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Viewpoint

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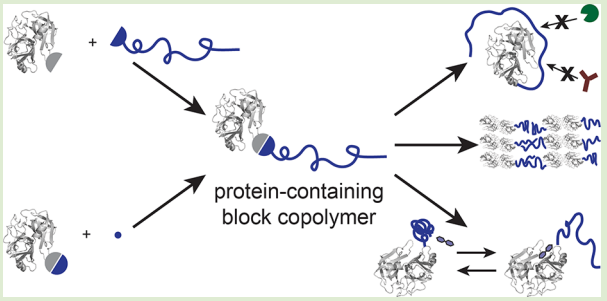
Synthesis and Application of Protein-Containing Block Copolymers

Allie C. Obermeyer and Bradley D. Olsen*

Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

ABSTRACT: Proteins possess an impressive array of functionality ranging from catalytic activity to selective binding and mechanical strength, making them highly attractive for materials engineering. Conjugation of synthetic polymers to proteins has the potential to improve the physical properties of the protein as well as provide functionality not typically found in native proteins, such as stimuli-responsive behavior and the programmable ability to self-assemble. This viewpoint discusses the design of protein−polymer conjugates, an important class of block copolymers. Use of these hybrid molecules in biological and catalytic applications is highlighted, and the ability of the polymer to direct the solution and solid-state self-assembly of the hybrid block copolymers is reviewed. Future challenges in polymer and material science that will enable these hybrid molecules to reach their potential as protein-based materials are outlined.



Proteins comprise one of the most impressive categories of polymers known: they produce extremely strong and tough materials,[1] they efficiently catalyze chemical transformations,[2] they selectively bind analytes within complex mixtures,[3,4] and they harvest light by converting it into chemical energy.[5] By combining the incredible diversity of structure and function of proteins with the stability, chemical diversity, and processability of synthetic polymers, materials with the advantages of both components can be accessed. For example, the modification of proteins with poly(ethylene glycol), known as PEGylation, has long been used as a strategy to modulate protein pharmacokinetics and stability.[6,7] The combination of the native protein function with the enhanced properties imparted by the polymer component has resulted in several new FDA approved protein-based therapeutics.[8] As the fields of bioconjugation and controlled radical polymerization advance, the complexity and diversity of these hybrid materials is ever increasing. New bioconjugation methods enable the synthesis of well-defined protein conjugates in high yield. Equally as important, the advent of controlled radical polymerization has enabled the synthesis of well-controlled polymers with diverse chemical functionality and end group functionality capable of protein conjugation. With these advances, protein−polymer materials based on bovine serum albumin,[9] cellulase,[10−12] and glucose oxidase[13−15] have found use in drug delivery, biocatalysis, and biofuel cells, respectively.

To harness the functional diversity of proteins for emerging technological applications, including catalysis, sensors, drug delivery, photovoltaics, and tissue engineering, their physical limitations often must be overcome through engineering in a material context. The sensitivity of some proteins to temperature, pH, organic solvents, and biodegradation limit their adoption for many applications. Furthermore, the majority of proteins of interest exist as soluble colloids (monomers or small aggregates) or membrane-associated complexes that are difficult to process, coat, or form into a mechanically robust material. Synthetic polymers typically possess mechanical strength, durability, and programmable self-assembly, but often lack the exquisite functionality found in sequence-specific protein biopolymers. Hybrids of protein biopolymers and synthetic polymers (hereafter referred to simply as polymers) will lead to new materials that dramatically expand the scope of protein-based technology. Conjugate materials have been created with improved stability and bioactivity,[16−19] with altered and recyclable enzymatic activity[10−12,20] and with the ability to self-assemble in a fashion similar to traditional block copolymers.[21−25] However, the combination of proteins and polymers does not necessarily result in a material with the additive properties of the two components. For example, the PEGylation of α-interferon results in >90% loss of activity of the protein, but the conjugate actually shows enhanced in vivo performance due to improvements in the pharmacokinetics.[16,26] Thus, to be able to rationally design materials capable of meeting these and emerging applications, a better physical understanding of bioconjugate materials is necessary.

The goal of this viewpoint is to discuss design criteria for the synthesis and application of protein-containing block copolymers. Well-established methods that satisfy a majority of the design criteria for the synthesis of these materials will be highlighted, and recently developed bioconjugation strategies that have the potential to improve the efficiency of protein−polymer bioconjugation will be explored. The successful demonstration of these promising hybrid materials in biological applications, catalysis applications, and self-assembly will be covered. Current approaches to material engineering, as well as challenges for the design of functional protein−polymer

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materials, will be discussed. As the synthesis of these hybrid materials becomes increasingly accessible, the role of the material scientist in understanding the physics of the conjugates has become essential, and particular fundamental challenges are highlighted throughout the discussion.

STRATEGIES FOR THE SYNTHESIS OF PROTEIN BLOCK COPOLYMERS

The application and study of protein-containing block copolymers relies on the synthesis of well-defined protein conjugates by site-selectively modifying the protein substrate. Several crucial factors must be considered when designing the synthesis of protein−polymer conjugates:

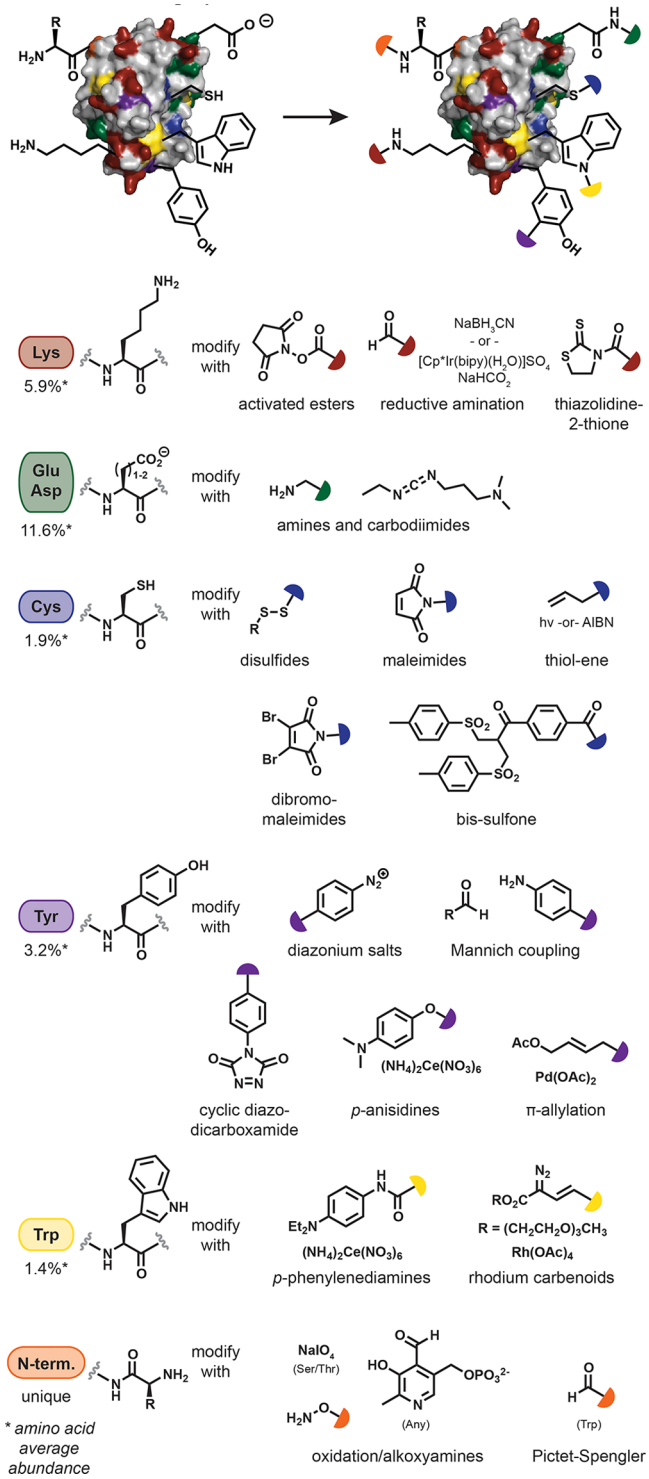
(1) The protein should have the desired functionality and be readily available either commercially or biosynthetically.
(2) The polymer should have appropriate physio-chemical properties, should be synthetically tractable, and should have known interactions with the protein component.
(3) The chemistry used to conjugate the protein and polymer should be mild, high yielding for both reagents, produce a stable, well-defined linkage and not adversely affect the structure or activity of the protein.
(4) The mass fraction of the polymer component should be optimized to produce a conjugate with maximal biological activity and the desired physical characteristics.

The goal of obtaining a well-defined linkage can be achieved by choosing a bioconjugation reaction that achieves high yield and site-specific modification even when only few equivalents of the polymer component are used. These bioconjugation reactions must take place in water, near neutral pH, and in the presence of a wide array of side chain functional groups and impurities. The synthesis of protein−polymer conjugates involves additional challenges for traditional bioconjugation reactions that must be addressed. Chiefly, the biomacromolecule-macromolecule coupling is highly dependent on the molar mass of the polymer. While the toolkit of bioconjugation reactions continues to expand, many of these methods have yet to be demonstrated using polymers. In addition, the performance of these new reactions should be evaluated with the addition of only a few equivalents of synthetic reagent. While the use of large excesses of polymer can result in high yield of the conjugation reaction, removal of the excess polymer can pose a significant challenge.

A number of different bioconjugation reactions have emerged that can meet many of the important criteria for protein−polymer conjugate synthesis. Here we focus on the relevant considerations for selecting a conjugation strategy and highlight the methods that have shown the greatest promise and success for protein−polymer conjugation. The reader is directed to in-depth reviews on the conjugation of synthetic components to biomolecules[27,28] and specifically on protein−polymer conjugation[29−32] for more details on all of the relevant reactions.

Typically, it is advantageous to perform conjugation reactions using only the 20 natural amino acids, as this helps to maximize protein yield and flexibility in protein synthesis. In order to achieve successful site-selective modification with native residues, they must be present on the protein surface in the desired location. Alternately, genetic engineering can be used to introduce a surface accessible residue in the location of choice; however, care must be taken in order to avoid perturbations that affect protein function. Methods for modifying the 20 canonical amino acids typically target the side chains of lysine, glutamate, aspartate, and cysteine (Scheme 1). However, the relatively high abundance[33] of lysine, aspartate, and glutamate, and the high solvent accessibility of their side chains makes it virtually impossible to modify a single site on the protein surface using these methods.

Scheme 1. Bioconjugation Methods for the Modification of Native Amino Acids with Polymers



Alternatively, the nucleophilic side chain of cysteine can be targeted for site-selective modification. At slightly basic pH, the thiolate moiety can be modified with disulfides, maleimides, or iodoacetamides.[34] Modification with disulfide and maleimide

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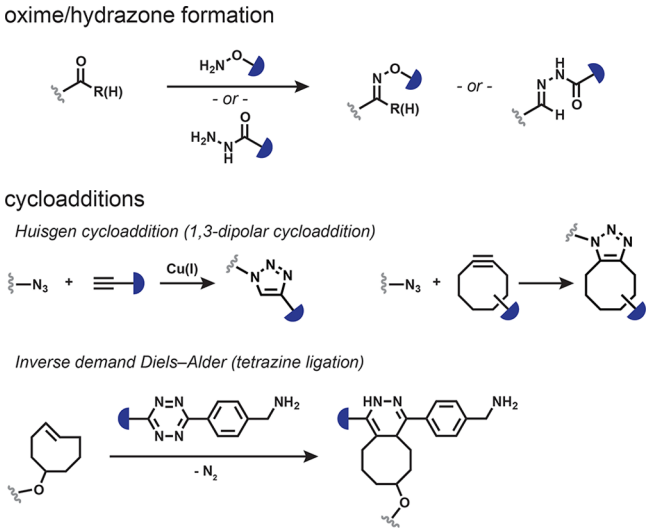
reagents produces linkages that are not stable in the presence of free thiols (such as those encountered under physiological conditions).[35,36] However, ring-opening hydrolysis of the succinimide thioether yields a stable conjugate. This hydrolysis can be promoted with specific maleimide reagents.[37] The relatively low abundance of cysteine facilitates genetic modification of the protein sequence to introduce a unique cysteine that can be modified site-selectively to create a well-defined bioconjugate. Due to the extraordinarily fast kinetics of these reactions and the commercial availability or straightforward synthesis of the reagents, these methods are widely utilized. However, cysteine residues are often found in disulfide bonds that are critical to the folding and structural integrity of proteins, and bioconjugation reactions may disrupt these bonds. To address this potential challenge, bioconjugation reactions that insert into disulfide bonds have been developed. Dibromomaleimide[38] and bis-sulfone reagents[39,40] can be used to modify cysteines in disulfide bonds without significantly perturbing the protein structure.

Methods for the modification of other nonabundant amino acids, such as tyrosine[41−43] and tryptophan,[44,45] have also been developed (Scheme 1). The phenolic side chain of tyrosine can be modified with electrophilic reagents via azo coupling with diazonium salts or Mannich-type coupling with in situ formed imines.[41] Tyrosine residues can also be modified with π-allylpalladium complexes[42] or cyclic diazodicarboxylate reagents.[43] Additionally, both tyrosine and tryptophan can be modified with phenylenediamine reagents in the presence of cerium(IV) ammonium nitrate (CAN).[45] By altering the electronics of the coupling substrate, tyrosine residues can be selectively targeted for oxidative coupling with CAN. These methods form stable linkages, with the exception of the Mannich coupling, which can undergo a retro-Mannich reaction, and the azo coupling, which can be selectively cleaved by reduction with sodium dithionite.[46] These methods have yet to be widely adopted for the synthesis of protein−polymer conjugates.

Another strategy for site-selective modification of proteins relies on the modification of a unique position on the protein (Scheme 1). The N-terminus can be selectively targeted for modification when it is sufficiently accessible and not post-translationally modified. These strategies can be general for any N-terminal residue, such as pyridoxal-5′-phosphate (PLP) mediated transamination,[47−49] or require a specific amino acid at the terminus, such as periodate oxidation of Ser/Thr[50] or Pictet−Spengler reactions with N-terminal Trp.[51] A powerful method for the modification of N-terminal Cys residues or C-terminal thioesters, termed "native chemical ligation", enables the synthetic modification of either protein terminus.[52−54]

Recent work on bioconjugation reactions has developed two alternative approaches, the use of enzymes and artificial amino acids, to site-selectively modify proteins. The ability of enzymes to modify proteins with small molecule substrates has been harnessed to attach non-native, synthetic substrates to proteins of interest. A minimal recognition sequence of amino acids can be genetically introduced as a tag on the protein of interest. Understanding the specificity of the enzyme for its small molecule substrate allows for perturbations to the substrate to be made. These enzymatic methods can be broken down into two categories: (1) enzymes capable of direct conjugation of synthetic molecules to the protein substrate and (2) enzymes capable of introducing a novel functional group for further elaboration. In the first category, transglutaminase[55] and Sortase A[56,57] have been used to label tagged proteins directly with non-natural substrates such as PEG.[58,59] Enzymes, such as lipoic acid ligase,[60] biotin ligase,[61] and farnesyltransferase,[62] which have stricter requirements for the synthetic substrate, fall into the second category and have been used to conjugate small non-native functional groups to the protein surface for further functionalization. Methodologies to modify these non-natural functional groups, or bioorthogonal moieties, are rapidly being developed (Scheme 2).

Scheme 2. Methods for the Modification of Bioorthogonal Functional Groups with Polymers



The ability to incorporate noncanonical amino acids biosynthetically has dramatically expanded the types of reactions that can be used to modify proteins. These bioorthogonal groups can be incorporated into proteins site-specifically via amber stop codon suppression[63] or in a residue-specific manner with global noncanonical amino acid replacement.[64] Discovery and evolution of tRNA and amino-acyl-tRNA synthetases that recognize the amber stop codon (TAG) have enabled the in vivo incorporation of artificial amino acids in *E. coli*,[63] yeast,[65] and mammalian cells.[66,67] An alternative, straightforward method relies on supplementing the growth medium with a noncanonical amino acid.[64] If the artificial amino acid is tolerated by the translation machinery it can be globally incorporated into proteins in place of the canonical amino acid.[68,69] This process is most efficient when the expression host is rendered auxotrophic for the amino acid being targeted and the media is depleted of the natural amino acid to be replaced. While the introduction of bioorthogonal functional groups provides access to a wide array of powerful chemistries, the incorporation of a non-natural amino acid increases the cost of protein production and decreases the amount of protein produced. Efforts are ongoing to improve the biosynthetic incorporation of bioorthogonal moieties.[70,71]

One of the first bioorthogonal reactions involved modification of introduced aldehydes or ketones. These electrophilic functional groups can be modified with hydrazines, hydrazides, and alkoxyamines, with the oxime linkage forming the most hydrolytically stable conjugation.[72−74] However, the oxime is still thermodynamically unstable to hydrolysis, with a half-life of approximately one month at neutral pH.[73] While

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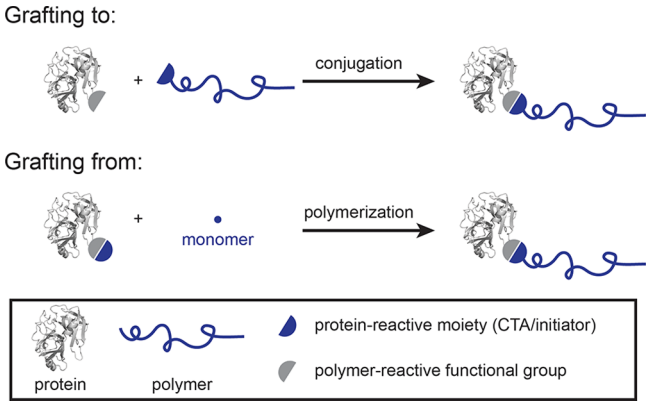
these reactions are very selective, they generally require long reaction times and large excesses of reagent. More recently, the introduction of artificial functionality to proteins has enabled the use of cycloaddition "click" reactions. The 1,3-dipolar cycloaddition of azides and alkynes has been widely adopted as it is both selective and high yielding when catalyzed by Cu(I)[75−78] or promoted by strain release.[79−81] The triazole linkage formed in the cycloaddition reaction is thermodynamically and hydrolytically stable.[77] Several other cycloaddition reactions, such as the inverse demand Diels−Alder reaction between tetrazines and strained alkenes[82,83] and the 1,3-dipolar cycloaddition of alkenes with photogenerated nitrile imines,[84] have been identified that have improved second order kinetics relative to the traditional azide−alkyne cycloaddition. In addition, many of these bioorthogonal reactions have been shown to be orthogonal to one another,[85] potentially enabling the synthesis of even more complex protein-containing block copolymers.

While the selection of a reaction for the synthesis of each protein−polymer conjugate should be carefully considered, taking into account the specific demands of the particular bioconjugate, a few methods have stood out as the most widely adopted and broadly successful.[86] To achieve site-selective modification, N-terminal modification, cysteine alkylation with maleimides, Sortase-catalyzed modification, and strain-promoted azide−alkyne cycloaddition are frequently successful. However, it is likely that several reactions and modification sites will need to be evaluated in order to achieve the desired level of selectivity, yield, and protein function.

Once a bioconjugation site and reaction have been identified, a strategy for protein-containing block copolymer synthesis must be developed (Scheme 3). The direct conjugation of polymers to proteins, "grafting to", has traditionally been used to attach end-functionalized polymers to proteins. Separating the polymer synthesis and protein modification steps allows for polymerization in organic solvents and at elevated temperatures, maximizing flexibility and control during polymerization. Additionally, the polymer can be characterized before attachment to the protein component. However, the coupling of a synthetic macromolecule to a biomacromolecule can prove very challenging. The efficiency of the coupling is dependent on the molar mass of the polymer, and to achieve high levels of conversion it is often necessary to use a large excess of the polymer component. This strategy also requires the installation of a protein-reactive handle on the polymer that must be preserved through the polymerization or introduced post-polymerization.[87−97] These challenges are further compounded by the difficulty of separating the unreacted macromolecular components from the desired bioconjugate.

Scheme 3. Methods for the Synthesis of Protein Block Copolymers



Alternately, polymer chains can be synthesized directly from the biomolecule in a strategy termed "grafting from". The development of controlled radical polymerization methodologies that are compatible with aqueous solution[98] and the wide array of functional groups present on the protein surface have enabled this strategy. Atom transfer radical polymerization (ATRP) and reversible addition−fragmentation chain transfer (RAFT) polymerization have been successfully used in the grafting from strategy.[97,99−109] Modification of the protein component with an initiator or chain transfer agent (CTA) to create a protein macroinitiator followed by polymerization in aqueous solution creates protein−polymer block copolymers. While the conjugation of a low molar mass compound in the first step often improves efficiency and yield, the aqueous polymerization conditions can be limiting and often result in higher polydispersities than the "grafting to" strategy.[109] Additionally, synthesis of the polymer directly from the protein makes the characterization of the polymer component difficult. The hydrophilic protein component makes traditional DMF or THF gel permeation chromatography (GPC) unfeasible, and the high molecular weight and differences in ionizability of the conjugate make MALDI-TOF characterization nontrivial. Two methods to address this difficulty have been developed. First, the protein component can be cleaved from the polymer, either by digestion of the protein or by incorporation of a cleavable moiety into the conjugation site. This method allows direct characterization of the polymer component. Alternately, indirect characterization of the in situ synthesized polymer can be accomplished by including a small amount of free initiator with the protein macroinitiator.

Regardless of the selected strategy, an appropriate purification must be coupled with the conjugation strategy. The purification of protein−polymer conjugates requires removal of unreacted polymer or monomer and unmodified protein. The selected purification strategy must be protein compatible and, thus, is limited to standard protein purification techniques such as affinity chromatography and precipitation.[110] Dialysis or ultrafiltration can also be used to remove free small molecules present in the grafting from strategy. The unreacted protein component can often be removed by selective precipitation of the protein−polymer conjugate and free polymer. By varying the concentration of ammonium sulfate it has been demonstrated that the higher molecular weight conjugate can be separated from the free protein.[22,111,112] Removal of unreacted polymer typically can be accomplished using ion exchange chromatography as many polymers are uncharged and thus minimally interact with the resin. Affinity chromatography, such as Ni-NTA, streptavidin, protein-A, and maltose-binding protein, can be used successfully to separate appropriately tagged proteins from the conjugation reaction. Finally, size exclusion chromatography can be used to separate the protein, polymer, and protein−polymer conjugate; however, this process is often low yielding and proves difficult to achieve the resolution necessary to separate the different species.

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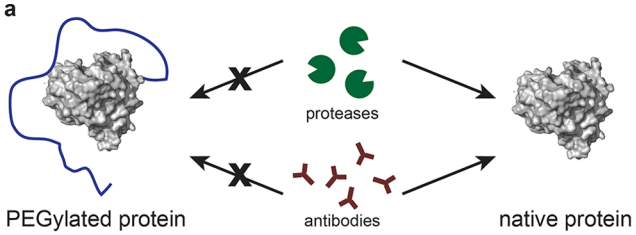
EMERGING APPLICATIONS OF PROTEIN BLOCK COPOLYMERS

Interest in protein−polymer conjugates stems from their ability to perform functions that unmodified proteins cannot. Conjugation of a polymer block to proteins can significantly alter the solution behavior of the protein. The polymer component can confer improved thermal stability, solubility in organic solvents, altered pharmacokinetics, and controllable bioactivity to the conjugate. In addition to modulating the solution behavior of the protein, addition of the polymer component can be used to direct the nanoscale self-assembly of proteins−a property not commonly found in native, unmodified proteins. In dilute solution, protein−polymer block copolymers can form structures such as micelles, micellar rods, and large vesicles. Significantly, the polymer component can also be used to direct the self-assembly of proteins in the solid state. This enables the immobilization of the protein component in a highly ordered, densely packed array. However, the design and application of such molecules also highlights the need for an improved physical understanding of their properties.

The most extensively studied aspect of protein-containing block copolymers is the effect of PEGylation on the biological properties of proteins (Figure 1a). Conjugation of PEG to

a



b

trade name	protein	PEG size	indication	company
adagen	adenosine deaminase	5 kDa	SCID	Enzon
oncaspar	asparaginase	5 kDa	ALL	Enzon
somavert	GH antagonist	5 kDa	excessive growth	Pfizer
PEG-intron	interferon α2b	12 kDa	hepatitis C	Schering-Plough
pegasys	interferon α2a	40 kDa	hepatitis C	Roche
neulasta	granulocyte CSF	20 kDa	neutropenia	Amgen
mircera	EPO	30 kDa	anemia	Roche
cimzia	anti-TNFα Fab'	40 kDa	rheumatoid arthritis	Nektar/UCB
krystexxa	uricase	10 kDa	chronic gout	Savient

Figure 1. Therapeutic protein−polymer conjugates. (a) Schematic of some of the biological effects of protein PEGylation. (b) Table of protein-PEG therapeutics with FDA approval (GH, growth hormone; CSF, colony stimulating factor; EPO, erythropoietin; TNFα, tumor necrosis factor α; SCID, severe combined immunodeficiency disease; ALL, acute lymphatic leukemia).

proteins results in an increase in the protein half-life in vivo.[7,113−119] Multiple mechanisms govern this increase in circulation time, including reduced renal clearance due to the increase in molecular weight and the "stealth" properties of PEG, which minimizes phagocytosis and blood clearance.[120] PEGylation also results in increased stability of the conjugate and decreased immunogenicity. The stability of the modified proteins is improved through two means: decreased protein aggregation and decreased proteolytic degradation.[121−123] PEGylation decreases protein immunogenicity by masking any protein epitopes in a well-hydrated polymer shroud. The number of modifications, polymer mass and branching all impact the changes in conjugate immunogenicity, with increased number of modifications, PEG molecular weight, and branching generally resulting in the greatest improvements to protein immunogenicity.[115] These properties have led to the FDA approval of several therapeutic protein-PEG conjugates (Figure 1b).[8] In addition to PEG, several other hydrophilic polymers are capable of modulating the interaction of proteins with biological systems.[124−126]

Protein modification with PEG can also be used to alter the solubility of the protein−PEG conjugate. PEGylation results in a conjugate that is soluble in both aqueous solution and organic solvent, improving the processability of the conjugate. These improvements in processability have been applied to PEG-modified viral capsids, among other proteins.[127] The hybrid materials can be transferred to nonpolar organic solvents and demonstrate high thermal stability. The ability to solubilize proteins in these organic solvents enables further functionalization with or incorporation into materials that are not soluble in aqueous systems. For example, the integration of proteins with conducting polymers, such as polythiophene or poly(phenylenevinylene),[128−130] is highly attractive for sensor applications but significantly challenging given their immiscibility; however, protein−PEG block copolymers can potentially enable solubilization of proteins with these electrically active polymers.

The conjugation of polymers that respond to small changes in environmental conditions, such as temperature, pH, specific ion concentration, or UV/visible light, was pioneered by Hoffman[131,132] and can be used to impart non-native responsive properties to the conjugate. These stimuli responsive, or "smart", polymers undergo dramatic, sharp changes in solubility in response to specific triggers. The ability to control the solubility of smart polymer protein conjugates has led to their application in affinity separations,[133−136] enzymatic reactions,[10−12,20,137−140] and biosensors.[141] The conjugation of a responsive polymer close to the active site of an enzyme can be used to gate the enzymatic activity in response to the particular trigger. This concept was elegantly demonstrated with the conjugation of azobenzene-dimethylacrylamide copolymers near the active site of endoglucanase 12A (Figure 2a).[11] By isomerizing the azobenzene using UV or visible light the activity of the cellulase could be regulated. This strategy has also been employed to modulate the binding properties of proteins, such as streptavidin, in response to specific stimuli.[142−144] An alternative use of stimuli responsive polymer enzyme conjugates relies on the significant decrease in polymer solubility upon a small change in the environmental conditions. The enzyme−polymer conjugate can be selectively precipitated from solution in order to simplify the recovery of the enzyme after catalysis (Figure 2b) while avoiding the loss of activity often observed with enzyme immobilization.[12] The potential for these smart protein conjugates to gate protein function and recycle protein substrates has been successfully demonstrated, yet the practical, scaled application of these useful conjugates with pharmaceutically or industrially relevant proteins remains underdeveloped.

Despite the myriad of possible new properties provided by polymer conjugation, our understanding of the physics of these increasingly important molecules remains poorly developed. The effect of the polymer on the solution properties of the conjugate is not very well understood. Currently, it is not

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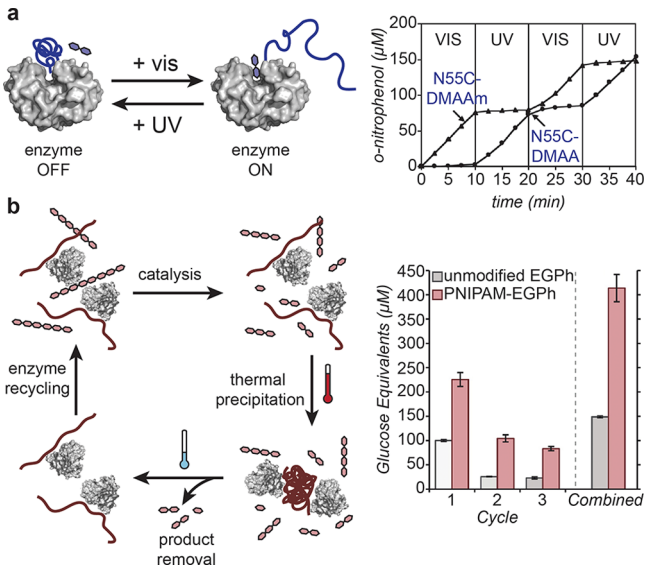

Figure 2. Conjugation of stimuli responsive polymers to proteins. (a) Conjugation of photoresponsive polymers (copolymers of *N*,*N*-dimethyl acrylamide and a photoresponsive acrylamide, DMAAm, or a photoresponsive acrylate, DMAA) close to the active site of endoglucanase 12A can be used to switch the activity of the enzyme on and off. Adapted with permission from ref 11. Copyright 2002 National Academy of Sciences, U.S.A. (b) Alternatively, conjugation of a thermoresponsive polymer, PNIPAM, far from the active site of endoglucanase EGPh can be used to recycle the enzyme for several catalytic cycles. Adapted with permission from ref 12. Copyright 2013 American Chemical Society.

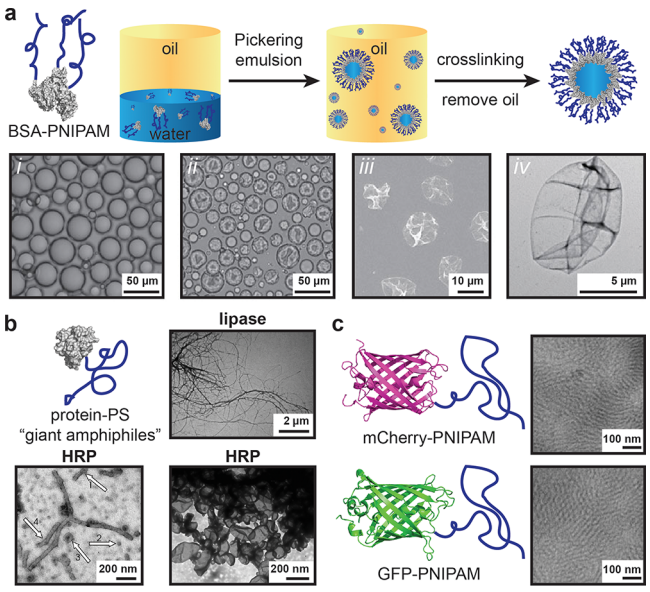

Figure 3. Self-assembly of protein−polymer conjugates. (a) Amphiphilic protein−polymer conjugates self-assemble at the water droplet/oil interface to produce proteinosomes. (*i*,*ii*) Optical microscopy images of the proteinosomes in oil (*i*) or after partial dyring (*ii*). Electron microscopy (scanning, *iii*, and transmission, *iv*) of the proteinosomes. Adapted with permission from ref 147. Copyright 2013 Nature Publishing Group. (b) Conjugation of hydrophobic polystryene to polar proteins results in self-assembly of the giant amphiphiles. Lipase conjugates self-assemble to form fibers of bundled micellar rods and HRP conjugates self-assemble to form a mixture of micelles, micellar rods, and vesicular aggregates as observed by TEM. Adapted with permission from refs 145 and 146. Copyright 2002 and 2007 American Chemical Society. (c) mCherry and GFP conjugated to PNIPAM exhibit rich phase behavior such as the lamellar phase shown in the TEM images. Adapted with permission from ref 25. Copyright 2014 American Chemical Society.

possible to predict a priori the size and shape of the conjugate in solution, and how that effects potential interactions with a biological system, or how the molecular level dynamics of the protein differ from the conjugate, and the effects that has on protein activity. The ability to design bioconjugate materials will benefit from additional understanding of fundamental polymer science questions. What is the conformation of a polymer conjugated to a protein? How is the polymer conformation influenced by interactions with the protein surface? And how does the presence of the polymer affect the dynamics of the molecule and stability of the protein fold? The answers to these questions will of course depend strongly upon the polymer chemistry and solution conditions for the polymer, and identifying the level of detail about the protein that must be captured to gain a predictive understanding of conjugate design poses an extremely large challenge for such a diverse class of molecules.

While polymers can be used to alter the solution properties of proteins, the polymer component can also be used to direct the assembly of proteins into nanomaterials (Figure 3). The attachment of hydrophobic polymers, such as polystyrene (PS) or PS-*b*-PEG, to proteins results in "giant amphiphiles" (Figure 3b)[145,146] that spontaneously aggregate into structures such as micellar rods, vesicles, and toroids. The ability to self-assemble in solution has been applied to make protein nanoparticles for drug delivery. Self-assembly of the protein−polymer conjugate into well-defined 50−100 nm micelles enables encapsulation of drug molecules in the core of the micelle. For example, a BSA-poly(methyl methacrylate) giant amphiphile has been used to form 100 nm micelles that encapsulate and deliver the chemotherapeutic camptothecin both in vitro and in vivo.[9] Alternately, amphiphilic protein−polymer conjugates have been shown to self-assemble into micron-sized (20−50 μm) vesicles, termed "proteinosomes", at the oil/water droplet interface (Figure 3a).[147] These vesicles were stably dispersed in oil or could be cross-linked and then transferred to aqueous solution. They were capable of guest molecule encapsulation and were selectively permeable. These properties enabled the proteinosomes to be used for cell free protein expression and membrane-gated enzymatic catalysis.

Bioconjugation may also be used to direct the self-assembly of protein-containing block copolymers to form nanostructured solid materials.[148−150] The incorporation of proteins in well-defined solid materials has applications in biosensors[151] and heterogeneous catalysis.[152,153] The folded shape of the protein is critical to both the function of the final material and to the physics of the self-assembly process, yet the specific three-dimensional folded shape of the protein, anisotropic charge, hydrophobicity and hydrogen-bonding capability add significant complexity to the self-assembly of protein-containing block copolymers. Initial fundamental studies on the direct self-assembly of conjugates with relatively simple polypeptide structures, such as α-helices and β-strands, revealed rich self-assembly behavior that differs from that observed in traditional coil−coil block copolymers.[154−157] The ability to form nanostructured solid materials from these conjugates has been extended to other simple polypeptide structures as well as larger globular proteins, such as the fluorescent proteins mCherry and GFP (Figure 3c).[22−25] The similar self-assembly

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behavior of these two structurally similar β-barrel shaped proteins suggests that coarse-grained properties such as shape, solubility, total charge, and second virial coefficient largely capture the self-assembly behavior of protein−polymer block copolymers. The two conjugates also demonstrated some key differences, such as the ability to form stable micelles, which highlights the need for the development of theories capable of predicting these differences.

Protein−polymer block copolymers have also been simultaneously coassembled with amphiphilic block copolymers. This was first demonstrated with the coassembly of PEG modified ferritin with P2VP-*b*-PEG,[158] and it has subsequently been used to produce catalytically active films of myoglobin.[21] By functionalizing the enzyme with poly(ethylene glycol), the modified protein can be processed with the block copolymer in organic solvents.

While conjugation of polymers to proteins has been shown to direct bioconjugate self-assembly, our understanding of the thermodynamics of bioconjugate self-assembly is currently underdeveloped. The ability to design de novo the self-assembly of these hybrid materials will be improved by an expanded understanding of fundamental polymer physics questions. How do protein electrostatics, hydrophobicity, and sterics effect protein−protein and protein−polymer interactions in dilute and concentrated solutions? How does the surfactancy of both the protein and the conjugate govern interactions with surfaces? And how does the shape of a protein affect the thermodynamics and kinetics of nanostructure formation? While these answers will be highly dependent on the protein substrate, a detailed understanding of these effects is required in order to rationally design stable self-assembled structures, both in solution and in the solid state.

SUMMARY AND OUTLOOK

As the synthetic methods to modify proteins and make well-defined polymers continue to improve, protein-containing block copolymers have become increasing accessible at scale. Recent advances in the field of bioconjugation have enabled the residue- and site-selective modification of proteins; however, the robustness of many of these new methods for demanding biomacromolecule−macromolecule coupling has yet to be demonstrated. In parallel, progress in controlled radical polymerization has allowed many of these new chemistries to be used for the bioconjugation of polymers to proteins. CRP has enabled the synthesis of polymers with highly functional end groups for selective bioconjugation or the use of proteins directly as macroinitiators.

Continued improvements in bioconjugation efficiency are needed in order to use the "grafted to" strategy for the synthesis of conjugates with increasingly complicated components, such as glycoproteins. Similarly, to achieve practical application of "grafted from" protein-containing block copolymers, further improvements to CRP in aqueous solution are needed to decrease the polydispersity of the resulting conjugates. The use of biosynthesis to create protein-containing block copolymers with two protein components offers a potential solution to the difficulties presented in the chemical synthesis of these hybrid materials; advances in the genetic construction and production of proteins with high molecular weight and highly repetitive sequences will continue to increase the attractiveness of this approach.[159] Additionally, identification of amino acid sequences that mimic the properties of the polymer component, such as PEG or "smart" polymers, will facilitate the adoption of fusion protein block copolymers.[160−162] To this end, fusion of an unstructured recombinant polypeptide, XTEN, to peptides and proteins has been shown to dramatically extend their plasma half-life in a manner similar to PEG.[163] The commercial success of this biopolymer demonstrates the great potential for biosynthetically produced protein block copolymers.

These synthetic and biosynthetic advances have enabled the application of protein-containing block copolymers to meet challenges in the fields of catalysis, sensing, biotechnology, and sustainability/energy. On the horizon, the utility of protein-polymer conjugates as therapeutic drugs will continue to grow rapidly−with a dramatic increase in marketed protein−polymer drugs expected. Beyond the use of PEG or XTEN to improve protein pharmacokinetic properties, the use of protein block copolymers for nanoparticle-based drug delivery is likely. The ability of synthetic and biosynthetic polymers to direct the self-assembly of proteins into micelles and vesicles enables the use of this technology for the straightforward delivery of protein or peptide drugs. In addition to the use of protein block copolymers for therapeutic applications, protein−polymer conjugates are anticipated to greatly impact the field of biocatalysis. Efforts to identify or evolve enzymes capable of performing difficult catalytic transformations combined with advances in the materials science of enzyme conjugates will advance the adoption of protein biocatalysts with tunable material properties.

However, a current limitation is that a majority of the conjugates synthesized to date have utilized model proteins. This is due, in part, to our inability to predict with confidence the success of both the conjugation chemistry and the physical effects of the polymer on the protein component. Going forward, to fully realize the potential of protein−polymer conjugates for a wider array of applications, a better understanding of the polymer physics of the hybrid materials is needed. Tackling applications with challenging protein substrates, such as membrane proteins, or those with quaternary structure, demands better design criteria for the properties and performance of the conjugate. Although the proteins are simple linear polymers of the 20 natural amino acids, the sequence specificity of the biopolymer leads to complex folded shapes and molecular interactions. A comprehensive understanding of the thermodynamics of the complex interactions between the protein and polymer components and predictive models for properties of interest are critical to the successful application of protein-containing block copolymers to meet current and future technological needs.

AUTHOR INFORMATION

Corresponding Author

*E-mail: bdolsen@mit.edu.

Notes

The authors declare no competing financial interest.

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