Synthesis of protein–polymer conjugates

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Protein–polymer conjugates are widely employed for applications in medicine, biotechnology and nanotechnology. Covalent attachment of synthetic polymers to proteins improves protein stability, solubility, and biocompatibility. Furthermore, synthetic polymers impart new properties such as self assembly and phase behavior. Polymer attachment at amino acid side-chains and at ligand binding sites is typically exploited. This Emerging Area focuses on synthetic methods to prepare protein-reactive polymers and also employing the protein itself as an initiator for polymerization.

1. Introduction

The inherent specificity of proteins in biological systems makes them ideal molecules for use as therapeutic agents or as molecular sensors and switches.[1–3] Applications of naturally occurring and recombinant proteins span the fields of biotechnology, nanotechnology and medicine, and covalent attachment of synthetic polymers to proteins has been shown to significantly improve properties such as stability, biocompatibility and solubility for performance in these areas.[4,5] Additionally, the attachment of a polymer chain can be used to modulate protein activity.[6] The first protein–polymer conjugates were based on modification of lysine side-chains with poly(ethylene glycol) (PEG), termed PEGylation,[7,8] and several PEGylated proteins are used clinically as therapeutics.[2] 'Smart' polymer bioconjugates have been employed for affinity separations, microfluidic protein analysis and capture, molecular sensors, and as switches to control protein activity.[6,9–11] The polymers in these cases respond to an external stimulus such as light, temperature or pH. The polymers undergo conformational changes in response to the stimulus and phase-separate from solution. In the absence of the stimulus the polymers are soluble in water.

The conventional approach to prepare polymer bioconjugates is to modify polymers with protein-reactive end-groups, such as activated esters, that facilitate coupling between the polymer and the amino acid side-chains. More recently, protein-reactive initiators have been used to synthesize polymers (Fig. 1); these polymers are reactive towards proteins directly after polymerization. Polymers with ligands for binding sites are also employed.

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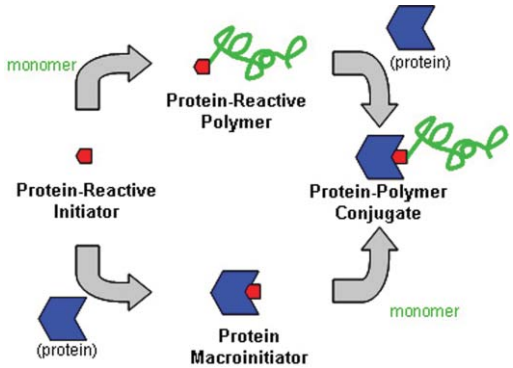


Fig. 1 Routes to protein–polymer conjugates.

A method to prepare conjugates that has also recently been introduced is to synthesize polymers directly from initiation sites on the protein (Fig. 1). This Emerging Area will focus on traditional ways to prepare polymers that react with amino acid side-chains to form heterogeneous and homogeneous conjugates. Newer approaches such as one-step synthesis of reactive polymers with bi-functional initiators and polymerization from protein initiators to form conjugates will also be discussed.

2. Postpolymerization modification strategies

2.1. Amine-reactive polymers

Amine side-chains of lysine have traditionally been targeted to synthesize protein–polymer conjugates. The excellent reactivity of amines toward a wide range of electrophiles, and the necessity that each protein contains at least one amine makes this an attractive method for bioconjugate formation. Amine-reactive polymers often contain activated esters such as *N*-hydroxysuccinimidyl (NHS) esters, and comprehensive reviews are available on this subject.[7,8] Polymers with alternative end-groups including mono- and dichlorotriazines, thioimidoesters, and aldehydes have also been synthesized for protein modification.

Early reports of protein PEGylation involved transformations of the terminal hydroxyl group of PEG; these methodologies can be employed for any polymer with a hydroxyl end-group. For example, dichlorotriazine end-functionalized monomethoxy PEG was synthesized for the modification of bovine serum albumin (BSA)[12] and bovine liver catalase.[13] One significant drawback of this reactive end-group is protein cross-linking. To circumvent this, 2,4-bis(methoxy-poly(ethylene glycol))-6-chloro-*s*-triazine was prepared by coupling the ω-hydroxyl groups with cyanuric chloride at 80 °C (Fig. 2a);[14] the resulting activated-PEG_2 was coupled to asparaginase. Thioimidoester end-groups are interesting because they form amidinated linkages at physiological pH, without loss of the positive charge at the conjugation site. This end-group was synthesized in two steps by reaction of a terminal alkoxide with 3-bromopropionitrile to form a cyano-terminated polymer.[15] Subsequent addition of ethanethiol in the presence of dry hydrochloric acid resulted in thioimidoester formation (Fig. 2b). Modification of the ribosome-inactivating protein, gelonin, was demonstrated employing this end-group.[15] Aldehydes couple with amine-functionalized residues *via* reductive amination and are readily installed by oxidation of a terminal hydroxyl group

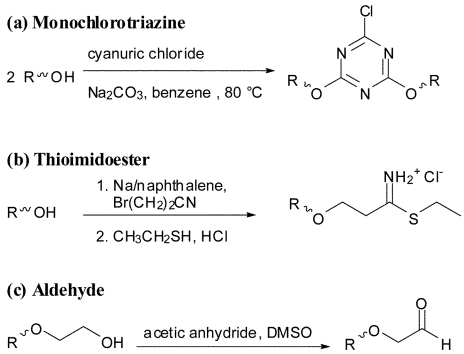


Fig. 2 Synthesis of amine-reactive end-groups.

with DMSO–acetic anhydride (Fig. 2c).[16] CD4 immunoadhesin– and human granulocyte colony-stimulating factor–PEG conjugates were prepared from aldehyde end-functionalized PEG.[17,18]

Each of these examples demonstrates straightforward routes to prepare protein-reactive polymer end-groups. However, the disadvantage of this approach is that a heterogeneous bioconjugate frequently results due to non-specific coupling with multiple and random amine residues. Heterogeneous conjugation often leads to a significant reduction in bioactivity. Site-specific modification of amines has been attempted by exploiting the lower pK_a of the N-terminal α-amine;[18] however, even when the coupling reaction is conducted under slightly acidic conditions, heterogeneity is often still observed.[7] Therefore, other approaches that result in well-defined conjugates are also explored.

2.2. Thiol-reactive polymers

In an effort to create well-defined protein–polymer conjugates, modification of the sulfhydryl group of cysteine is employed. Targeted cysteines include those present in the native proteins, as well as those introduced by site-directed mutagenesis. Cysteines not participating in disulfide bonds occur with low frequency, and therefore modification of this residue results in site-specific bioconjugates. Numerous postpolymerization modifications have been described to install thiol-reactive end-groups into polymers. Vinyl sulfone, maleimide, and activated disulfide end-groups are among the most popular and have been demonstrated to be effective for direct modification of cysteines in proteins.

Hoffmann, Stayton and co-workers have carried out pioneering work on 'smart' polymer bioconjugates and have illustrated site-specific modification of various genetically engineered proteins by employing vinyl sulfone terminated polymers.[19–25] For example, free-radical polymerization of *N*-isopropylacrylamide (NIPAAm) initiated by 2,2′-azobisisobutyronitrile (AIBN) with β-mercaptoethanol as the chain-transfer agent resulted in the thermoresponsive, α-hydroxyl-functionalized polyNIPAAm. The α-hydroxyl group was then converted to the vinyl sulfone through coupling with divinyl sulfone in the presence of base (Fig. 3a).[20] Protein modification with these 'smart' polymers resulted in the ability to control ligand binding events in streptavidin[19–23] and endoglucanase 12A[24,25] by attachment of the polymers to an unnaturally occurring cysteine introduced near the active site of the proteins.

Maleimides react selectively with the thiols of cysteine residues in the pH range 6.5–7.5 *via* Michael addition, and can be added to

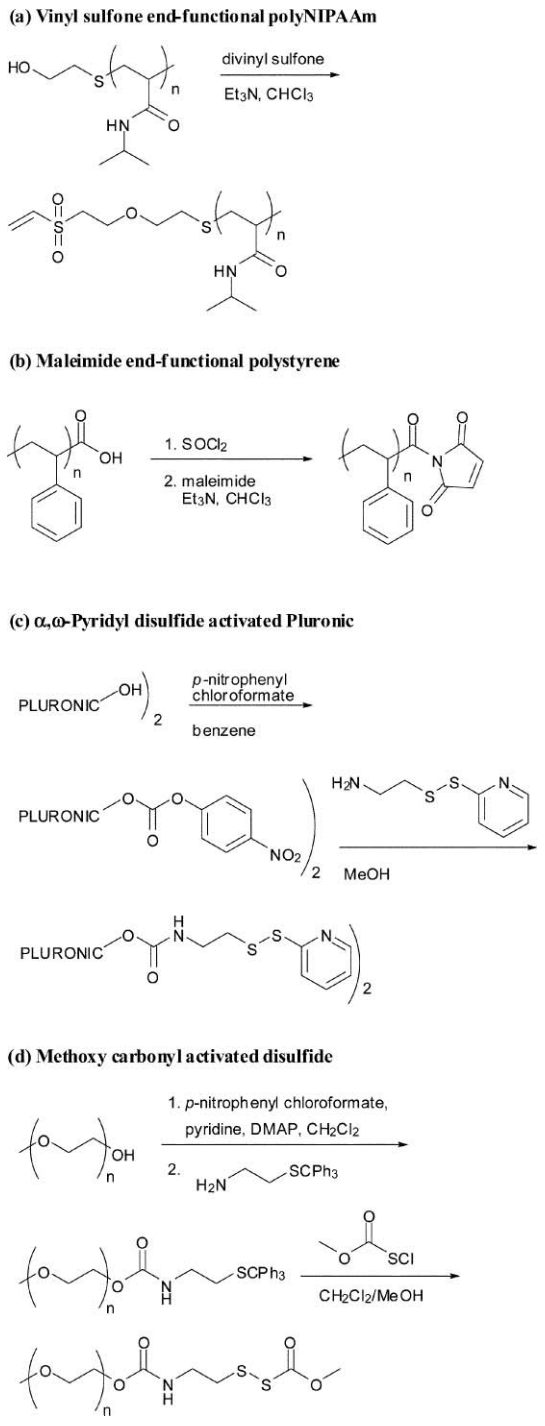


Fig. 3 Synthesis of thiol-reactive end-groups.

polymer end-groups using a variety of approaches. In one example, Kogan demonstrated conversion of an amine-terminated PEG to a maleimide by reaction with maleic anhydride. The intermediate maleamic acid was dehydrated with acetic anhydride/sodium acetate to facilitate ring-closure.[26] Velonia *et al.* demonstrated the synthesis of a maleimide end-functionalized polystyrene in two steps: conversion of the terminal carboxylic acid of the polymer to the acid chloride followed by coupling with maleimide in the presence of base (Fig. 3b).[27] This polymer was covalently attached to the free cysteine of lipase B from *Candida antarctica* (CAL B) that was generated by reduction of an external disulfide bond on the enzyme. Modification of CAL B with polystyrene resulted in a giant amphiphile where the enzyme provided the hydrophilic headgroup and the polystyrene provided the hydrophobic tail. In a separate report, Chilkoti *et al.* synthesized polyNIPAAm by free-radical polymerization employing 2-aminoethanethiol as the chain-transfer agent. The α-amine of the resultant polyNIPAAm was subsequently coupled with a NHS-activated maleimide derivative to form an α-maleimide polyNIPAAm for modification of cytochrome b5.[28] A similar approach was demonstrated by Pennadam *et al.*, where an α-functional maleimide polyNIPAAm–EcoR124I endonuclease conjugate was prepared.[29] In this example, the thermoresponsive nature of polyNIPAAm was exploited to allow for control over enzyme activity.

Activated polymers with pyridyl, alkoxycarbonyl and *o*-nitrophenyl disulfide end-groups form protein–polymer conjugates *via* disulfide formation. One appeal of this approach is that the bond is reversible and the protein can be released from the polymer under reducing conditions. A report by Li *et al.* demonstrated the synthesis of an α,ω-pyridyl disulfide functionalized Pluronic® (PEG-*b*-poly(propylene oxide)-*b*-PEG) for protein immobilization on a surface.[30] The terminal hydroxyl groups of Pluronic® were first activated with *p*-nitrophenyl chloroformate, followed by coupling with 2-(2-pyridyldithio)ethylamine (Fig. 3c). The functionalized Pluronic® was adsorbed onto a polystyrene resin and immobilization of β-galactosidase was demonstrated. Methoxy- or ethoxycarbonyl disulfide and *o*-nitrophenyl disulfide end-functional polymers can be synthesized by activation of a terminal hydroxyl group with *p*-nitrophenyl chloroformate, followed by reaction with 2-tritylthioethylamine, to afford a thiotrityl ether terminated polymer. Subsequent reaction with methoxycarbonyl sulfenyl chloride (Fig. 3d) or 2-nitrophenyl sulfenyl chloride forms polymers terminated with methoxycarbonyl or *o*-nitrophenyl disulfide, respectively.[31] These activated disulfide end-groups showed excellent reactivity with the model tripeptide, glutathione.

A new approach to site-selectively modify cysteines has recently been introduced whereby a native disulfide bond was targeted with an end-functionalized PEG containing a bisalkylating agent to form a three-carbon bridge between the two sulfur atoms.[32] This method employs a thiol-specific, cross-functionalized monosulfone PEG: amino-PEG was modified by reaction with 4-[2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl]benzoic acid NHS ester. The resulting bis-sulfone was dissolved in phosphate buffer and incubated to eliminate *p*-toluenesulfinic acid, forming the desired PEG mono-sulfone. Modification of human interferon α-2b and a human CD4 receptor-blocking antibody fragment (Fab) was demonstrated. This approach offers an opportunity to target natural disulfide bonds while retaining the protein's three-dimensional structure.

2.3. Polymers for oxime formation

Oxime formation between a hydroxylamine and a ketone/aldehyde-functionalized polymer or protein is another method to form site-specific conjugates. It may not seem obvious to use this approach due to the absence of either functional group within the naturally occurring amino acids; however, aminooxy or ketone moieties can be incorporated into a protein.[33–35] Due

to the excellent chemoselectivity of the reacting partners, this is a desirable approach to obtain well-defined conjugates.

In an elegant report by Kochendoerfer and co-workers, a synthetic erythropoiesis protein (SEP) was prepared by native chemical ligation, and N^{ε}-levulinyl lysines were incorporated into the protein.[33] Coupling of a branched α-functional hydroxylamine polymer with ketone groups of the N^{ε}-levulinyl residues formed a site-specific PEG–SEP conjugate. To prepare the polymer, the end-group with an aminooxy-functionalized amino acid and the linear PEG moiety were each prepared on solid-phase resin. Subsequent coupling of the fragments using amidation chemistry afforded the branched α-aminooxy PEG. The resultant SEP bioconjugate was homogeneous, and unlike typical PEGylated bioconjugates, bioactivity was equal to that of native erythropoietin. In the opposite approach, Kochendoerfer and co-workers demonstrated site-specific incorporation of a hydroxylamine moiety into the chemokine CCL-5 (RANTES) analogue at amino acid 45. Employing native chemical ligation, the protein was synthesized containing a protected aminooxy group. Upon deprotection, the free hydroxylamine was exposed for conjugation with an aldehyde end-functionalized linear PEG (Fig. 4).[34] Schlick *et al.* demonstrated oxime-linked bioconjugates using modified tyrosine residues of tobacco mosaic virus (TMV).[35] In their approach, tyrosine residues of proteins were selectively modified using ketone-functionalized diazonium salts. The introduced ketone moieties were then targeted with an aminooxy end-functionalized PEG. This polymer was synthesized by displacement of the terminal alcohol of monomethyl ether PEG with *N*-hydroxyphthalimide under typical Mitsunobu reaction conditions, followed by hydrazinolysis to afford the terminal hydroxylamine.

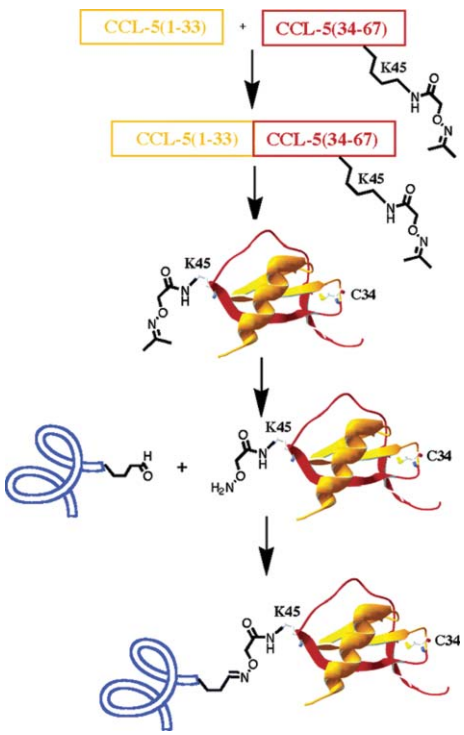
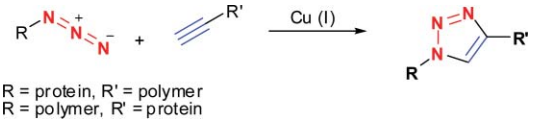


Fig. 4 CCL-5–PEG conjugate *via* oxime formation. (Reprinted with permission from Shao *et al.*,[34] copyright 2005 American Chemical Society).

2.4. Polymers for "click" conjugate formation

The high yields and mild conditions of azide–alkyne 1,3-dipolar cycloaddition reactions present an attractive approach to prepare bioconjugates (Scheme 1). To prepare conjugates in this way, the protein must be first modified with an azide or alkyne moiety.



Scheme 1 Bioconjugate formation *via* [3 + 2] cycloaddition.

In a recent report, an amphiphilic bioconjugate was synthesized by the [3 + 2] cycloaddition of an azide-terminal polystyrene with alkyne-functionalized BSA.[36] Bromine-terminated polystyrene was synthesized by atom transfer radical polymerization (ATRP), and subsequently reacted with azidotrimethylsilane and tetrabutylammonium fluoride (TBAF) to install a terminal azide. The free thiol of BSA was modified with a maleimide-functionalized alkyne, to produce the alkyne-modified protein. Deiters *et al.* employed a site-specific cycloaddition reaction on an azide-functionalized human superoxide dismutase-1 (SOD) using an alkyne end-functionalized PEG.[37] The protein was prepared by incorporation of *p*-azidophenylalanine into SOD *via* site-directed mutagenesis. The polymer was synthesized by coupling propargylamine with NHS ester-activated PEG. The resulting PEGylated SOD had approximately the same bioactivity as the native enzyme.

2.5. Ligand-modified polymers

An alternate route to prepare well-defined protein reactive polymers involves using polymers containing ligand end-groups. The near-covalent bond between (strept)avidin and biotin has generated much interest in the synthesis of polymers with biotin end-groups for applications in biotechnology. The naturally occurring carboxylic acid of biotin presents an easy handle for modification. Biotinylated 'smart' polymers have been synthesized for conjugation to both streptavidin and avidin. Kulkarni *et al.* synthesized a biotinylated polyNIPAAm *via* reversible addition–fragmentation chain transfer (RAFT) polymerization of NIPAAm.[38] Hydrolysis of the dithioester end-group in a methanol–aqueous sodium hydroxide solution formed a thiol-terminated polyNIPAAm. Subsequent coupling with a maleimide-functionalized biotin formed the biotinylated polyNIPAAm. Aggregation of mesoscale streptavidin–polyNIPAAm particles prepared from these conjugates was investigated for potential use in microfluidic devices for capture and release of biomolecules.[38] Giant amphiphiles comprised of a streptavidin–polystyrene conjugate were synthesized by Hannink *et al.* through use of a biotinylated polystyrene. The biotin functionality was introduced *via* coupling of an amine-functionalized biotin derivative with a carboxylic acid terminated polystyrene.[39]

Nolte has also demonstrated cofactor reconstitution of horseradish peroxidase (HRP) to prepare amphiphilic, bioactive protein–polymer conjugates.[40,41] A cofactor-terminated polymer was synthesized *via* amidation between a carboxylic acid terminated polystyrene and a mono-protected diamine. Subsequent

deprotection to form the free amine, coupling with one carboxylic acid group of protoporphyrin IX, and addition of ferrous chloride tetrahydrate, resulted in a polystyrene-cofactor (Fig. 5a). HRP was reconstituted with the polymeric cofactor (Fig. 5b), resulting in vesicular aggregates that displayed enzymatic activity.[40] This approach has great potential for producing controlled nanostructures of bioactive enzymes and proteins for biotechnology applications.

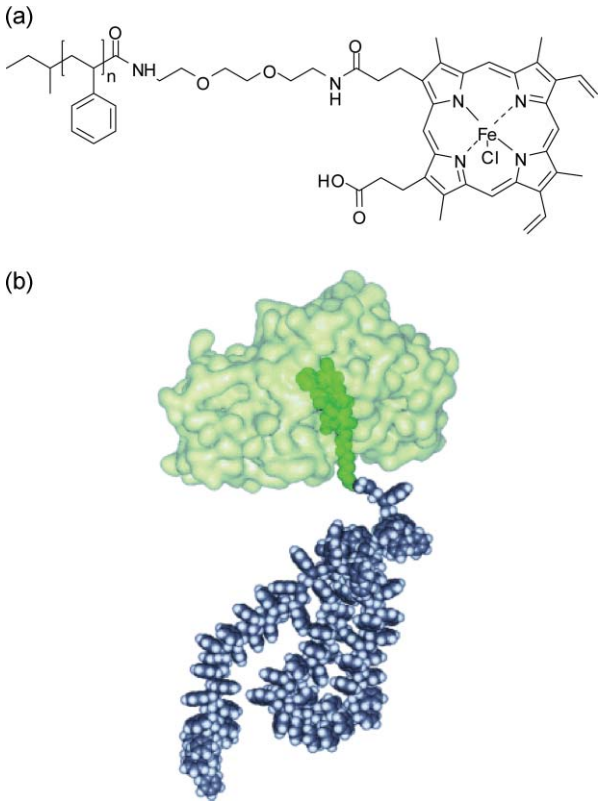


Fig. 5 Giant amphiphile prepared by cofactor reconstitution: a) polystyrene-cofactor and b) computer-generated model of HRP–polystyrene giant amphiphile. (Reprinted with permission from Boerakker *et al.*,[40] copyright 2002 Wiley Interscience).

3. New approaches to prepare bioconjugates

3.1 Functionalized initiators

Polymerization directly from protein-reactive initiators circumvents postpolymerization modifications and is a straightforward and less time-consuming approach to synthesize protein-reactive polymers. It virtually guarantees that each polymer chain contains one reactive end-group and does not rely on optimization of postpolymerization modification reactions. Similar to the strategies described above that incorporate end-groups reactive toward amino acid side-chains or binding sites, initiators with these same reactive groups have been synthesized for free-radical polymerization, as well as controlled/'living' radical polymerizations such as ATRP and RAFT. The latter two provide polymers with narrow molecular weight distributions.

Amine-reactive polymers have been synthesized with NHS-[42,43] and acetal-[44] functionalized initiators for ATRP in order to target lysine side-chains in proteins (Fig. 6a and Fig. 6b). The NHS-functionalized initiator was synthesized in one step by coupling *N*-hydroxysuccinimide with 2-bromopropionic acid to form the 2-bromopropionate NHS ATRP initiator.[42] Polymerization of PEG methacrylate (PEGMA) was conducted to form α-NHS-activated polyPEGMA with narrow polydispersities. The reactivity of the resultant polymers toward proteins was investigated, and it was found that 6–7 chains conjugated to the lysozyme. Interestingly, the analogous polymer prepared from the 2-bromoisobutyrate initiator was unreactive; the authors attributed the lower reactivity mainly to steric hindrance due to the extra methyl group. Acetal-functionalized initiators are also employed because the resulting polymer can be hydrolyzed, exposing aldehyde groups for reaction with amines *via* reductive amination.[44] This functionalized initiator for ATRP was synthesized by reaction of 2-chloro-1,1-dimethoxyethane with ethylene glycol, followed by esterification with 2-bromoisobutyryl bromide.[44] Copper-mediated ATRP of PEGMA was conducted. After hydrolysis of the α-acetal, conjugation to side-chain lysines was demonstrated.

Pyridyl disulfide[45] and maleimide[46] functionalized initiators for ATRP have been synthesized to enable conjugation to the free cysteines of proteins. Unlike targeting amines, this approach allows for the formation of well-defined conjugates. We have demonstrated the use of a pyridyl disulfide-functionalized 2-bromoisobutyrate initiator for ATRP of 2-hydroxyethyl methacrylate (HEMA) (Fig. 6c).[45] This functionalized initiator was synthesized in two steps by the reaction of 2,2′-dithiopyridine with 3-mercapto-1-propanol to form pyridyl disulfide propanol. Subsequent esterification yielded the ATRP initiator. Narrow molecular weight distribution polyHEMA with an activated disulfide group was synthesized and employed for direct conjugation to the free cysteine residue of BSA. An advantage of this approach is that no postpolymerization modification of the chains was necessary prior to conjugate formation and that the conjugate formation was reversible. Mantovani *et al.* prepared a protected maleimide initiator for ATRP (Fig. 6d), which was synthesized in three steps beginning with a Diels–Alder reaction between maleic anhydride and furan to form 3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride.[46] Subsequent reaction with ethanolamine formed the protected maleimide alcohol, which was then esterified with 2-bromoisobutyryl bromide to afford the initiator. PolyPEGMA was synthesized, and although a postpolymerization modification was required, it was straightforward. Refluxing the polymer in toluene resulted in a retro-Diels–Alder reaction to afford the α-maleimide group. Modification of BSA and the tripeptide glutathione was demonstrated with maleimide-functionalized polyPEGMA.

An azide-functionalized ATRP initiator (Fig. 6e) was employed for the polymerization of the glycomonomer, methacryloxyethyl glucoside. The azide-functionalized end-group enabled conjugation to a fluorescein dialkyne, which was used as a spectroscopic reporter molecule in successive manipulations. The resultant alkyne-fluorescein-glycopolymer was employed in a [3 + 2] cycloaddition reaction with azide-functionalized cowpea mosaic virus, formed by coupling of 5-(3-azidopropylamino)-5-oxopentanoic acid NHS ester to the amine side-chains. The glycopolymer–virus conjugate was evaluated for its ability to interact with the glucose-binding protein concanavalin A.[47]

Biotinylated initiators for a range of polymerization techniques have been synthesized and employed for direct conjugation to

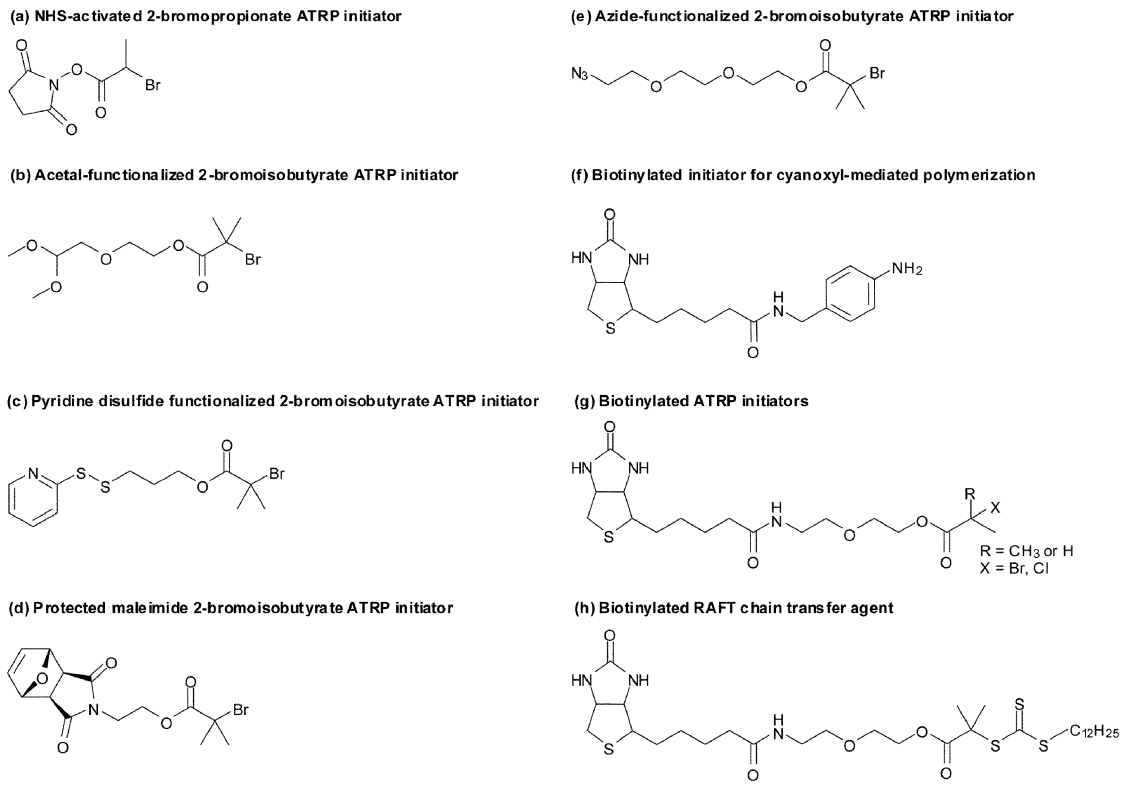


Fig. 6 Protein-reactive initiators.

streptavidin. Chaikof and co-workers employed a biotinylated initiator for cyanoxyl-mediated free-radical polymerization of glycomonomers.[48,49] The arylamine initiator (Fig. 6f) was synthesized in two steps after coupling a NHS-activated biotin with *p*-nitrobenzylamine and reducing the nitro group with hydrogen/palladium. Formation of the diazonium salt, followed by addition of sodium cyanate and glycomonomer, generated the biotinylated polymer. Streptavidin–glycopolymer conjugates were readily achieved by the interaction of the two species. In recent reports Wooley[50] and our group[51,52] have demonstrated the use of biotinylated ATRP initiators to synthesize low polydispersity polymers. The biotinylated ATRP initiators (Fig. 6g) were synthesized by activating the carboxyl group of biotin with *N*,*N*′-disuccinimidyl carbonate, followed by addition of 2-(2-aminoethoxy)ethanol.[51,52] Esterification of the resulting alcohol with 2-chloropropionic acid or 2-bromoisobutyric acid formed the biotin ATRP initiators. Biotinylated poly(acrylic acid)-*b*-poly(methyl acrylate),[50] polyNIPAAm,[51] and polyHEMA functionalized at the side-chains with *N*-acetylglucosamine (GlcNAc)[52] were synthesized from these initiators; facile conjugate formation with avidin or streptavidin was demonstrated. A biotinylated RAFT chain-transfer agent (CTA, Fig. 6h) has recently been reported.[53] The biotinylated CTA was synthesized by esterification of a carboxylic acid terminated trithiocarbonate CTA with a biotinylated alcohol. AIBN-initiated RAFT polymerization produced homo and block 'smart' polymers. In this case, streptavidin–polymer conjugates were not demonstrated; however, they are expected to form readily upon interaction of the two species.

3.2 Grafting from proteins

Generating polymers directly from proteins at defined initiation sites provides the opportunity to evade all postpolymerization modification strategies and protein–polymer coupling reactions. Some additional advantages of polymerizing directly from proteins are that purification of the final bioconjugate from unreacted monomer or catalyst is simplified. Also, the precise number and placement of polymer chains is predetermined, thereby facilitating the synthesis and characterization of well-defined conjugates. Reports in the literature have described methods of initiating polymerization of monomers in the presence of gelatin,[54–63] casein,[64,65] and ovalbumin[66] with peroxides or potassium persulfate. In these examples however, grafting of the polymers occurs at random and at multiple sites on the protein, resulting in poorly-defined materials. A route we have developed in our laboratory is to first modify the protein with an initiator for ATRP to form the protein macroinitiator and then polymerize directly from defined sites on the protein.

In the first report that we employed this methodology, polymerization was conducted directly from a streptavidin-macroinitiator (Fig. 7). The protein-initiator was formed by coupling a biotinylated ATRP initiator with streptavidin in phosphate buffered saline (PBS)–methanol, forming a streptavidin macroinitiator.[67] Polymerization of NIPAAm and PEGMA in water at ambient temperature with copper bromide and 2,2′-bipyridine (bipy) formed the streptavidin–polymer conjugates. Polymerization was conducted in the presence of 2-bromoisobutyrate-functionalized Wang resin in order to increase the concentration of initiation sites, while

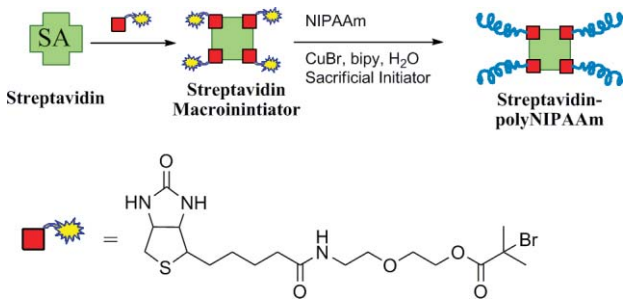


Fig. 7 Synthesis of streptavidin macroinitiator and *in situ* polymerization. (Reprinted with permission from Bontempo *et al.*,[67] copyright 2005 American Chemical Society).

utilizing a small amount of protein. Proteins are often unavailable in large quantities, and thus flexibility in this respect is critical. Streptavidin–polymer formation was confirmed by size exclusion chromatography (SEC) and SDS polyacrylamide gel electrophoresis (SDS-PAGE). Denaturation of the conjugate by heating in DMF–water at 90 °C resulted in dissociation of the streptavidin tetramer and release of the biotinylated polymer. Analysis of the released polymer by surface plasmon resonance (SPR) verified the presence of the biotin end-group, confirming that polymerization occurred specifically from the biotin initiation sites.[67]

In a separate report, we described modification of cysteine residues of BSA and a mutant T4 lysozyme with initiators for polymerization.[68] To form the macroinitiators, an ATRP initiator functionalized with pyridyl disulfide or maleimide was treated with the proteins in PBS–methanol, forming the disulfide or thioether bonds, respectively (Fig. 8a). Polymerization of NIPAAm formed the thermosensitive BSA– and T4 lysozyme–polyNIPAAm conjugates *in situ*. Protein–polymer conjugate formation was confirmed by gel electrophoresis and SEC, as evident by shifts to higher molecular weights. It was demonstrated that "sacrificial" resin-bound initiator was not necessary when large amounts of protein macroinitiator were available. The polyNIPAAm polymerized from BSA was isolated by reduction with dithiothreitol, and gel permeation chromatography indicated a polydispersity index (PDI) as low as 1.34. Bioactivity of the lysozyme–polyNIPAAm conjugates was evaluated (Fig. 8b) and found to be completely retained after initiator attachment and polymerization, indicating that this methodology is amenable for bioactive protein–polymer conjugate formation. Recently, Matyjaszewski and Russell demonstrated modification of lysine residues with 2-bromoisobutyramide groups for the formation of various chymotrypsin–polymer conjugates.[69]

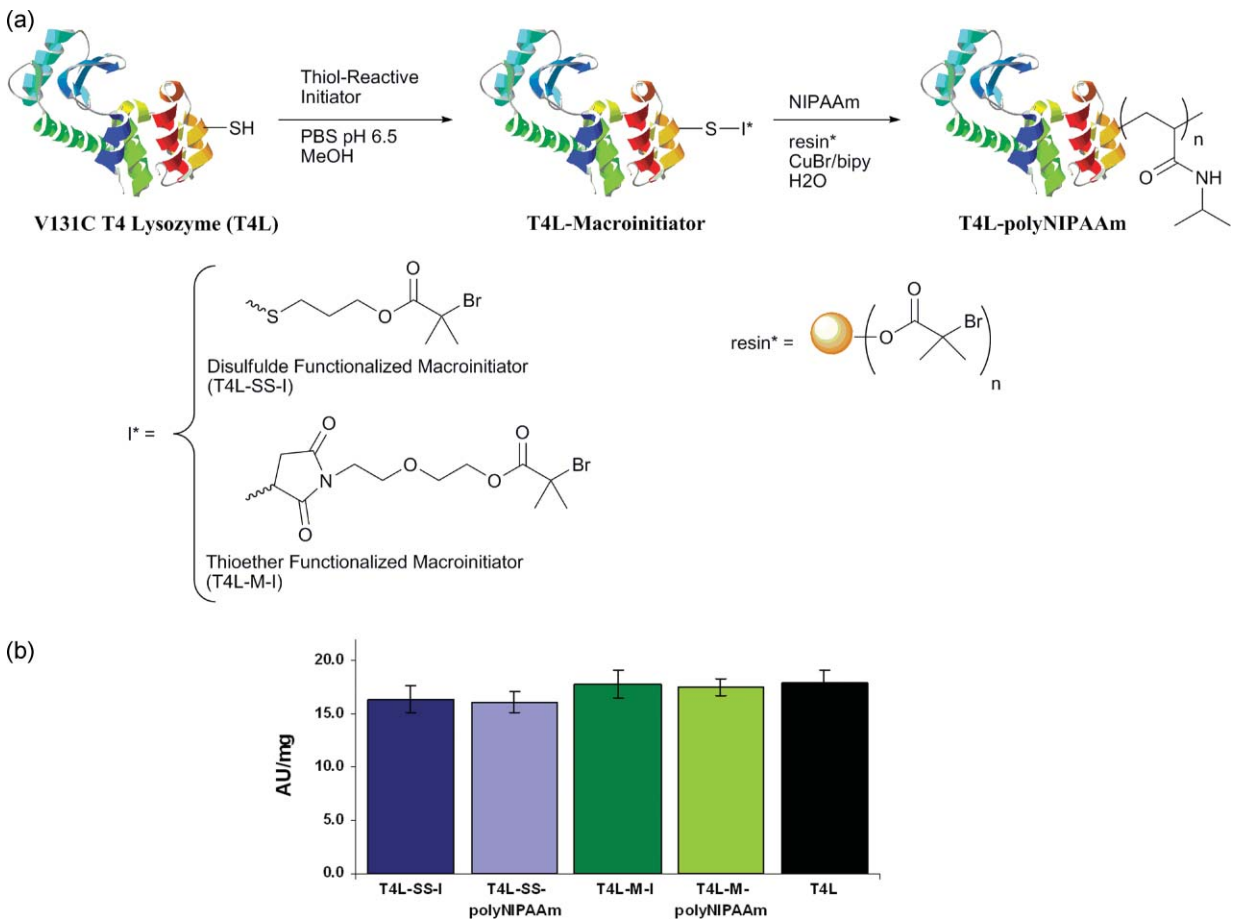


Fig. 8 a) Synthesis of V131C T4-lysozyme macroinitiator and *in situ* polymerization; b) UV-Vis bioactivity assay. (Reprinted with permission from Heredia *et al.*,[68] copyright 2005 American Chemical Society).[71]

4. Conclusions and outlook

Protein–polymer conjugates undoubtedly are an important biomaterial for a wide range of applications in the areas of medicine and biotechnology. Perhaps the most critical aspect of preparing protein–polymer conjugates for applications in these areas is to synthesize well-defined conjugates. This has generally been accomplished by targeting free cysteines or ligand binding sites. Traditionally the reactive polymers were prepared by modification of pre-formed chains. However, with the advent of new controlled/'living' polymerization techniques that are tolerant to a wide range of functional groups, the use of protein-reactive initiators to form well-defined polymers is now possible. This latter strategy is less time-consuming and results in polymers amenable to coupling to proteins without any further modification. Recently the use of protein macroinitiators to prepare protein–polymer conjugates *in situ* has been described. This route eliminates the necessity to form a reactive polymer-chain altogether.

Native chemical ligation, tRNA engineering methods, and other advances in protein engineering allow for the synthesis of proteins containing non-natural amino acids.[70] So far, the incorporation of functional groups that react orthogonally to natural amino acids has been under-exploited to prepare polymer bioconjugates. Controlled radical polymerization techniques such as ATRP and RAFT can be used to produce a number of end-functionalized polymers for chemoselective reaction with non-natural proteins. In addition, thus far, protein macroinitiators have been prepared by modification of cysteines, amines, or ligand binding sites in proteins. However, it may be possible to incorporate artificial amino acids containing polymerization initiators directly into proteins. These proteins could then be tailor-made to contain initiators and subsequent polymers at predetermined locations. This route to prepare protein–polymer conjugates has yet to be explored. Various monomers, proteins and alternative controlled polymerization techniques such as RAFT or ROP can be envisioned. The combination of controlled radical polymerization with non-natural protein engineering could result in unprecedented control over polymer conjugation, resulting in precise bioconjugates for a variety of applications.

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