to control the residue and site selectivity of the conjugation reaction. Some of these problems can be overcome by using what are referred to as second-generation PEGylation agents. Examples include PEG-aldehyde derivatives, which react selectively with amine groups, as well as PEG-maleimide, PEG-vinylsulfone, PEG-iodoacetamide, and PEG-*o*-pyridyl disulfide reagents that have been developed for the PEGylation of cysteine thiol groups.[84] These second-generation PEGylation reagents result in stable linkages, do not suffer from PEG impurities, and are not restricted to low molecular weight PEG derivatives. Second-generation PEGylating agents also improve control over the site selectivity of the PEGylation reaction. PEG aldehyde derivatives selectively react with amine groups, and by appropriately adjusting the pH, these reagents can be used to selectively PEGylate the N-terminus of proteins.[85] Cysteine selective PEGylation agents are attractive due to the relatively low overall natural abundance of this α-amino acid, which facilitates the synthesis of well-defined protein−synthetic polymer conjugates. While the second-generation PEGylating agents represent a significant advancement, the site and extent of PEGylation are still predominantly controlled by the natural occurrence of the target amino acid in the protein of interest. The possibility of modern biosynthetic methods to produce proteins that contain noncanonical α-amino acids with bioorthogonal side-chain functional groups at precisely defined positions in the protein primary structure opens the way to truly well-defined protein−synthetic polymer conjugates. A wide range of bioorthogonal coupling reactions is available for the synthesis of such precision protein−synthetic polymer conjugates,[43] and the use of several of these reactions has already been reported in the literature. Chaikof et al., for example, used the Staudinger ligation reaction to site-specifically PEGylate a C-terminal azido-methionine thrombomodulin mutant using a triarylphosphine-functionalized PEGylation agent.[86] Bovine serum albumin (BSA) is an attractive candidate for the synthesis of well-defined protein−synthetic polymer conjugates since it exposes a single free thiol group at Cys-34. This thiol group has been used to prepare acetylene-functionalized BSA derivatives, which were subsequently coupled in a convergent fashion with azide-functionalized synthetic polymers that were obtained via ATRP or RAFT polymerization.[49,87] Francis et al. reported the convergent bioorthogonal synthesis of protein−synthetic polymer conjugates via oxidative coupling of an aniline-modified protein and a phenylenediamine-functionalized PEG derivative.[88] Since techniques have been developed to produce proteins that contain the noncanonical α-amino acid 4-aminophenylalanine,[89] this represents another strategy to produce precision protein−synthetic polymer conjugates. Given the possibilities to produce proteins that contain noncanonical α-amino acids, however, it seems that the full potential of modern bioorthogonal

Table 4. Overview of Selected Functions and Applications of Peptide−Synthetic Polymer Conjugates

drug and gene delivery	• peptide−synthetic hybrid block copolymers as carriers for drug and gene delivery[98−100] • functionalization of synthetic polymers with peptide sequences to allow targeted delivery[104,105] and guide intracellular trafficking[106,107,109]
mechanical properties	• silk-inspired peptide−synthetic hybrid multiblock copolymers[112,113] • modulation of the mechanical properties of synthetic polymers by blending self-assembled peptide−synthetic polymer hybrid nanofibers[114]
hydrogels	• hydrogel formation driven by peptide self-assembly; coiled coil formation,[115−119] entanglement of β-sheet peptide based peptide−synthetic polymer nanotubes[120] • bioactive hydrogels: incorporation of cell-adhesion peptide sequences and protease substrates[121,122]
directing mineralization	• PEG-*b*-PAsp and PEG-*b*-PGlu as additives for the mineralization of $CaCO_3$ and $BaSO_4$[127−129] • peptide−synthetic polymer nanotapes as a template for the formation of silica composite nanofibers[130]

coupling techniques for the synthesis of protein−synthetic polymer conjugates has not yet been fully explored.

The divergent synthesis of protein−synthetic polymer conjugates has become possible with the advent of controlled radical polymerization techniques such as ATRP and RAFT. ATRP requires protein macroinitiators, which are usually obtained by direct acylation of the protein's lysine amine groups with 2-bromoisobutyric acid bromide or by modification of cysteine thiol groups with appropriate maleimide- or pyridyl disulfide-functionalized ATRP initiators.[90−92] Instead of covalent postmodification, noncovalent interactions can also be used to prepare protein-based ATRP macroinitiators. One example is the streptavidin−biotin interaction, which has been used to generate protein macroinitiators via binding of biotinylated ATRP initiators by streptavidin.[93] The divergent RAFT synthesis of protein−synthetic polymer conjugates requires protein chain transfer agents, which can be obtained e.g. by modification of the free thiol groups of cysteine residues with pyridyl disulfide-functionalized RAFT agents.[94,95]

Most of the protein−synthetic polymer conjugates that have been reported so far consist of a single protein that is modified with multiple copies of a synthetic polymer. While this has proven to be a useful strategy to modify the properties of therapeutic proteins, there are a variety of biological processes that involve protein dimers or higher multimers. Multivalent protein−synthetic polymer conjugates would be of interest to study and/or modulate such processes. Maynard et al. recently reported the use of RAFT polymerization to prepare α,ω-homobifunctional or α,ω-heterobifunctional poly(*N*-isopropylacrylamide) (PNiPAm).[96,97] These telechelic polymers were subsequently used to synthesize homo- or heterodimeric protein−synthetic polymer conjugates in a convergent fashion. Homodimeric protein−synthetic polymer conjugates were obtained by reaction of an α,ω-bismaleimide PNiPAm derivative with a cysteine-modified lysozyme mutant.[96] Heterodimeric protein−synthetic polymer conjugates were obtained following a two-step strategy, which started with the modification of the maleimide group of an α-biotin,ω-maleimide heterobifunctional PNiPAm with BSA followed by conjugation of streptavidin via the biotin label.[97]

Peptide−Synthetic Polymer Conjugates

Peptide−synthetic polymer conjugates have attracted interest for a number of reasons. First of all, the peptide segment can endow these materials with unique self-assembly properties and induce the formation of hierarchically organized nanoscale structures, both in solution and in the solid state, with a much higher level of complexity as compared to e.g. ordinary block copolymers. In several instances, the sensitivity of the peptide secondary structure to environmental parameters such as temperature, pH, or ion strength allows to reversibly manipulate the nanoscale structure formation of these hybrid materials. The literature on this subject is vast and has been summarized in various review articles.[26,27] Instead of focusing on structure formation, the objective of this section is to highlight some of the opportunities that are offered by peptide−synthetic polymer conjugates to generate functional materials. Table 4 provides an (incomplete) overview of selected functions, and applications of peptide−synthetic polymer conjugates that have been reported in the literature.

Drug and Gene Delivery. Peptide−synthetic polymer conjugates that are designed as therapeutic agents to transport and release drugs or genes can be subdivided in two groups depending on the function of the peptide segment. In the first class of peptide−synthetic polymer therapeutics, the peptide segment merely serves to bind the drug or gene of interest. In the second group, the peptide segment serves to mediate targeted delivery and to control intracellular trafficking and release. The first class of conjugates are peptide−synthetic hybrid block copolymers that are typically comprised of PEG as the synthetic polymer and are obtained via NCA ring-opening polymerization. The use of these block copolymers in drug and gene delivery has been pioneered by the group of Kataoka.[98] Functionalization of the carboxylic acid groups of the peptide segment of poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-*b*-PAsp) with the anticancer drug doxorubicin (Dox) results in an amphiphilic block copolymer that self-assembles to form stable micelles, which can physically entrap additional Dox.[99,100] Another example for the use of peptide−synthetic polymer conjugates for cancer therapy are cisplatin-loaded micelles that are spontaneously obtained by complexation of cisplatin with the carboxylic acid groups of PEG-*b*-PAsp.[101] The second class of peptide−synthetic polymer conjugate therapeutics does not (only) use the peptide segment to bind drugs/genes but also attempts to take advantage of the ability of various bioactive peptide sequences to target delivery[102] and/or control intracellular trafficking and release.[103] To allow targeted delivery of radiotherapy to tumor neovasculature, Ghandehari et al. synthesized poly(*N*-(2-hydroxypropyl)-methacrylamide)) (PHPMA) conjugates containing ~16 copies of a cyclic derivative of the $\alpha_v\beta_3$ integrin targeting RGD peptide.[104] Hubbell and co-workers used phage display to discover short peptides that bind collagen II α1 and which were subsequently used to construct polymer nanoparticles that allowed intra-articular targeting.[105] In addition to directing the drug/gene carrier to the appropriate cell or tissue, peptides can also be used to guide intracellular trafficking and target subcellular organelles.[103] Peptide−synthetic polymer conjugates containing fusogenic peptide

sequences e.g. have been used to facilitate endosomal escape and enhance cytosolic delivery.[106,107] Another class of peptides that has found use for the development of therapeutic peptide−synthetic polymer conjugates are cell penetrating peptides (CPPs). CPPs are short peptide sequences that can mediate the cellular uptake of a broad range of cargos.[108] Kopeček et al. elegantly demonstrated that conjugation of a cell penetrating peptide sequence derived from the HIV Tat transactivator domain represents an effective strategy to control intracellular trafficking of synthetic macromolecules.[109] Upon internalization by human ovarian cancer cells, PHPMA−Tat peptide conjugates were transported to the cytoplasm and nucleus via a nonendocytotic and concentration-independent pathway, whereas PHPMA copolymers lacking the Tat peptide were taken up via endocytosis.

Mechanical Properties. Silks are unique protein-based biomaterials with mechanical properties that are equal to or better than that of many man-made materials.[110,111] Silks are semicrystalline materials composed of amorphous protein segments that are reinforced with β-sheet crystalline segments. Sogah and co-workers have reported the synthesis and characterization of peptide−synthetic hybrid multiblock copolymers that were inspired by these basic silk design principles.[112,113] These multiblock copolymers consisted of soft PEG segments that alternated with crystalline, β-sheet forming AlaGlyAlaGly or $(Ala)_x$ ($x=4, 6$) sequences. The mechanical properties of films and fibers of these polymers were investigated with tensile measurements. These experiments revealed that polymers based on the spider silk inspired $(Ala)_4$ peptide sequence showed better mechanical properties than the *B. mori* inspired $(AlaGly)_2$-containing multiblock copolymers. For the spider silk inspired multiblock copolymers, increasing the length of the $(Ala)_x$ segment from an average of 4−6 residues was found to result in an increased modulus and tensile strength. Interestingly, the mechanical properties of films of the spider silk inspired multiblock copolymers were considerable better than those of regenerated spider silk films. In another, more recent example, Hentschel and Börner reported the use of β-sheet forming peptide−synthetic polymer conjugates to modulate the mechanical properties of synthetic polymers.[114] These authors found that blending 5 wt % of nanofibers formed by self-assembly of a poly(*n*-butyl acrylate)-*b*-$(ThrVal)_5$nPheGly conjugate with high molecular weight poly(*n*-butyl acrylate) resulted in a significant stiffening compared to the pure poly(*n*-butyl acrylate).

Hydrogels. Hydrogels are of high interest for applications such as drug delivery and tissue regeneration. Conjugation of peptides to synthetic polymers is a powerful approach, to both induce and control hydrogel formation, as well as to endow hydrogels with specific biological properties. The use of biologically inspired folding motifs to develop peptide/protein−hybrid hydrogels has been pioneered by Kopeček and co-workers.[115] These authors have primarily concentrated on the coiled coil motif, which is a superhelical, quaternary assembly composed of 2−5 helical peptide strands, to control hydrogel formation. These self-assembled hybrid hydrogels are attractive as they can be formed in situ without chemical cross-linking. Furthermore, the sensitivity of the secondary structure and self-assembly properties of the coiled coil peptides toward e.g. temperature, ion strength, and chaotropic agents provides opportunities to control the formation and dissociation and/or swelling and collapse of these materials. Kopeček et al. have described the formation of hydrogels from a variety of graft-type peptide−synthetic polymer conjugates that consisted of a PHPMA backbone functionalized with multiple copies of a coiled coil-forming peptide. These graft copolymers were obtained by postpolymerization modification of maleimide-functionalized PHPMA precursors[116,117] as well as via copolymerization of the appropriate methacrylate-functionalized peptides.[118] Hydrogel formation was induced either by self-association of the peptide grafts[116,118] or by mixing two PHPMA graft copolymers modified with complementary peptide sequences.[117] Another example of a coiled coil-based hybrid hydrogel was recently reported by Collier et al.[119] Instead of graft-type polymers, these authors used triblock copolymers composed of a central PEG block flanked by two fibrin-inspired coiled coil sequences to produce self-assembled peptide−synthetic polymer hybrid hydrogels. In addition to coiled coil-forming peptide sequences, β-sheet forming peptides can also be used to generate peptide−synthetic polymer conjugate hydrogels. This was recently demonstrated by Adams et al., who prepared poly(ethylene glycol)−tetraphenylalanine conjugates that were found to self-assemble in nanotubes driven by the formation of antiparallel β-sheets and $\pi\pi$-interactions.[120] At sufficiently high concentrations, entanglements between the nanotubes resulted in the formation of soft hydrogels. Besides inducing and modulating hydrogel formation, the integration of short peptide sequences in synthetic polymer hydrogels also provides opportunities to produce biologically active hybrid hydrogels. Hubbell et al., for example, have developed hybrid hydrogels based on 4-arm, vinylsulfone-functionalized star PEGs, which contained two different peptide sequences that served to mediate cell adhesion and can act as substrates for matrix metalloproteinase (MMP).[121,122] The synthesis of these gels was a two-step process that started with the functionalization of the star PEGs with a small fraction of RGD peptide ligands. The RGD-functionalized star polymers were subsequently cross-linked by a bis-cysteine-functionalized peptide that could serve as MMP substrate. When loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2), these gels could be successfully used to repair bone defects in rat crania. The MMP-mediated degradation of the gels facilitates cell ingrowth, allowed rhBMP-2 release, and resulted in efficient and highly localized bone regeneration.

Directing Mineralization. Many biominerals possess extraordinary properties and are characterized by complex, hierarchically ordered structures, and there is a great interest in mimicking the strategies that nature uses to prepare organic−inorganic composite materials. Since proteins play an important role in directing biological mineralization processes, a broad variety of peptide-based additives, including block copolypeptides,[123] peptide amphiphiles,[124,125] and peptide−synthetic polymer conjugates, have been explored to control crystal growth. The peptide−synthetic polymer conjugates that have been explored have an architecture that is referred to as double hydrophilic and are composed of a PEG block that is attached to a peptide segment. Double hydrophilic peptide−synthetic polymer conjugates (block copolymers) are of interest since they can self-assemble to provide a confined crystallization environment with a high density of functional groups and the ability to direct anisotropic crystal growth.[126] Antonietti and Cölfen investigated the use of poly(ethylene glycol)-*b*-poly(L-aspartic acid) (PEG-*b*-PAsp) and poly(ethylene glycol)-*b*-poly(L-glutamic acid) (PEG-*b*-PGlu) as additives for the crystallization of $CaCO_3$ and $BaSO_4$.[127] In contrast to the expectations, these peptide−synthetic polymer conjugates were most effective in crystallization control when the peptide segment adopted a random coil conformation. Yu et al. have investigated the use of PEG-*b*-PGlu as additive for the crystallization of

Table 5. Overview of Selected Functions and Applications of Protein−Synthetic Polymer Conjugates

enzymes	• separation, recovery, recycling and modulation of activity via attachment of thermosensitive polymers[92,131−134]
viral capsids and protein cages	• polymer-modified viral capsids as vectors for gene delivery[140−144]
hydrogels	• hybrid hydrogels formed by self-assembly of recombinant coiled coil protein−synthetic polymer conjugates[115,147] • bioactive hydrogels based on recombinant proteins containing cell adhesion sequences and protease substrates[148,149] • "dynamic" hydrogels based on proteins that undergo large conformational changes upon binding of a substrate[150−153]
therapeutic proteins	• modification of therapeutic proteins to improve stability and solubility, reduce renal clearance, enhance circulation half-life, reduce immunogenicity and antigenicity, and prevent proteolytic degradation[82,156,157] • reversible PEGylation and polymer masking−unmasking protein therapy (PUMPT) to avoid loss of activity upon polymer conjugation and obviate the need for site-specific modification[159−161]

$CaCO_3$ in organic and mixed organic/aqueous media. In organic/aqueous mixtures containing approximately equal amounts of DMF and water, highly monodisperse vaterite microspheres were produced.[128] Crystallization in organic or mixed organic media resulted in $CaCO_3$ superstructures with complex forms and hierarchical surface textures.[129] In another example, Kessel and Börner used poly(ethylene glycol)-*b*-peptide nanotapes to direct the formation of silica composite nanofibers.[130] The authors speculated that the formation of the nanofibers was due to the preferential enrichment of silicic acid mono- and oligomers on the PEG-peptide nanotapes, resulting in an increase in local concentration of silicic acid and a concomitant increase in the rate of condensation.

Protein−Synthetic Polymer Conjugates

Covalently combining proteins and synthetic polymers is of interest for numerous reasons. The conjugation of an appropriate synthetic polymer can be used, for example, to modulate the biological activity of a protein. From the polymer perspective, the attachment of a protein may endow a synthetic polymer with unique functional and structural properties. Table 5 lists several selected examples of the wide range of attractive properties and diverse applications of protein−synthetic polymer conjugates.

Enzymes. The synthesis and study of well-defined enzyme−synthetic polymer conjugates, i.e., conjugates in which the number of attached synthetic polymer chains and their attachment site are well-defined, has been pioneered by Hoffman and Stayton and co-workers. In an early example, these authors reported the coupling of an *N*-hydroxysuccinimide (NHS) end-functionalized PNiPAm oligomer with the enzyme β-D-glucosidase and demonstrated that the thermosensitive properties of the PNiPAm chain allow separation, recovery, and recycling of the enzyme via small temperature changes.[131] In another example, the temperature-induced collapse of PNiPAm was used to modulate the substrate (biotin) binding properties of streptavidin.[132] To this end, a vinylsulfone end-functionalized PNiPAm derivative was coupled to a streptavidin mutant with a cysteine conjugation site in close proximity to the protein's binding site. While streptavidin is not an enzyme, this example does demonstrate the power of thermoresponsive polymers to control protein−ligand binding. More recently, using similar conjugation chemistry, Górecki and Alexander demonstrated that the activity of a multisubunit DNA restriction−modification enzyme can be controlled by the attachment of a thermoresponsive PNiPAm segment.[133] The enzyme−synthetic polymer conjugates discussed so far have all been prepared via convergent coupling of a presynthesized polymer with the protein of interest. The development of a broad range of controlled radical polymerization techniques over the past 15 years also enables the synthesis of enzyme−synthetic polymer conjugates in a divergent fashion. Maynard et al., for example, reported the synthesis of lysozyme−PNiPAm conjugates via ATRP of NiPAm using a lysozyme-based ATRP initiator.[92] The polymer-modified lysozyme derivative was found to show lytic activities that were equal to that of the unmodified enzyme. Sumerlin and co-workers used RAFT polymerization to prepare BSA−PNiPAm conjugates.[134] These authors demonstrated that the activity of the enzyme could be regulated by thermal cycling between 25 and 40 °C. At 40 °C, the BSA−PNiPAm conjugates possessed 90% activity compared to native BSA. In a recent study, the *in vitro* serum stability and enzymatic activity of a PEGylated chymotrypsin conjugate, which was prepared via grafting an NHS PEG derivative, were compared with those of chymotrypsin−PHPMA and chymotrypsin−poly(2-methacryloyloxyethylphosphorylcholine) (PMPC) conjugates that were obtained via ATRP.[135] In human serum, PEGylated chymotrypsin was found to deactivate within 4 days of incubation, whereas native chymotrypsin and the chymotrypsin−PHPMA and chymotrypsin−PMPC conjugates retained $>25\%$ catalytic activity after 5 days of incubation. These results highlight the importance of choosing or designing the appropriate conjugation chemistry in order to avoid changes in structure, stability, or activity of the protein in the final conjugate.

Viral Capsids and Protein Cages. Viral capsid proteins represent a unique class of biomolecular building blocks due to their ability to self-assemble into protein cages (viral capsids) that are uniform in size and shape. Among others, viral capsids have been used as scaffolds for the synthesis of various materials, as building blocks for the formation of nanowires and liquid crystals, as nanoreactors, and as nanocontainers for drug and gene delivery and for diagnostics.[136−139] A number of reports have been published that describe the synthesis and properties of polymer-modified viral capsids. In most cases, the viral capsids were intended to be used as vectors for gene delivery and the polymer modification served to mask the immunogenic properties, extend the plasma circulation time, and decrease the toxicity of the viral capsid. Traditional, first-generation PEGylation chemistry has been used to modify cowpea mosaic virus (CPMV) as well as adenoviruses.[140−142] Immunogenicity studies indicated that the PEG coating can shield the viral capsids from inducing a primary antibody response.[140] Ogawara et al. reported the preparation of targeted adenoviruses, which were obtained by modification of the adenovirus with a heterobifunctional (NHS and vinylsulfone) PEG followed by conjugation with a thiol-modified RGD peptide.[142] Seymour and colleagues have used PHPMA copolymers containing reactive active ester side-chain functions to prepare polymer-coated adenoviruses.[143,144] The PHPMA-coated viruses showed extended plasma circulation times

and decreased toxicities compared to the unmodified virus. These authors further showed that residual active ester groups on the surface of the PHPMA coated viruses can be used to introduce targeting ligands.[143] Wang et al. have reported a divergent strategy for the modification of the protein cage derived from ferritin with PPEGMA.[145] PPEGMA was grafted from the horse spleen apoferritin by modification of the surface amine groups of the cage with 2-bromoisobutyrate following by copper-catalyzed ATRP of PEGMA. So far, most of the work on polymer-modified capsids has concentrated on applications in drug and gene delivery. Due to their well-defined size, shape, and surface chemistry, viral capsids and other protein cages, however, also represent unique scaffolds to construct catalytically active systems or nanoreactors, and appropriate polymer surface modification may be useful to improve the solubility and stability of these conjugates. A final remark concerns the synthesis of polymer-modified capsids and protein cages. In all of the work that has been reported until now these constructs were obtained by modification of the complete protein cage. There are several capsids, including the cowpea chlorotic mottle virus,[146] for example, which can be reversibly assembled and disassembled into the individual viral coat proteins. Modification of individual viral capsid proteins and successful reconstitution of these building blocks would represent a new bottom up strategy toward functional viral capsids and protein cages and may also enable modification of the interiors of these particles.

Hydrogels. In addition to peptides, proteins have also been used to fabricate hybrid hydrogels. Although in many instances peptides have the advantage that they can be easily prepared in large quantities via chemical synthesis, the use of protein-based building blocks allows the preparation of hybrid hydrogels of greater structural and functional complexity. Kopeček et al. have prepared hybrid hydrogels by mixing genetically engineered His-tagged coiled coils with a Ni^{2+}-iminodiacetate PHPMA copolymer.[115,147] The coiled coil proteins self-assemble into superhelical quaternary structures, which leads to the formation of a noncovalent network. These hydrogels underwent a temperature-induced collapse, which was ascribed to the cooperative unfolding of the coiled coil protein domains. Hubbell and co-workers have reported the preparation of bioactive protein−synthetic hybrid polymer hydrogels, which were obtained via Michael-type conjugate addition of the vinylsulfone end groups of α,ω-bifunctional PEGs and the cysteine thiol groups of recombinant proteins.[148,149] The protein used in these studies served two functions: (i) to act as a cross-linker and (ii) to provide biological functionality. By including peptide sequences derived from fibrinogen and collagen, the recombinant protein provided both ligands for cell-surface integrin receptors and substrates for plasmin and matrix metalloproteinases (MMPs), which are proteases involved in wound healing. Another class of proteins that is of interest for the fabrication of hybrid hydrogels are those that can undergo conformational changes in response to stimuli such as light, pH, and the binding of a substrate. Such proteins provide opportunities for the preparation of stimuli-responsive hybrid hydrogels. Murphy et al., for example, have prepared and studied hybrid hydrogels based on the protein calmodulin (CaM), which undergoes a rapid transition from an extended dumbbell to a collapsed conformation in response to binding of various ligands including the small molecule drug trifluoperazine (TFP).[150−152] These hydrogels were prepared from a genetically engineered CaM mutant that contained cysteine residues at the ends of the dumbbell-shaped protein and which was reacted with bis- or tetra-acrylate-functionalized PEG derivatives. Kopeček et al. prepared hybrid hydrogels based on the enzyme *Escherichia coli* adenylate kinase (AKe).[153] These hydrogels were obtained by reacting a maleimide-functionalized PHPMA copolymer with a bis-cysteine-functionalized Ake mutant. Upon binding of a substrate, e.g. ATP, the AKe undergoes a large conformational change, which is translated into shrinkage of the gel. Francis and co-workers have prepared hybrid hydrogels by reacting alkoxyamine-functionalized synthetic polymers with proteins containing aldehyde/ketone groups at the N- and C-termini.[154,155] Hybrid hydrogels based on metallothionein were able to bind heavy metal ions from water and underwent large reductions in volume upon ion binding, which is attractive for sensory applications.

Therapeutic Proteins. PEGylation is a well-established technology to modify therapeutic proteins (*vide supra*). In many cases, PEGylation targets lysine amine groups, which are relatively abundant in most proteins. While the attachment of PEG chains offers many benefits, the random nature of the modification may lead to a loss or decrease in the activity of the protein. The wide range of bioorthogonal reactions that has become available the past years,[43] however, now makes it possible to PEGylate proteins in a site-specific manner. By introducing a specific, unique natural or nonnatural amino acid at the site of interest in the protein using either chemical or recombinant techniques, the degree and site of PEGylation can be precisely controlled.[82,156,157] A nice example of the use of site-specific PEGylation was reported several years ago by Kochendoerfer et al.[158] These authors prepared a PEGylated erythropoiesis protein via total chemical synthesis. While the *in vitro* activity of this synthetic erythropoiesis protein (SEP) conjugate was similar to that of human erythropoietin (Epo), SEP showed superior *in vivo* potency compared to Epo. A very interesting technique that may either complement or replace site-specific PEGylation is reversible or releasable PEGylation.[159] Reversible PEGylation involves the covalent modification of the protein of interest with PEG chains that are tethered via a cleavable linker. Hydrolytically sensitive linkers are most widely used, but other linkers, for example, disulfide based, have also been used. Among several other benefits, reversible PEGylation allows to avoid loss of activity due to the modification reaction without the need for site-selective attachment since the PEG chains are gradually cleaved with time. As a recent example, Shechter et al. reported the reversible PEGylation of insulin using a heterobifunctional 9-hydroxymethyl-7(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysuccinimide linker. Under physiological conditions, the PEG grafts underwent spontaneous hydrolysis to release the nonmodified protein with a $t_{1/2}$ of 30 h.[160] In contrast, nonreversible, conventional PEGylation was found to lead to inactivation of insulin. An interesting extension of the concept of reversible PEGylation is the polymer masking−unmasking protein therapy (PUMPT) that was recently reported by Duncan et al.[161] Instead of using PEG, PUMPT involves modification of the protein of interest with a biodegradable, hydrophilic polymer (dextran) that protects the protein and masks its activity while in transit but allows release of the protein with complete installment of biological activity by triggered enzymatic degradation. The use of the biodegradable dextran instead of the nonbiodegradable PEG avoids the possible risk of accumulation following chronic administration. In addition to its nondegradability, another possible drawback of the use of PEG is that it only possess two reactive (end) groups, which can restrict further functionalization. As a consequence, there is an interest in alternative synthetic polymers

that can be used for protein modification. Interesting candidates are PMPC and PPEGMA, which can be synthesized via ATRP using active ester, bis-sulfide, aldehyde, or maleimide containing initiators to afford α-functional polymers that can subsequently be conjugated to the protein of interest.[162−166] In addition to the greater flexibility offered by controlled radical polymerization techniques to engineer the composition and architecture of the grafted synthetic polymers, the use of alternative monomers, such as MPC, also provides opportunities to further tailor the pharmacokinetic properties of therapeutic proteins. Lewis et al., for example, compared the pharmacokinetics of a 20 kDa PEG equivalent PMPC interferon-α2a (IFN) conjugate with that of a 20 kDa PEG-IFN conjugate and found that the elimination half-life of the PMPC conjugate was significantly increased compared to the PEGylated IFN analogue.[162]

Conclusions and Outlook

This Perspective has attempted to capture the state-of-the-art of the field of peptide/protein−synthetic polymer conjugates. Over the past decade or so, impressive advances have been made in the synthesis of these hybrid materials. New strategies for the ring-opening polymerization of α-amino acid *N*-carboxy anhydrides now provide easy access to peptide−synthetic hybrid block copolymers and other more complex architectures with precise control over polymer molecular weight and chain architecture. In parallel, the development of a range of chemoselective, bioorthogonal coupling reactions in combination with the increased possibilities to prepare artificial proteins containing noncanonical amino acids has revolutionized the synthesis of peptide/protein−synthetic polymer conjugates. Although the incorporation of new noncanonical α-amino acids is not always trivial, additional noncanonical α-amino acids that can be introduced via recombinant techniques would be desirable and further (even more chemoselective) bioorthogonal coupling reactions useful, these developments have enormously facilitated the synthesis of well-defined peptide/protein−synthetic polymer conjugates, i.e., hybrid constructs in which the number of conjugation sites as well as the position of the conjugation site(s) are exactly defined. The examples given in the last two sections of this paper only give a flavor of the possibilities offered by these synthetic advances, and it is obvious that these new developments have not yet been fully explored. In particular, for the development of novel biomolecular drugs, new (bio)sensory materials as well as bioactive hybrid hydrogels that can aid in wound healing and tissue regeneration, the enhanced possibilities for the precision synthesis of peptide/protein−synthetic polymer conjugates provide new and unique opportunities to control the structure and properties of these materials.

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