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Protein-Polymer Conjugates: Synthetic Approaches by Controlled Radical Polymerizations & Interesting Applications

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Abstract

Protein-polymer conjugates are of interest to researchers in diverse fields. Attachment of polymers to proteins results in improved pharmacokinetics, which is important in medicine. From an engineering standpoint, conjugates are exciting because they exhibit properties of both the biomolecules and synthetic polymers. This allows the activity of the protein to be altered or tuned, a key aspect in therapeutic design, anchoring conjugates to surfaces, and utilizing these materials for supramolecular self-assembly. Thus, there is broad interest in straightforward synthetic methods to make protein-polymer conjugates. Controlled radical polymerization (CRP) techniques have emerged as excellent strategies to make conjugates because the resulting polymers have narrow molecular weight distributions, targeted molecular weights, and attach to specific sites on proteins. Herein, recent advances in the synthesis and application of protein-polymer conjugates by CRP are highlighted.

Introduction

Conjugation of synthetic polymers to proteins yields macromolecular constructs that synergistically merge the properties of the individual components.[1,2] Covalent attachment of polymers to proteins is known to improve protein efficacy by increasing lifetimes *in vivo* and decreasing immunogenicity.[3–6] For patients, this translates to fewer doses of the drug. Depending on the properties of the polymer, directed self-assembly of the conjugate to higher ordered nanostructures can also occur.[7] Additionally, protein-polymer conjugates can exhibit higher biological activities compared to unmodified proteins when attached to surfaces.[8] As a result, synthesis and characterization of these materials is of interest to researchers in the fields of medicine, biotechnology, nanotechnology, mechanical engineering, polymer chemistry, and bioengineering. Examples of peptide-, oligonucleotide-, and side chain polymer-conjugates have been summarized elsewhere.[9–12] Conjugation of linear poly(ethylene glycol) (PEG) to biomolecules (PEGylation) has also been extensively reviewed.[13,14] Herein, we highlight representative examples of the application of controlled radical polymerization (CRP)[15,16] techniques in the synthesis of well-defined, protein-polymer conjugates. Since the point of attachment of the polymer is crucial to the resulting properties, such as bioactivity, we focus on site-specific conjugation strategies.[17] The CRP methods that will be discussed are reversible addition-fragmentation chain transfer (RAFT) polymerization[18,19] and atom transfer radical polymerization (ATRP).[20,21]

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Controlled radical polymerizations

ATRP and RAFT polymerization are ideal methods to prepare bioreactive polymeric scaffolds. With ATRP, polymer growth occurs from an initiator. An ATRP initiator consists of a halogenated molecule capable of undergoing a reversible redox reaction with a transition metal catalyst to produce a reactive radical center. It is this reversible redox reaction that produces polymers with narrow molecular weight distributions (low polydispersity indices, PDIs) and pre-determined molecular weights. At the end of the reaction, ATRP polymers have the initiator fragment at the alpha chain end and a halogen at the omega chain end (Figure 1). RAFT polymerization is mediated by a reversible chain transfer agent (CTA), which typically contains a thiocarbonylthio group. The polymerization is often initiated with a small amount of a sacrificial initiator such as 2,2′-azobis(2-methylpropionitrile) (AIBN). Control during RAFT polymerization results from the chain transfer of the radical center to and from the thiocarbonylthio reactive group. Polymers produced by RAFT possess the thiocarbonylthio moiety covalently bonded to a radical stabilizing functional group (“Z” fragment) at the omega termini of the polymer. The alpha termini of the polymer contains the “R” fragment (site of polymer growth) of the CTA (Figure 1). Both techniques perform well in the presence of a wide range of solvents, functional groups, and reaction conditions. It is the high retention of the halogen and thiocarbonylthio groups at the omega termini postpolymerization for ATRP and RAFT, respectively, that allow further functionalization of these materials, as well as the ability to design protein-reactive initiators and CTAs to create bioreactive materials.

General synthetic approaches to protein-polymer conjugates

Synthesis of protein-polymer conjugates via CRP can be simplified into three distinct approaches (Figure 2).[22] *Grafting to* is the most common. The appropriate end-functionalized polymers are prepared either by modifying the chain postpolymerization to render it bioreactive or by growing the chain from a protein-reactive handle. *Grafting from* entails functionalization of the protein with a small molecule from which polymer growth can occur, such as an ATRP initiator or RAFT CTA, followed by polymerization. *Grafting through* involves biomolecular monomers and will not be discussed in this review.[12,22] The ultimate goal is to synthesize well-defined protein-polymer conjugates with high retentions of bioactivities relative to unmodified proteins. In order to achieve this, the targeted polymers have low PDIs (usually <1.3) and high retention of the end-groups. The sites of polymer conjugation to the protein or polymer growth from the protein are predetermined. The chemistry is mild, chemospecific, and efficient to maintain the tertiary and quaternary structure of the polypeptide, which is essential for activity. Therefore, three conjugation strategies are generally exploited for site-specific conjugations: 1) reaction with free cysteine residues, 2) modification of the *N*-terminal amine, or 3) use of protein-specific ligands.

Grafting polymers to proteins

As mentioned above *grafting to* can be segregated into two approaches. The first entails postpolymerization bioactivation of the polymer by end-group transformation. ATRP polymers retain the halogen at the omega-termini postpolymerization. Therefore, the halogen can be displaced with an appropriate nucleophile to generate the semitelechelic polymer, and then reacted with a protein containing the complimentary functional group. [23,24] For example, van Hest and coworkers utilized nucleophilic displacement of the bromine on polystyrene (pS) with an azide.[24] This facilitated the synthesis of pS-b-PEG-acetylene polymer upon subsequent Huisgen cycloaddition with a bis-acetylene-PEG. These block copolymers where then self-assembled into polymersomes ranging from 90 to 180 nm

in diameter with glucose oxidase (GOx) encapsulated in the lumen and lipase from *Candida antarctica* (Cal B) embedded in the bilayer. Diazo transfer was performed on horse radish peroxidase (HRP) to transform surface amines on the lysine residues into azides. HRP was conjugates onto the acetylene coated polymersome (Figure 3) by Huisgen cycloaddition. The three enzyme-cascade was initiated by incubation of the enzyme functionalized polymersomes with 2,3,4,6-tetra-O-benzyl–D-glucopyranose acetylated at the anomeric position and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The sugar was deacetylated by Cal B and then oxidized by GOx. This produced hydrogen peroxide, which in turn caused the oxidization of ABTS to $ABTS^{\bullet+}$ successfully demonstrating a three-enzyme cascade made possible by the polymersome architecture.

There are two approaches utilized for postpolymerization bioactivation of RAFT polymers, and both involve reaction with the thiocarbonylthiol group located at the chain end. Radical coupling with functionalized azoinitiators[25–28] and reduction of the thiocarbonylthio group to generate the free thiol, followed by further modification are both employed. Functionalization using azoinitiators will be discussed later in this review. Reduction of the thiocarbonylthio group is facilitated with reducing agents or good nucleophiles such as sodium borohydride and butylamine, respectively. The subsequent free thiol can then be adsorbed onto gold particles or surfaces,[29] trapped as an activated disulfide,[30] or modified using Michael acceptors.[31,32] For example, we synthesized Boc-protected α-aminooxy-poly *N*-isopropylacrylamide (pNIPAAm) ω-trithiocarbonate by RAFT. The Boc-aminooxy-pNIPAAm-trithiocarbonate was then reduced with butylamine and adsorbed onto a gold surface. After removal of the BOC group, oxidized heparin was immobilized on the surface via oxime bond formation. Bulmus, Davis and coworkers have recently demonstrated the ability to synthesize a variety of polymers by RAFT with both trithiocarbonate and dithioester CTAs that were then reduced with hexylamine.[30] The resulting thiols were trapped with 2,2'-dithiopyridine in order to produce activated disulfides. The versatility of the resulting semitelechelic polymers was demonstrated by reaction with a thiol containing peptide, oligonucleotide, and carbohydrate. We also recently demonstrated the *in situ* reduction of branched PEG polymers and reaction with divinylsulfone (Figure 4).[31] Conjugation of branched polymers to proteins is of interest because it has been shown that these types of scaffolds can result in conjugates with improved pharmacokinetic properties over linear polymer derivatives.[4–6,33] Specifically, PEG acrylate (M_n = 454 g/mole) (PEGA) was polymerized in the presence of a benzyl-dithiobenzoate CTA to generate a range of well-defined polymers.[31] The dithioester end-groups were reduced and incubated with divinyl sulfone, resulting in well-defined semitelechelic Michael acceptor pPEGAs. Conjugation of these polymers to bovine serum albumin (BSA), which contains a free cysteine residue (C34), resulted in conjugates with 92% esterase activity compared to the unmodified protein.

Polymers suitable for protein conjugation can also be synthesized in one step using reactive initiators or CTAs. We and others have published a number of papers that take this approach. For example, ATRP and RAFT were utilized to synthesize α-pyridyl disulfide-poly(*N*-acetyl-D-glucosamine)-ω-bromine and α-pyridyl disulfide-pNIPAAm-ω-trithiocarbonate, respectively.[34,35] Additionally, α-aminooxy polymers were synthesized by both ATRP and RAFT and conjugated to N^{ε}-levulinyl lysine-modified BSA via oxime bonds.[29,36] PEG methacrylate (M_n = 1,100 g/mole) (PEGMA) was polymerized from an aldehyde initiator by ATRP to synthesize a range of well-defined poly(PEGMA)s (pPEGMAs).[37] polyPEGMA was then conjugated to salmon calcitonin (sCT) via Schiff base formation with the *N*-terminus and subsequent reduction with sodium cyanoborohydride (Figure 5). The sCT conjugate was able to elevate cyclic adenosine monophosphate (cAMP) 85% compared to the unmodified protein that demonstrated 92% elevation in T47D cells, a human ductal breast epithelial tumor cell line.

Grafting polymers from proteins

The *grafting from* strategy involves functionalization of a protein followed by polymerization. One advantage of this approach is that it does not require separation of unreacted polymer and protein from conjugate. Another advantage is that hydrophobic polymer conjugates are more readily synthesized. We pioneered this approach by demonstrating ATRP of thermoresponsive NIPAAm from streptavidin (SAv) and T4 lysozyme (T4L) V131C.[38,39] In the first example, a biotinylated ATRP initiator was synthesized and conjugated to SAv (Figure 6).[39] Polymer growth occurred only from the ATRP functionalized ligand bound by the protein, and the protein maintained activity postpolymerization. Thermoresponsiveness of the SAv-pNIPAAm conjugate imparted by the polymer was also demonstrated. In our second example, reversible and nonreversible ATRP macro-initiator of T4L V131C were synthesized upon conjugation of a pyridyl disulfide and maleimide ATRP initiator to the protein, respectively (Figure 7).[38] The ability of resulting T4L- pNIPAAm conjugates to lyse *Micrococcus lysodeikticus* was statistically the same as unmodified T4L. Both examples showed that this methodology is robust and able to synthesize protein-polymer conjugates with a high retention of bioactivity.

This technique of growing polymer chains directly from proteins has recently been applied to recombinant human growth hormone (rh-GH) by Caliceti and coworkers.[4] In this example *N*-hydroxysuccimide (NHS) bromoisobutyrate was coupled to rh-GH via the *N*-terminal amino acid and lysine residues and polymerization of PEGMA (M_n = 475 g/mol) was conducted. Conjugation of polymer to approximately nine out of ten available amine residues was confirmed by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Maintenance of structural integrity of protein was verified by circular dichroism. Administration of 40 μg daily doses of the rh-GF-pPEGMA and unmodified rh-GF to female Sprague Dowley rats over six days resulted in similar weight growth profiles. When the animals were administered 120 μg dose of rh-GF or rh-GF-pPEGMA at day 0 and day 3 and monitored for nine days, only the group given rh-GF-pPEGMA showed increased mass gains over an extended period compared to rh-GF (Figure 8). This result nicely demonstrates that *grafting from* produces conjugates that are viable *in vivo*. This approach has also been applied by Chilkoti and coworkers for the polymerization of branched PEG monomers from the *N*-terminus of myoglobin and the *C*-terminus of green fluorescent protein (GFP).[5,6] Both conjugates demonstrated better pharmacokinetics than the unmodified proteins.

It was demonstrated that RAFT polymerization can also be used to graft polymers from proteins.[40–42] The Centre of Advanced Macromolecular Design (CAMD) group conjugated a pyridyl disulfide trithiocarbonate CTA to BSA (Figure 9). In their approach the protein was attached to the Z-group of the CTA followed by polymerization and subsequent capping by the protein (the Z-group approach). This BSA-macroCTA was utilized to prepare well-defined, pPEGA and pNIPAAm conjugates via gamma irradiation and thermal initiation with a sacrificial azoinitiator, respectively. The thermoresponsive nature of the pNIPAAm conjugates was confirmed, and 94% of esterase activity was maintained compared to unmodified BSA.[41] Sumerlin and coworkers synthesized a BSA-macroCTA in which the polymer chain was grown directly from the protein (R-group approach).[42] They demonstrated the ability of this BSA-macroCTA to mediate the polymerization of NIPAAm to generate well-defined protein-polymer conjugates that respond to changes in temperature. The conjugate exhibited 95% esterase activity of the native protein.

Besides improving conjugate pharmacokinetic properties, polymers can also transfer their properties to the protein. For example, as was described above, conjugates containing

thermoresponsive pNIPAAm also respond to temperature. By tailoring the characteristics of the synthetic component it is possible for self-assembly of protein-polymer conjugates into higher order nanostructures to occur.[7] Nolte and coworkers synthesized the first giant amphiphile. Cal B was conjugated to maleimide poly(styrene) (pS).[43] The amphiphilic conjugate self-assembled into nanofibers in solution that maintained some of the activity of the protein. This approach has since been refined to generate the biopolymersome nanoreactors described above.[24] Le Droumaguet and Velonia recently utilized the *grafting from* approach to polymerize styrene from BSA by ATRP. The resulting conjugates self-assembled into spherical nanoparticles (NPs) 20 to 100 nm in diameter.[44] *In situ* NP formation was also demonstrated with human serum albumin (HSA) and reduced human calcitonin (rhCT). The authors were able to encapsulate fluorescently labeled papain and HRP into the NPs. Confocal fluorescence microscopy confirmed the presence of fluorescent papain. Incubation of the BSA-pS encapsulated with HRP NPs with hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine indicated that active HRP was present inside the NP. The above examples, demonstrate the versatility of CRP resulting in a range of materials with interesting properties.

Multimeric protein-polymer conjugates

Until recently most of the protein-polymer conjugates that have been reported were composed of one protein attached to one or more polymer chains. However, multimeric protein-polymer conjugates, where two or more proteins are attached to a single chain, are of interest. Linking multiple proteins can generate materials with higher affinity for a biological receptor, ability to target multiple biological processes or pathways, and/or provide a scaffold that contains a targeting group.[45] To obtain well-defined multimeric protein-polymer conjugates it is necessary to site-specifically link multiple proteins to a polymer scaffold.

Homodimeric and starred protein-polymer conjugates

We recently demonstrated a general approach for the construction of homodimeric, protein-polymer conjugates using CRP.[28] NIPAAm was polymerized in the presence of a two-armed CTA resulting in a well-defined polymer. Postpolymerization, the thiocarbonylthiol groups were exchanged for furan-protected maleimides via radical coupling with a functionalized azo-initiator (Figure 10). T4L V131C was then incubated with the deprotected, telechelic, maleimide-pNIPAAm resulting in the formation of dimeric T4L-polymer conjugates. This approach was expanded upon to produce protein star polymer conjugates.[27]

Heterodimeric protein-polymer conjugates

Multiple methodologies have also been developed for the synthesis of heterotelechelic polymers for bioconjugation.[25,26,46–48] The CAMD group performed polymerization of a series of monomers via RAFT in the presence of a CTA with an azide on the R-group and pyridyl disulfide on the Z-group.[46] Postpolymerization, a biotinylated alkyne was clicked to the well-defined polymers. Incubation of these polymers with SAv and BSA resulted in starred BSA conjugates with a SAv core. Theato and coworkers mediated the RAFT polymerization of diethyleneglycol monomethylether methacrylate (DEGMA) with an activated ester CTA. Thyroxin (a ligand for transthyretin) was then conjugated to the polymer via the activated ester. The thiocarbonylthiol group was then reduced with butylamine and the free thiol trapped with *N*-biotinylaminoethyl methanethiosulfonate resulting in heterotelechelic α-thyroxin, ω-biotinpNIPAAm. Heterodimer formation was demonstrated upon incubation with SAv and transthyretin as well as conjugation to SAv coated surfaces. We used the radical coupling approach described above for homodimers/

stars in the synthesis of heterodimeric protein-polymer conjugates.[25,26] RAFT polymerization of NIPAAm was performed in the presence of biotinylated CTAs with or without a cleavable disulfide bond in the “R” group. Postpolymerization, the thiocarbonylthiol group was exchanged, and deprotection of the end-group afforded the α-biotin, ω-maleimide-pNIPAAm. Heterodimer formation with T4L V131C or BSA and SAv was demonstrated. Both maleimide-pNIPAAm-biotin and maleimide-pNIPAAm- disulfide-biotin (Figure 11) were used to tether proteins to surfaces. Enzyme linked immunosorbant assays (ELISAs) on SAv-coated surfaces demonstrated the reversibility of the disulfide containing conjugate indicating that the polymer will be useful for capture and release-type applications.

Conclusions

Controlled radical polymerizations enable the synthesis of protein-polymer conjugates with interesting macromolecular structures and tailored properties. The ability to finely tune the chemical composition and conjugation site of the polymers provides access to new properties. PEG-graft polymer conjugates are promising as longer circulating therapeutics. Multi-protein encapsulated polymersomes have the potential for drug delivery and as cell/organelle mimics. Multimeric protein-polymer conjugates should result in more effective therapeutics and better biosensors. These materials, made possible by controlled radical polymerization techniques, stand to revolutionize the fields of biotechnology, nanotechnology, and medicine.

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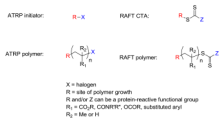

Figure 1.
General structure of ATRP initiators, RAFT CTAs, ATRP polymers, and RAFT polymers.

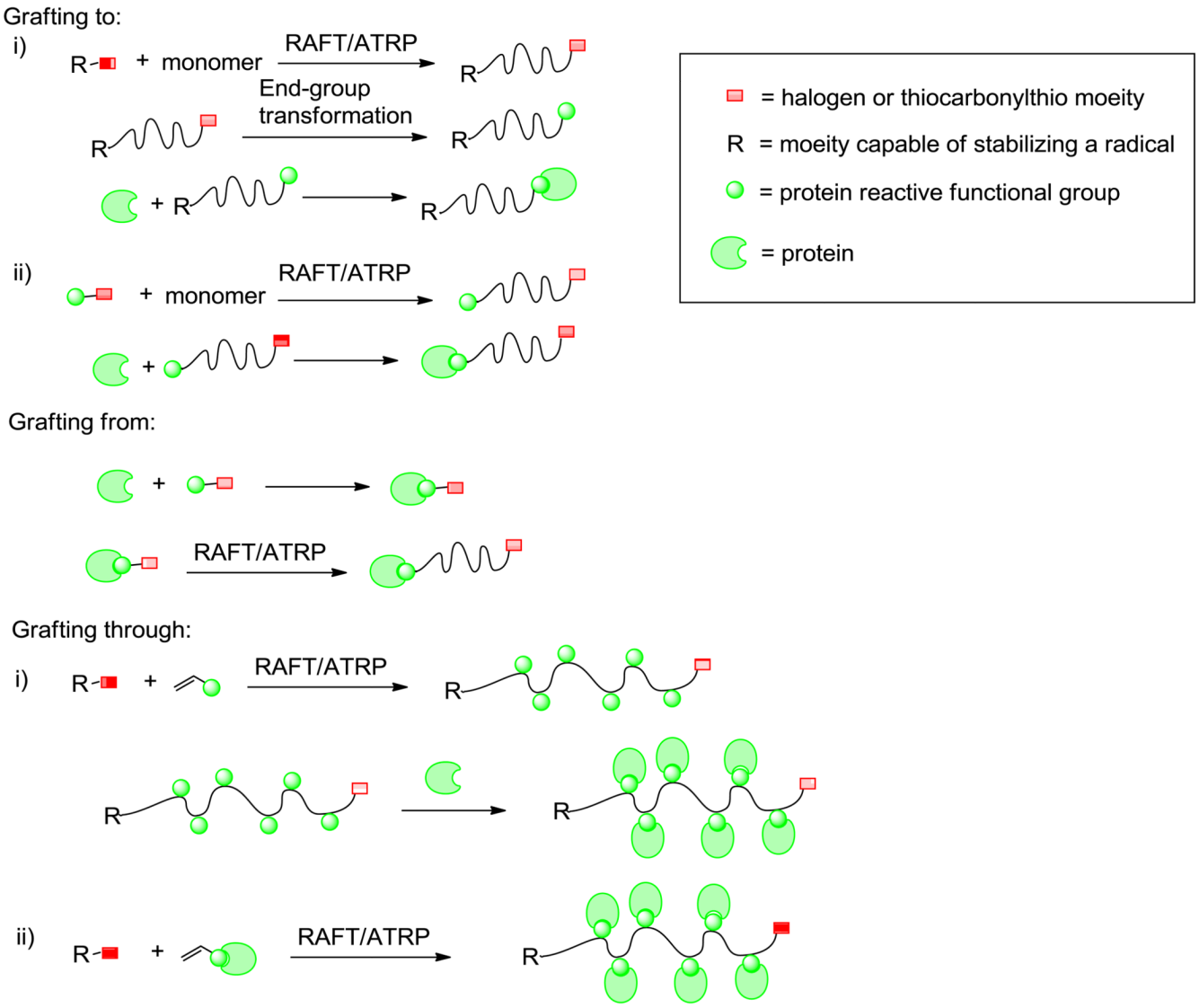

Figure 2.
General approaches used to synthesize protein-polymer conjugates.

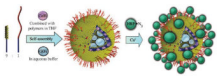

Figure 3.
Synthesis of a three enzyme functionalized nanoreactor.[24] *Reprinted with permission from S.F.M. van Dongen, et. al, (2009),* Chemistry-A European Journal, 15, 1107–1114. Copyright 2009 Wiley-VCH.

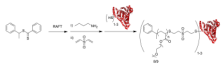

Figure 4.
Synthesis and conjugation of semitelechelic vinyl sulfone pPEGA to BSA (PDB # 1E7H). [31] Reproduced with permission from *Macromolecules* **2009**, *42*, 7657–7663. Copyright 2009 American Chemical Society.

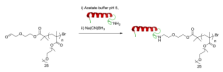

Figure 5.
Conjugation and reduction of $pPEGMA_{1100}$ to the *N*-terminus of salmon calcitonin (protein structure from PBD # 2GLH).[37]

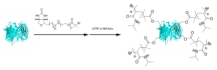

Figure 6.
Synthesis of streptavidin macroinitiator and polymerization of *N*-isopropylacryamide (protein structure from PBD # 3MG5).[39]

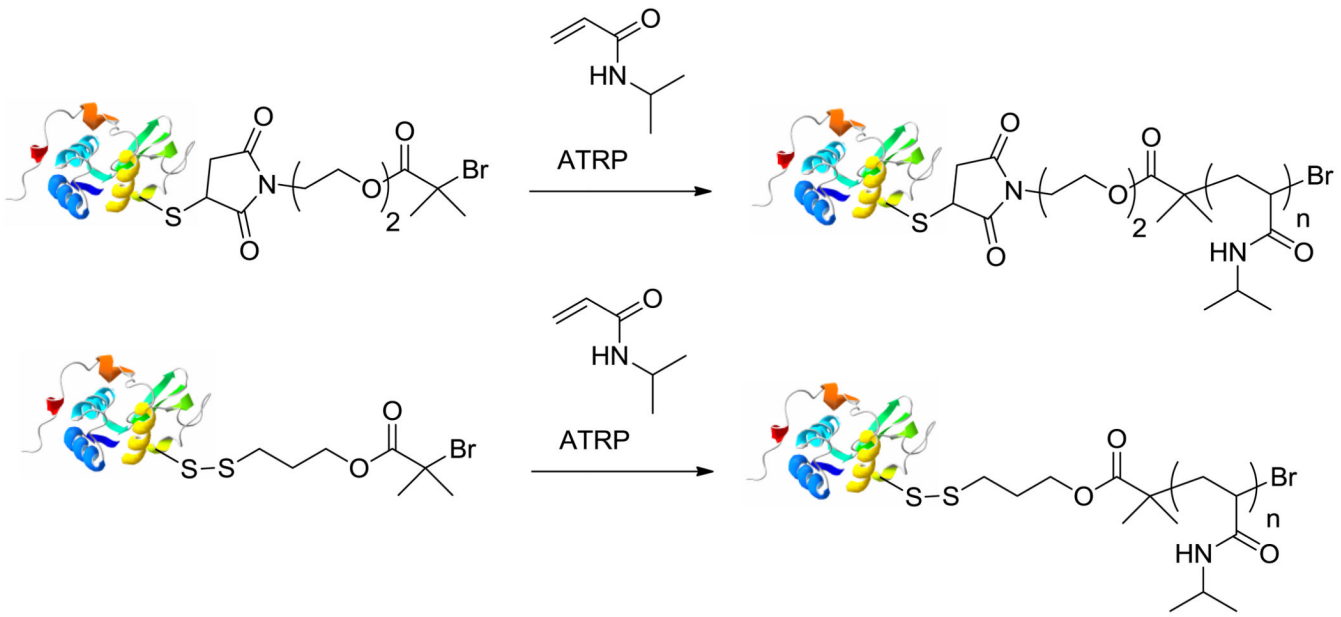

Figure 7.
Synthesis of reversible and nonreversible T4L-pNIPAAm conjugates (protein structure from PBD # 132L).[38]

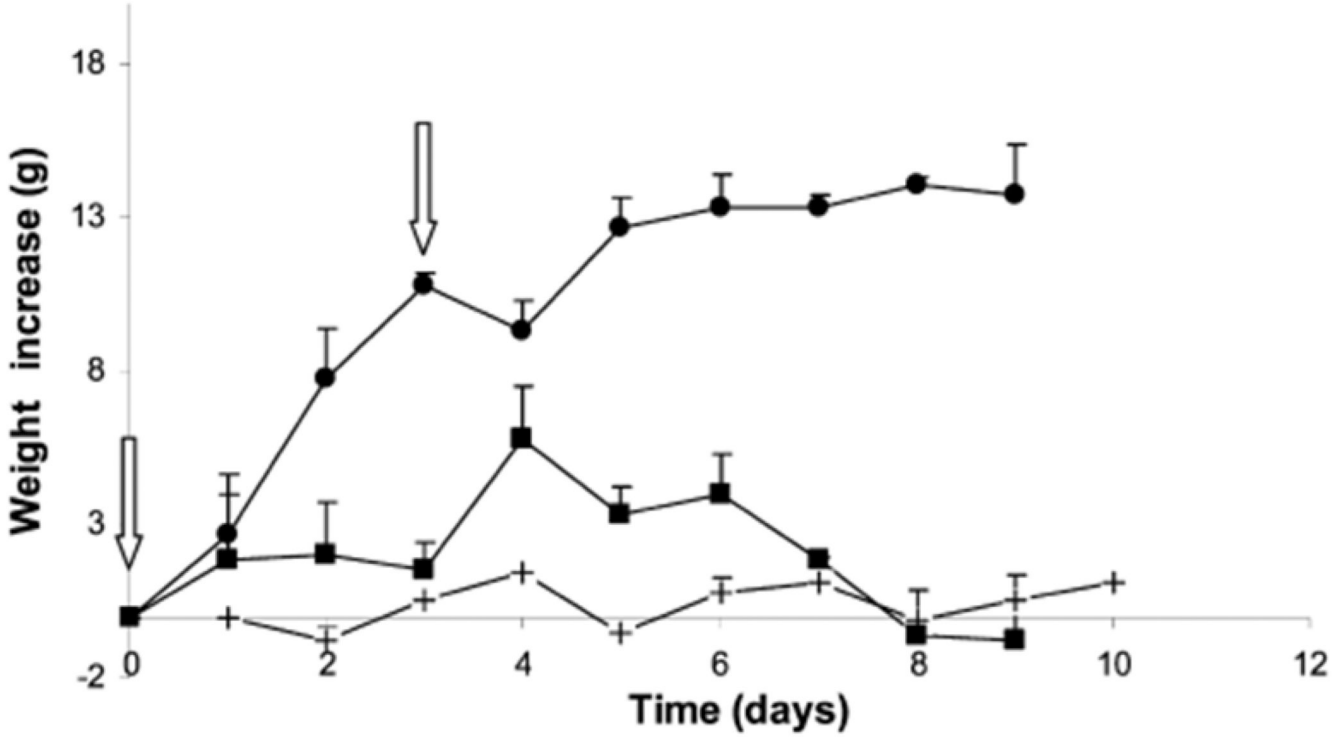

Figure 8.
Increase of rat weight (%) after administration of rh-GH and rh-GH-pPEGMA. Arrows represent injection 40 μg of rh-GH (black squares), rh-GH-pPEGMA (black circles), and buffer (crosses).[4] Reproduced with permission from *Bioconjugate Chemistry* **2010**, *21*, 671–678. Copyright 2010 American Chemical Society.

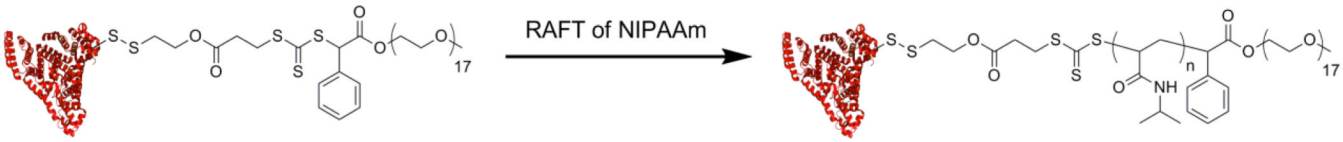

Figure 9.
Polymerization of NIPAAm from BSA (protein structure from PBD # 1E7H) with RAFT (Z-group approach).[41]

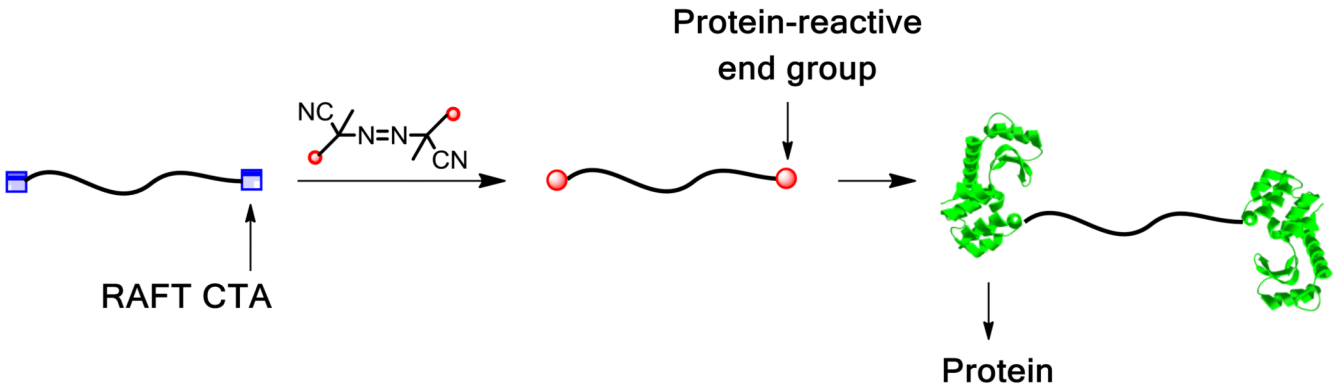

Figure 10.
Synthesis of homodimeric protein-polymer conjugates (protein structure from PBD # 1P56). [28] Tao, et al., *Chem. Commun.* **2009**, 2148–2150 – Reproduced by permission of The Royal Society of Chemistry.

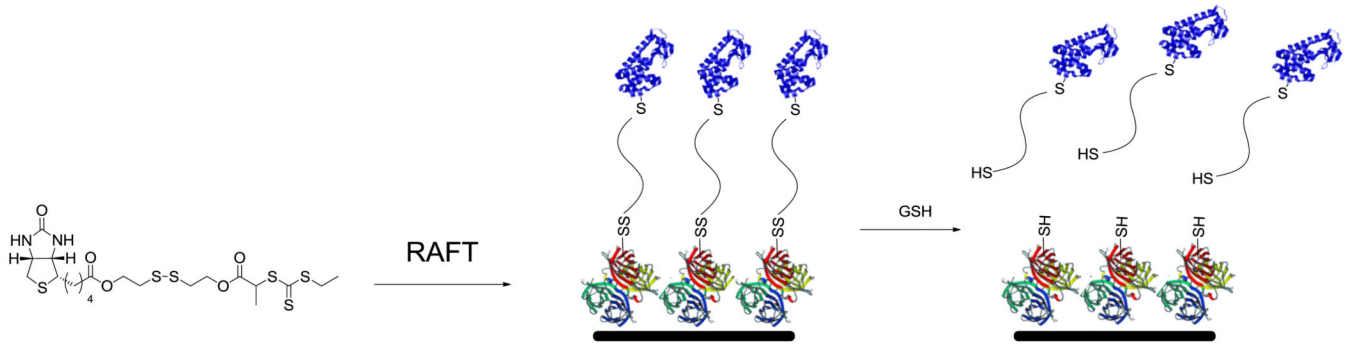

Figure 11.
Synthesis of heterotelechelic polymers for reversible surface immobilization of protein-polymer conjugates (protein structure from PBD # 1P56 and 1SWA).[26] Heredia, et al., *Polym. Chem.* **2010**, *1*, 168–170 – Reproduced by permission of The Royal Society of Chemistry.