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Preparation of Biomolecule-Polymer Conjugates by Grafting-From Using ATRP, RAFT, or ROMP

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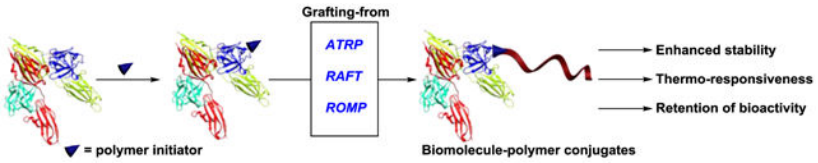
Abstract

Biomolecule-polymer conjugates are constructs that take advantage of the functional or otherwise beneficial traits inherent to biomolecules and combine them with synthetic polymers possessing specially tailored properties. The rapid development of novel biomolecule-polymer conjugates based on proteins, peptides, or nucleic acids has ushered in a variety of unique materials, which exhibit functional attributes including thermo-responsiveness, exceptional stability, and specialized specificity. Key to the synthesis of new biomolecule-polymer hybrids is the use of controlled polymerization techniques coupled with either grafting-from, grafting-to, or grafting-through methodology, each of which exhibit distinct advantages and/or disadvantages. In this review, we present recent progress in the development of biomolecule-polymer conjugates with a focus on works that have detailed the use of grafting-from methods employing ATRP, RAFT, or ROMP.

Graphical abstract



Keywords

Grafting-from; bioconjugate; protein-polymer conjugates; peptide-polymer conjugates; DNA-polymer conjugates

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

Biomolecule-polymer conjugates represent an important class of macromolecular architectures that combine the advantageous properties inherent to both the biomolecule and synthetic polymer(s) appended to it. The attachment of poly(ethylene glycol) (PEG) to biomolecule therapeutics represents the most utilized form of biomolecule-polymer conjugates in the pharmaceutical realm, and there currently exist 17 PEGylated peptide and protein therapeutics which have garnered approval from the United States Food and Drug Administration (FDA).[1-8] In this instance, PEG acts to increase the *in vivo* biomolecule half-life by protecting the therapeutic from recognition by the immune system and/or reduces clearance.[2] The development of controlled polymerization techniques has equipped the scientific community with the ability to prepare specially tailored polymers of controlled molecular weights and well-defined architectures from a wide array of monomers.[9-11] This has led to the development of diverse polymers able to invoke unique characteristics such as pH responsiveness, increased storage and *in-vivo* stability, thermo-responsiveness, and in PEG alternatives which exhibit lower immunogenicity.[1, 12-16] Commonly used controlled polymerization techniques for biomolecule-polymer modification are atom-transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer polymerization (RAFT), and ring opening metathesis polymerization (ROMP), which are the focus of this review.[11, 16-18] It is important to note that nitroxide-mediated radical polymerization (NMP) has also been used to great extent to prepare bioactive polymers and conjugates, and interested readers are directed to the many interesting reviews and manuscripts which detail the use of NMP. [19-22]

Biomolecule-polymer conjugates prepared using controlled polymerization techniques are typically accessed through three different routes: grafting-to in which a polymer is first synthesized, purified, and subsequently coupled to the biomolecule, grafting-from in which a small-molecule reactive handle is attached to the biomolecule and used as an initiation site to grow the polymer from the surface of the protein, and grafting-through in which monomers functionalized with a specific payload are polymerized (Figure 1).[15, 23, 24] Grafting-through has been used to a great extent in the preparation of bioactive polymers synthesized using ROMP and we will briefly cover a few examples in that area towards the end of the review. The grafting-to strategy has been employed to a greater extent most likely due to the great advances that have been made in coupling chemistry and the introduction of so-called “click” reactions which are highly versatile and efficient methods to generate bioconjugates (Figure 1A). However, the grafting-from technique offers many advantages, especially in regard to the purification of the prepared biomolecule-polymer conjugate (Figure 1B). [15, 23] Using an excess amount of a small-molecule for bioconjugation is a straight-forward process, as any unreacted material can easily be removed either by dialysis or size-exclusion chromatography (SEC) due to the large disparity in molecular weights between the protein and small-molecule. In contrast, using an excess amount of polymer to couple onto a biomolecule can be problematic due to the time intensive nature of polymer synthesis. Purification of polymers from the bioconjugates is also challenging due to similarities in molecular weight. Additionally, in grafting from limitations due to

sterics, which are inherent to the coupling of polymers to biomolecules, are avoided and characterization of the locations of the polymer chains on the conjugate is easier. However, grafting-from techniques require polymerization conditions which are suited to the stability of the biomolecule and it remains challenging to generate low dispersity conjugates.

Over the course of this review, we will cover recent progress in the formation of biomolecule-polymer conjugates which have been prepared using the grafting-from technique. For reading ease, the review is sectioned in terms of the controlled polymerization technique used. The initial section will cover select works using ATRP, the second section will cover RAFT polymerization, and the third will be devoted to ROMP with a brief discussion on grafting-through techniques to develop bioactive polymers (Figure 1C).

2. Conjugation of reactive groups onto biomolecules

Key to the successful implementation of the grafting-from technique is the installation of small molecule reactive handles capable of initiating or mediating polymerization processes from the biomolecule surface. Throughout the years this has taken many forms, with lysine (Lys) and cysteine (Cys) amino acid residues being the most frequently targeted sites for modification on proteins (Figure 2).[1, 25] For the purposes of grafting-from, Cys amino acid modifications have taken the form of pyridyl disulfide (PDS) exchange and Michael addition using maleimide-modified substrates. The weaknesses of these linkages lie in their inherent instability with disulfide bridges being reversible in the presence of external reductants and the thiol-ether bond from the maleimide being susceptible to retro-Michael processes *in vivo.* However, Cys remains a popular site for conjugation due to the low relative abundance on proteins, thereby allowing for site selectivity.[25] Lys is also a popular site for conjugation of reactive groups with several research groups taking advantage of conjugation through acid-halides, *N*-hydroxysuccinimide (NHS)-esters, nitrophenyl carbonates, and squaric acid containing functionality (Figure 2).[1, 25, 26] However, Lys residues typically have a high abundance on proteins and conjugation methods are generally non-selective, and as a result there are groups currently working on selective Lys conjugation techniques.[27-29]

Other methods of conjugating reactive handles onto protein substrates include non-covalent modification taking advantage of the streptavidin (SAv)-biotin interaction, genetic incorporation of non-native amino acids, sortase A (SrtA)-mediated modifications targeting LPGXTG/A sequences on the C-terminus of proteins, and oxime formation after treatment by pyridoxal-5-phosphate (PLP) targeting the protein N-terminus.[1, 23, 30, 31] Given the expansion of amino acid modification techniques along with the introduction of transition-metal-mediated conjugation techniques, there remains a wealth of opportunities to modify proteins with reactive handles capable of mediating polymerization.[32-41]

Typical peptide conjugation for grafting-from has been performed *via* on-resin reactions targeting the amine terminus of the peptide by either acid-halide conjugation or *N,N*-dicyclohexylcarbodiimide (DCC) coupling of reactive handles.[42, 43] Deoxyribonucleic acid (DNA) modification for grafting-from has typically made use of either PDS exchange

on a thiol terminated DNA strand, NHS-ester or pentafluorophenyl (PFP)-ester conjugation on amine terminated DNA, and phosphoramidite coupling which targets the phosphoester backbone on DNA (Figure 3).[24, 44, 45]

3. Biomolecule-polymer conjugates accessed through ATRP

ATRP is a living and controlled radical polymerization technique, first reported independently by the Matyjaszewski group and the Sawamoto group in 1995, which falls under the category of reversible deactivation radical polymerization (RDRP).[18, 46, 47] In the traditional ATRP mechanism, homolytic cleavage of a C(sp^3)-X (where X = Br or Cl) bond on an ATRP initiator is imposed by a reduced metal halide catalyst thereby initiating polymerization (Figure 4). The then oxidized metal catalyst can reversibly deactivate the radical propagation of the polymer chain transfer of the halide to the end of the propagating polymer chain. This reversible deactivation ultimately imposes control over the polymerization process by decreasing the amount of active radicals in solution thereby minimizing unproductive termination pathways (Figure 4).[9, 18]

There are many alternatives to typical ATRP procedures which differ in the method of initiation used. A few commonly used alternative ATRP strategies for grafting-from processes include initiators for continuous activator regeneration (ICAR), activators generated by electron transfer (AGET), activators regenerated by electron transfer (ARGET), and photo-induced ATRP among others.[18] Though there exist many other ATRP strategies, we will only focus on those used for the grafting-from polymerization.[9, 18]

3.1 Traditional ATRP to access protein-polymer conjugates by grafting-from

Workers at Biocompatibles Ltd synthesized hydroxysuccinimide ester substituted ATRP initiators which were then appended onto Lys residues of the protein lysozyme (Lyz).[48] Protein functionalization was performed in borate buffer at room temperature and the resulting protein-macroinitiator was used without purification. A variety of olefin substituted monomers and zwitterionic monomers were polymerized from the protein surface under traditional ATRP conditions in the presence of a copper bromide catalyst and bipyridyl ligand at room temperature. The resulting protein-polymer conjugates could be isolated via capillary electrophoresis.[48]

Our group published grafting-from methodology by employing traditional ATRP to polymerize *N*-isopropylacrylamide (NIPAAm) from both SAv and T4 lysozyme (T4-Lyz) (Figure 5A and B).[49, 50] In our initial disclosure targeting SAv bioconjugates, we developed a biotin functionalized ATRP initiator. The strong binding affinity of biotin to SAv (K_d = 10^{-15} M) was utilized to modify the protein with up to four of the biotin ATRP initiators thereby forming the SAv-macroinitiator (Figure 5A).[49, 51] The room temperature polymerization of NIPAAm using the SAv-macroinitiator was carried out in an aqueous solution in the presence of copper (I) bromide (CuBr) and the 2,2'-bipyridine ligand.[49] In a follow-up article that same year, our group modified a Cys amino acid residue (Cys-34) on bovine serum albumin (BSA) as well as a Cys residue (Cys-131) on a genetically engineered T4-Lyz protein with an ATRP initiator.

The BSA- or T4-Lyz-macroinitiators were prepared using either pyridyl disulfide exchange to modify the proteins with reversible disulfide linkages or by maleimide conjugation (Figure 5B).[50] Grafting-from polymerization of NIPAAm was carried out similarly to the previous report grafting-from SAv (Figure 5B).[49, 50] The polymer-conjugates were purified via preparative SEC and characterized using gel permeation chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, the T4-Lyz-polymer conjugate retained activity as determined by a commercially available fluorescence assay which monitored lysis of the *Micrococcus lysodeikticus* substrate. Though these works provided a foundation for the introduction of the grafting-from concept, polymerization from the SAv and T4-Lyz bioconjugates required the addition of a 2-bromoisobutyryl-functionalized resin which was introduced in order to increase the concentration of initiating sites (Figure 5A and B).[49, 50] The addition of a sacrificial initiator is not needed when there is an abundance of the protein substrate, as is the case with cheaper proteins such as BSA.

Matyjaszewski, Russell, and co-workers also disclosed an example of grafting-from using traditional ATRP from chymotrypsin functionalized at Lys amino acid residues with 2-bromoisobutyramide initiators (Figure 5C).[52] The authors expanded the monomer scope to include polymerization of monomethoxy poly(ethylene glycol)-methacrylate (MPEGMA), sodium 4-styrenesulfonate, and 2-(dimethylamino)ethyl methacrylate (DMAEMA) from the chymotrypsin macroinitiator, which was carried out in the presence of phosphate buffered saline (PBS) (pH 6.0), CuBr, and 2,2’-bipyridine (Figure 5C). All of the protein-polymer conjugates retained their bioactivity to varying degrees dependent on the nature of the polymer, with the poly(MPEGMA) conjugated protein retaining the highest at 76%, as determined by a spectrophotometric assay monitoring enzymatic hydrolysis of an oligopeptide sequence. [52] An important feature represented in this manuscript is that uniform protein-polymer conjugates could be accessed when attaching one initiator onto the protein.[52] Taking further advantage of grafting-from using traditional ATRP techniques, the groups have gone on to publish manuscripts detailing the tuning of enzyme activity and stability based on the polymers attached to the enzyme.[53] Additionally, redox active polymers have been polymerized from the surface of glucose oxidase (GOx) and exhibited an increase in the efficiency of current generation when testing the GOx-polymer conjugates on the surface of an anode.[54]

The initial reports on grafting-from were followed-up extensively with reports, which demonstrated expansions in the conjugation chemistry, monomer scope, variance in polymer architectures, and the protein substrates used. The Haddleton group developed protein macroinitiators using maleimide conjugation chemistry to target the Cys-34 on BSA and NHS-ester conjugation to target Lys residues on Lyz.[55] They were able to co-polymerize methacrylate-functionalized fluorescent monomers based on either rhodamine B or hostasol along with PEGMA.[55, 56] An impressive example of grafting-from is a report by the Velonia group in which they combined copper-catalyzed azide-alkyne cycloaddition (CuAAC) and ATRP which resulted in the polymerization, from a Cys-34 modified BSA, of alkyne-functionalized methacrylate monomers and subsequent attachment of azide-functionalized small-molecules for the *in-situ* production of *giant amphiphiles*.[57] The

Klok group has also shown success in using squaric acid mediated conjugation techniques to install ATRP initiators on Lys amino acid residues of BSA.[26]

Mehl, Matyjaszewski and co-workers devised an elegant approach to installing ATRP initiators within proteins by genetically encoding the ATRP initiator containing unnatural amino acid 4-(2'-bromoisobutyroamido)phenylalanine within green fluorescent protein (GFP).[58] The authors evolved a *Methanococcus jannaschii* tyrosyl-tRNA synthetase/ $tRNA_{CUA}$ pair that installed the novel amino acid in the presence of an amber codon. Incredibly, up to 420 mg of the ATRP initiator modified GFP was purified from one liter of medium. Upon purification of the GFP-macroinitiator and characterization by ESI-mass spectrometry, which showed an increase in mass from the wild type GFP corresponding to addition of the ATRP initiator, polymerization was carried out using traditional ATRP techniques in PBS with methoxy OEG methacrylate monomer (MOEGMA) at room temperature.[58]

The scientific community also gained interest in grafting-from larger types of biomolecule constructs. Wang and co-workers modified Lys residues on horse spleen apoferritin (apo-HSF) with bromoisobutyrate initiators via NHS-ester bioconjugation.[59] Matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis following tryptic digestion of the apo-HSF macroinitiator revealed modification of Lys-83, Lys-97, Lys-104, and Lys-143, all of which are highly exposed on the surface of the biomolecule. Polymerization of PEGMA from the apo-HSF macroinitiator was carried out in a 4:1 mixture of PBS (pH 8.5) and *N*,*N*-dimethylformamide (DMF) at 4 °C upon treatment with CuBr and bipyridine. Hydrogels could be formed in the presence of high monomer loadings (up to 1800 equiv to the macroinitiator) which could be subsequently solubilized in dichloromethane (DCM). The biomolecule-polymer conjugates were characterized via fast protein liquid chromatography (FPLC) and transmission electron microscopy (TEM).[59] The Russell and Emrick research groups also used grafting-from to access horse spleen ferritin (HSF) and poly(methacryloyloxyethyl phosphorylcholine) poly(MPC) conjugates.[60] Poly(MPC) is a biocompatible zwitterionic polymer which is resistant to nonspecific protein adsorption, similarly to PEG, though it is much more hydrophilic. The authors used NHS-ester chemistry to conjugate up to 45 bromoisobutyryl ATRP initiators onto Lys residues of HSF and accessed HSF-poly(PEGMA) conjugates in a similar manner to what was previously reported.[59] Notably, the HSF-poly(MPC) and HSF-poly(PEGMA) conjugates exhibited distinct changes in recognition properties due to the presence of the polymers on the protein surface. This was apparent in the decreased ferritin antibody binding as determined by an enzyme-linked immunosorbent assay (ELISA)-based assay. [60] The Ye group has also utilized NHS-ester bioconjugation to develop temperature and pH responsive hybrid biomaterials made by polymerizing NIPAAm from the surface of amelogenin.[61]

Finn and co-workers demonstrated an elegant example of grafting-from virus-like nanoparticles (VLP's) using traditional ATRP (Figure 6).[62] The group developed bacteriophage Qβ VLP macroinitiators by reacting azide functionalized NHS-ester linkers to Lys residues on the biomolecule and using CuAAC to add on the alkyl bromide ATRP initiator. It was found by protein digestion and subsequent mass spectrometry analysis that ~180 Lys residues were modified (Figure 6). Polymerization of oligoethylene glycol

methacrylate (OEGMA) was carried out by treating the macroinitiator and monomer solution with 2,2'-bipyridine, CuBr, and $CuBr_2$ in water. The purified conjugates were characterized via dynamic light scattering (DLS) and SEC which showed that the VLP-polymer conjugates were much larger in size to the Qβ wild-type. Notably, the bromine end-groups present on the polymers of the VLP-poly(OEGMA) conjugates could be modified further through treatment with sodium azide to form azide terminated polymers which could undergo CuAAC with an alkyne bearing AlexaFluor488 fluorescent dye (Figure 6). Additionally, the authors developed VLP-poly(OEGMA-N_3) conjugates in which each OEGMA repeat unit was modified with a pendent azide. This allowed for post-polymerization modification using CuAAC with the AlexaFluor488 dye, doxorubicin (Dox), or a gadolinium (Gd) complex (Figure 6). This is a notable achievement given the importance of Gd-modified nanoparticles in magnetic resonance imaging. Through inductively coupled plasma optical emission spectroscopy (ICP-OES), the authors found that the VLP-poly(OEGMA-Gd) particles contained 500-650 Gd complexes per conjugate. Additionally, the authors conjugated Dox via 'click' chemistry on the pendent poly(OEGMA-N_3) and found about 150 Dox molecules appended onto the particle. The drug could be released in the presence of pH 5.5 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer through degradation of the hydrazone linkage present on Dox and were also found to be cytotoxic towards HeLa cells at the same concentrations as free Dox. Interestingly, control experiments in which Dox was loaded directly on the VLP through triazole linkages and subjected to pH 5.5 MES buffer led to complete degradation and subsequent precipitation of the biomolecule indicating that the presence of poly(OEGMA) conjugated to the protein surface is required for stability.[62]

The Chilkoti group has targeted the N-terminus of myoglobin (Mb) to attach an ATRP initiator by way of oxime ligation chemistry. [31] This modification resulted in a single reactive site on the protein surface. The site-specific conjugation of the ATRP initiator was confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) of peptide fragments after trypsin digestion of the Mb-macroinitiator. In-situ ATRP of OEGMA from the singly modified Mb-macroinitiator in aqueous conditions resulted in the formation of a Mb-poly(OEGMA) bioconjugate which retained bioactivity and exhibited an increased in vivo half-life compared to native Mb.[31] Chilkoti and coworkers have also used SrtA-mediated conjugation to attach an ATRP initiator on the C-terminus of GFP. [30] GFP harboring a SrtA recognition site was expressed and purified then subsequently treated with an amine functionalized ATRP initiator in the presence of SrtA. This reaction resulted in a singly modified GFP-macroinitiator which was modified exclusively at the C-terminus of the protein. Notably, this methodology proved to be highly efficient with the GFP-macroinitiator purified in >85% yield.[30] SrtA mediated installation of a reactive handle has also been used in the attachment of an ATRP initiator onto the C-terminus of interferon-α (IFN-α) by the Gao group.[63] Using traditional ATRP techniques they were able to polymerize OEGMA thereby developing IFN-α-poly(OEGMA) conjugates which exhibited increased *in-vivo* half-life compared to native IFN-α. Additionally, IFN-α-poly(OEGMA) showed increased anti-proliferative activity in mice compared to IFN-α-PEG conjugates used clinically.[63] The Gao group has also made use of SrtA mediated transformations to produce a cyclized GFP (*c*GFP) derivative and subsequently coupled

an ATRP initiator using maleimide chemistry to target an engineered Cys residue on the protein.[64] Polymerization of OEGMA to produce *c*GFP-poly(OEGMA) conjugates resulted in a hybrid protein with increased thermal stability and enhanced tumor retention relative to either native GFP or the non-cyclized GFP-poly(OEGMA) conjugates.[64]

3.2 Peptide-polymer conjugates

Traditional ATRP techniques have also been used to access peptide-polymer conjugates via grafting-from. Though it is important to note that peptide-polymer conjugates had been previously prepared by grafting-from using NMP and additionally, ATRP had been previously shown to operate using substrates attached to a Wang resin. [65, 66] The Washburn group used ATRP to prepare peptide-polymer conjugates via grafting-from to prepare GRGDS-poly(2-hydroxyethyl methacrylate) (poly(HEMA)) conjugates. [67] GRGDS is an oligopeptide sequence within the protein fibronectin, which has been shown to interact with cell surface integrins. The GRGDS sequence was prepared through solid-phase peptide synthesis (SPPS) using a Wang resin. The ATRP initiator, 2-bromopropionyl bromide, was introduced in the final coupling step thereby producing an initiator-terminated protected oligopeptide on resin. Polymerization of HEMA was carried out by treatment of the GRGDS-resin initiator with the monomer, CuCl, and 2,2'-bipyridine in the presence of butanone and 1-propanol. Cleavage from the resin and subsequent deprotection of the prepared GRGDS-poly(HEMA) was carried out by treatment with a standard peptide cleavage cocktail (trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and H2O). A cell adhesion assay was carried out using mouse NIH-3T3 fibroblasts and the results indicated successful adhesion of cells to the GRGDS-poly(HEMA) conjugate and cell spreading while no adhesion was detected using the poly(HEMA) control thereby indicating the biocompatibility of the peptide-polymer conjugate and retention of the cell adhesive peptide moiety.[67] The Börner group also disclosed a report detailing their preparation of peptide-polymer conjugates via grafting-from soon after.[43] Incorporation of an ATRP initiator on the amine terminus of an oligopeptide during SPPS using DCC coupling chemistry and subsequent cleavage of the oligopeptide from the resin afforded an oligopeptide-macroinitiator. The authors carried out polymerization of *n*-butyl acrylate (*n*BA) in the presence of the oligopeptide-macroinitiator and performed an extensive study on the polymerization kinetics. Though the CuBr catalyst was found to interact with the amine functionality on the oligopeptide, this interaction was found not to have an effect on the controlled synthesis of monodisperse polymers.[43]

The Wooley group was also able to utilize ATRP of protected peptides on-resin, in addition to NMP.[42, 65] They prepared the 13 amino acid residue tritrpticin peptide, an oligopeptide which exhibits anti-microbial activity, using SPPS on Wang resin (Figure 7). The tritrpticin-resin was treated with bromoisobutyryl bromide to install the ATRP initiator on the oligopeptide end. The group was able to polymerize block co-polymers made from *tert*-butyl acrylate (*t*BA) and styrene monomers by treatment of the tritrpticin-resin with CuBr and *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA) in the presence of the monomers. Cleavage and deprotection were carried out to afford the tritrpticin-*b*-poly(acrylic acid-*b*-styrene) conjugate (Figure 7). Micelles from the peptide-polymer conjugates were generated and found to have improved anti-microbial activity at

lower concentration towards *Staphylococcus aureus* and *Escherichia coli* in comparison to tritrpticin alone (Figure 7).

3.3 DNA-Polymer Conjugates

Grafting-from to access DNA-polymer conjugates using traditional ATRP was used by the He group for the amplification of signal to specific DNA sequences.[68] Hybridization and ligation of an ATRP-functionalized single-stranded DNA (ssDNA) onto complementary ssDNA sequences modified on a gold surface led to the formation of DNA-macroinitiators. Polymerization of HEMA from the DNA-macroinitiators led to the surface formation of DNA-poly(HEMA) conjugates which were characterized by atomic force microscopy, ellipsometry, and contact angle measurements. Notably, the formed polymer films could be detected visually without the need for an optical microscope, and the technique allowed for the detection of single point mutations as ATRP modified ssDNA exclusively hybridizes to complementary sequences on the surface.[68] An interesting observation was found that initial polymerization rates from DNA surfaces were considerably accelerated.[69] The authors have attributed this initial rate enhancement to an interaction of Cu with the highly charged phosphate backbones on DNA.[69] Using similar methodology to their previous reports, the He group was also able to develop DNA-polymer conjugate coated core-shell Au nanoparticles using traditional ATRP under aqueous conditions.[70] Again, only nanoparticles harboring the complementary ssDNA strands to the ssDNA-macroinitiator were able to undergo polymerization leading to the visual detection of DNA-polymer conjugate loaded nanoparticles.[70]

3.4 AGET and ARGET ATRP

While the effectiveness of traditional ATRP to prepare biomolecule-polymer conjugates via grafting-from has been well established, the main challenges with traditional ATRP techniques include sensitivity to oxygen which may limit its implementation by novice users and low efficiency in aqueous solutions.[71, 72] Activator generated by electron transfer (AGET) ATRP relies on the introduction of an external reducing agent to reduce the transition metal species in solution thereby promoting activation of the initiator or halide terminated polymer chain. AGET ATRP is much more tolerant to low oxygen concentrations which allows for better control over polymerization in aqueous media at lower temperatures. The higher oxygen tolerance arises from the use of oxidatively stable transition metal-based catalysts coupled with the ability for the introduced reductant to remove dissolved oxygen and to reduce the transition metal catalyst (Figure 8).[18] This important characteristic makes AGET ATRP a more feasible technique for the novice user and a popular approach to access protein-polymer conjugates via ATRP.

In an early example using AGET ATRP to graft-from biomolecules, He and co-workers expanded on their previous work (*vide supra*) but instead used AGET ATRP with water soluble ascorbic acid as a reducing agent, thereby employing an oxygen-tolerant and homogenous method to make DNA and protein sensors based on a unique amplification-by-polymerization strategy.[73] Polymerization of HEMA from an ssDNA macroinitiator following hybridization and ligation to the immobilized complimentary ssDNA strand allowed for detection and determination of DNA concentration, which was proportional

to the film thickness. The sensitivity of this method was comparable to that of sensors using traditional ATRP techniques but the turnaround time was reduced by 2.5 times and a purging step to remove dissolved oxygen was eliminated, making it more attractive for portable applications.[73] The authors extended this work further by the implementation of electrochemical sensors based on polymers harboring pendant ferrocene-based monomers. Post-polymerization modification of ssDNA-polymer conjugates made from either glycidyl methacrylate (GMA) or HEMA was carried out by coupling aminoferrocene to the pendant chains on the polymer thus generating electrochemically active polymers. This method allowed for detection of unlabeled DNA or proteins with sensitivity modulated by the length of the polymer chains and amount of aminoferrocene added to the polymers.[73]

Other groups have also utilized AGET ATRP to graft from oligonucleotides. Das, Matyjaszewski, and co-workers incorporated an acid-stable amide initiator during solid-phase DNA synthesis using phosphoramidite coupling chemistry.[44] A variety of methacrylate monomers were polymerized from the DNA macroinitiator which either remained attached on the solid support or was polymerized from while in solution after cleavage from the solid support. DNA remained stable under the mild polymerization conditions and was able to hybridize with complementary strands. The DNA-polymer hybrids were characterized via gel permeation chromatography (GPC) and fluorescence analysis.

Lin and Maynard prepared polymer conjugates with small interfering ribonucleic acid (siRNA) using AGET ATRP (Figure 9).[74] A macroinitiator was prepared by modifying a 5’-thiol siRNA with a PDS-functionalized ATRP initiator. Ethylene glycol methacrylate monomers, which varied in length, were polymerized in the presence of $CuCl_2$, tris(2-pyridylmethyl amine (TPMA), and ascorbic acid (AA) from siRNA in PBS using a sacrificial resin, due to the low quantities of the siRNA-macroinitiator available. The authors compared the grafting-to strategy to prepare siRNA-polymer conjugates by conjugating pyridyl disulfide terminated polymers to siRNA through disulfide exchange reactions. Ultimately, the grafting-from process exhibited improved conjugation efficiency and a more facile purification process (Figure 9). All siRNA-polymer conjugates were characterized by PAGE analysis.

The groups of Wu and Weil used a bottom-up approach to construct nanoscale polymer patterns by grafting-from ATRP initiator modified DNA origami.[75] Different DNA origami patterns were formed on a surface and underwent hybridization with ATRP-initiator functionalized complementary strands. AGET ATRP of MPEGMA in the presence of a sacrificial initiator was then performed from the DNA origami initiating sites and polymer growth was followed using atomic force microscopy (AFM). The purified polymer products were characterized by agarose gel electrophoresis which showed a molecular weight increase from the native DNA origami and AFM which showed an increase in the height from the surface. The surface height could be controlled depending on the ratio of monomer to initiator employed which led to differences in the molecular weight of the polymer. Interestingly, after the preparation of cross-linked polymers using similar techniques and the degradation of the DNA origami template under heat stress, the polymers retained their shape. Ultimately, this work showcased the great potential of pairing grafting-from

techniques using AGET ATRP with DNA origami templates to create unique 2D and 3D polymer shapes.[75]

Enzyme conjugates have also been prepared by the grafting-from method using AGET ATRP. Liu and coworkers prepared an enzyme-polymer conjugate by polymerizing acrylamide from horseradish peroxidase (HRP) in the presence of air, thus taking advantage of the oxygen tolerance inherent to AGET conditions.[76] Molecular weight was controlled by the ratio of the HRP macroinitiator to monomer and the polymers exhibited low dispersity values. Modification of HRP with the small-molecule ATRP initiators decreased the activity of the protein by nearly 50%, likely due to the use of dichloromethane during the initial modification step. However, no change in activity after polymerization was detected and the stability of the conjugate to elevated temperatures (55 °C) and enzymatic degradation by trypsin was improved. Magnusson, Alexander, and co-workers prepared thermo-responsive trypsin conjugates using AGET ATRP.[77] The Lys residues on trypsin were modified using a heterobifunctional NHS-ester functionalized tetraethylene glycol ATRP initiator and MALDI characterization of the trypsin macroinitiator determined there to be an average of 5 initiating sites per protein. Trypsin proteins harboring PEGMA-based copolymers or block copolymers which varied in molecular weight were synthesized through grafting-from under typical AGET ATRP conditions in aqueous buffer solutions at low temperature (4 °C) to form trypsin-polymer conjugates which were characterized by SDS-PAGE. The polymers were analyzed by GPC after cleavage from trypsin using tetra-n-butylammonium fluoride (TBAF) to validate differences in molecular weight. The trypsin-polymer conjugates exhibited different self-assembly behaviors above the lower critical solution temperature (LCST) dependent on the molecular weight of the polymers appended to the protein. Conjugate activity could also be regulated through the phase transition of the conjugated polymers.[77]

Conjugates with therapeutically relevant proteins have also been prepared by grafting-from using AGET ATRP conditions. As an alternative to traditional grafting-to PEGylation, Caliceti and coworkers utilized AGET ATRP to polymerize PEGMA from recombinant human growth factor (rh-GH), a hormone used as a therapeutic for growth deficiency (Figure 10A).[78] Conjugation of Lys amino acid residues on rhGH was carried out by treatment of the protein with a tetraethylene glycol based heterobifunctional ATRP initiator harboring an NHS ester functional group in the presence of pH 7.5 PBS. MALDI-ToF analysis revealed rh-GH functionalized with 6-8 initiating sites. Polymerization from the rh-GH macroinitiator was performed in phosphate buffer at 4 °C using standard AGET ATRP conditions. This conjugate exhibited improved stability to heating at 37 °C, mechanical agitation, and enzymatic degradation compared to the native protein (Figure 10A). The rh-GH-polymer conjugates were characterized via SDS-PAGE which indicated a large increase in molecular weight from the native rh-GH. Additionally, acidic hydrolysis to liberate the polymers from the protein allowed for GPC analysis which showed relatively monodisperse ($Đ = 1.2$) polymers. The stability of native rh-GH and the rh-GH-polymer conjugate during exposure to heat stress or in the presence of the enzyme pepsin was tested. In each case, the rh-GH-polymer conjugates exhibited a higher degree of stability over native rh-GH. In vivo studies were carried out wherein rats were injected with either native rh-GH or the rh-GH-polymer conjugates. It was found that the rh-GH-polymer conjugates retained

biological activity similarly to the native rh-GH as indicated by weight increase following dosage in rats with a daily dosage schedule. Notably, the rh-GH-polymer conjugate retained bioavailability when following a twice weekly dosage schedule as indicated by a weight gain in rats, in contrast to rats injected with native rh-GH which maintained their weight.[78]

We have previously used AGET ATRP to prepare a trehalose glycopolymer conjugate of insulin (Figure 10B). Using the grafting-from approach simplified purification and characterization of the insulin-macroinitiator.[79] A site-specific insulin macroinitiator was prepared by treatment of insulin with a nitrophenyl carbonate modified ATRP initiator in the presence of pH 11.0 borate buffer (Figure 10B). Basic conditions were used in order to favor modification at the Lys residues due to the residues higher nucleophilicity over other N-terminal amines. Purification by of the insulin-macroinitiator by HPLC and characterization by MALDI and LC-MS/MS after reduction of the conjugate with DTT revealed a single modification at the LysB29 residue on insulin. A methacrylate trehalose monomer was polymerized from the insulin-macroinitiator in pH 7.4 PBS using AGET ATRP conditions in the presence of a sacrificial resin and the insulin-polymer conjugate was purified via HPLC after polymerization. The purified insulin-polymer conjugate was characterized by SDS-PAGE which appeared as a higher molecular weight band to that of native insulin, and the polymer was characterized by GPC after digestion of insulin with proteinase K and found to be well defined. Though a 3-fold increase in dosage was needed in order to achieve similar decreases of blood glucose to native insulin in mice, the insulin-polymer conjugate retained bioactivity to a greater extent than nonspecific insulin-polymer conjugates prepared by grafting-to.[79]

Other groups have prepared nanostructures with covalently incorporated proteins by grafting-from ATRP macroinitiators in the presence of cross-linkers to encapsulate the protein over the course of polymerization. Matyjaszewski and co-workers prepared GFP nanogels wherein GFP was incorporated into a polymer matrix through the course of AGET ATRP in a water-in-oil inverse miniemulsion. GFP containing a genetically incorporated ATRP initiator was expressed through site-directed mutagenesis. Polymerization from the GFP macroinitiator was carried out in the presence of a PEGMA monomer and a bifunctional methyl methacrylate PEG cross-linker.[80] The GFP-nanogels were characterized by DLS and confocal microscopy. Importantly, it was found that grafting-from was essential for keeping the protein covalently entrapped in the nanogel. Polymerization using similar conditions to form the nanogel in the presence of wt-GFP, which did not contain a genetically incorporated ATRP initiator, resulted in GFP leaching out of the nanogel, as determined by the UV-Vis absorption spectrum of the purified product. Liu, Zhao, and coworkers prepared core-corona type nanoparticle with a BSA decorated corona and a cross-linked poly(HEMA) core.[81] The BSA-macroinitiator was prepared through disulfide exchange with a PDS-functionalized ATRP initiator to modify Cys-34 on BSA. Polymerization from the BSA-macroinitiator was carried out in aqueous solutions using typical AGET ATRP conditions in the presence of HEMA and the cross-linker *N,N'*-methylene diacrylamide. Because the protein macroinitiator functioned as a colloidal stabilizer, particle size was controlled by the amount of the BSA-macroinitiator employed: loading of a smaller amount of BSA-macroinitiator resulted in larger nanoparticles. Interestingly, upon treatment of the nanoparticles with dithiothreitol (DTT) in order to

reduce disulfide linkages thereby removing BSA from the particle coronas, the nanoparticles underwent aggregation as determined by TEM. This indicated that the BSA-macroinitiator was also important for the stabilization of the nanoparticles in addition to its role in initiating polymerization. Additionally, the authors found that covalently immobilized BSA on the nanoparticle surface retained up to 76% activity in comparison to native BSA as determined by a spectrophotometric assay measuring the hydrolysis of 4-nitrophenyl acetate. The BSA-nanoparticles were also found to be non-toxic when incubated with HepG2 cells and were internalized in the same cell line. These examples illustrate the utility of AGET ATRP grafting from in preparing more complex architectures with retention of protein structure and activity.[81]

Matyjaszewski and coworkers systematically screened conditions to polymerize PEGMA from BSA using ATRP in aqueous conditions resulting in conjugates with good control over molecular weight and dispersity.[71] Conditions were first screened with traditional ATRP methods, then AGET ATRP was then investigated to prepare BSA conjugates. Interestingly, different CuX/ligand conditions were optimal for traditional ATRP compared to AGET ATRP. Additionally, controlled polymerization in PBS buffer, which can be challenging due to formation of insoluble phosphate salts and displacement of ligands by chloride, was achieved with different CuX/ligand conditions than was optimal in water. The authors also observed that slow feeding of the ascorbic acid reducing agent resulted in the highest molecular weight and narrowest molecular weight distribution by minimizing termination with very active ligands like TPMA for polymerization of PEGMA under AGET ATRP conditions.

This observation of improved control with slow feeding of reducing agent, or activators regenerated by electron transfer (ARGET) ATRP, was then investigated in depth by the Matyjaszewski group to polymerize from proteins.[82] This technique allows for low catalyst loading and an extra handle of control to start/stop polymerization based on the feed of reducing agent. Systematic optimization of polymerization conditions with OEGMA was first accomplished with a small molecule initiator; addition of halide salt (30-100 mM), increased equivalents of ligand (4/1 L/Cu), and altering copper concentration (300 to 100 ppm) resulted in the best control with acceptable rates of polymerization. Additionally, a block copolymer could be prepared with the optimized method and polymerization could be stopped and started with the ascorbic acid feed. Polymerization of OEGMA from a BSA macroinitiator was accomplished in PBS buffer with 300 ppm Cu. Matyjaszewski and coworkers also utilized this technique to polymerize OEGMA from GFP with a genetically encoded, base-cleavable initiator.[83]

3.5 ICAR ATRP

During initiators for continuous activator regeneration (ICAR) ATRP, addition of a conventional thermal radical initiator (typically an azo-initiator) generates the active Cu(I)/L complex in situ, enabling use of low (<100 ppm) levels of copper catalyst.[84] Similar to ARGET ATRP, oxidatively stable Cu(II) reagents are used and the technique allows for some tolerance to oxygen. however, continuous feeding to regenerate the active species is not required due to the slow decomposition of the radical initiator. Polymerization

conditions are generally mild, although with slightly elevated temperatures to activate the thermal initiator, this method may not be appropriate for more sensitive biomolecules. The Matyjaszewski group was the first to report the use of ICAR ATRP to polymerize from a biomolecule.[85] The group initially optimized aqueous polymerization conditions for homopolymers of oligo(ethylene glycol) acrylate (OEGA) with a small molecule initiator in the presence of TPMA, $CuBr_2$, an excess of tetraethylammonium bromide (TEA-Br, 20-300 mM) to maintain the concentration of the deactivator complex, and 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) as the thermal initiator. Cu catalyst concentrations as low as 20 ppm could be employed while maintaining control over molecular weight and dispersity. Optimized conditions (100 ppm Cu, 44 °C, 8 h) were then used to polymerize OEGA from a BSA macroinitiator, resulting in a conjugate with polymers of controlled molecular weight and molecular weight distribution.[85]

ICAR ATRP has also been used by the Averick group to polymerize homo- and block-copolymers of acrylamide monomers.[86] Conjugates of BSA with a block copolymer of acrylamide, *N*,*N*-dimethylacrylamide (DMA), and *N*-vinylimidazole (VI) were prepared by ICAR using PBS buffer as the halide source as opposed to TEA-Br. BSA conjugates harboring block copolymers of VI and OEGA underwent cross-linking in the presence of palladium through the metal interaction with the imidazole containing block, thus forming a biohybrid nanoparticle capable of serving as a catalyst for suzuki-Miyaura cross-coupling reactions.[86] Further work used ICAR ATRP to explore the effect of conjugation site on *Thermomyces languginosa* lipase (TL)-poly[*N*-(3-(*N*,*N*-dimethylamino)propyl) acrylamide] conjugates.[87] TL was modified with an ATRP initiator at either Lys residues with an NHS-ester or acidic (glutamic and aspartic acid) residues with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. Enzyme activity was increased by 50% for conjugates grafted from acidic residues compared to from Lys residues, despite 6 initiating sites for the former compared to 3 for the latter.[87]

3.6 SET-LRP

Single electron-transfer living radical polymerization (SET-LRP) or Cu(0)-mediated LRP involves disproportionation of Cu(I) in polar solvents to generate active Cu(0) species and Cu(II) species which deactivate the propagating polymer chains; thus aqueous conditions are well suited for this polymerization method. Haddleton and coworkers utilized SET-LRP to polymerize water soluble monomers from a small library of proteins and peptides.[56] BSA, bovine hemoglobin (Hb), human Lyz, salmon calcitonin (sCT), and bovine insulin were chosen as mode, proteins and peptides with a variety of molecular weights and properties. Macroinitiators were prepared by reacting ATRP initiators with proteins/peptides through NHS-ester coupling at Lys amino acid residues. Conditions were screened to polymerize NIPAAm, DMA, and OEGA from the protein/peptide-macroinitiators. Synthesis of well-defined BSA conjugates were achieved through an excess of CuBr relative to Me_6TREN ligand to improve control. Conjugates of Hb were obtained, though with broad dispersities ($Đ$ >1.6) likely due to interactions with Cu(II) with the Fe(II) containing heme centers present on the protein. Lyz and sCT macroinitiators were insoluble in aqueous conditions, but the addition of 0.5% SDS denaturant to solubilize the protein-macroinitiators and sodium bromide (NaBr) to minimize loss of halide anions from SDS resulted in well-defined

protein-polymer conjugates. The insulin-macroinitiator formed a stable colloid in aqueous solution but polymerization could be accomplished in this heterogeneous system with the SET-LRP conditions. These conjugates also exhibited interesting self-assembly behavior in solution. Demonstration of the range of conjugates prepared by SET-LRP indicates that this is a versatile method to prepare polymer conjugates by the grafting from method.[56]

In collaboration with the Haddleton group, Joensuu, Milani, Linder and coworkers synthesized protein-polymer conjugates by the grafting-from method using Cu(0)-mediated LRP to make antifouling surfaces.[88] A Cys mutant of hydrophobin (NCysHFBI), a protein that self-assembles on hydrophobic surfaces, was used and conjugation with a maleimide modified initiator afforded a site-specific macroinitiator. Growth of poly(PEGA) from the macroinitiator already self-assembled on a hydrophobic surface resulted in a surface with decreased nonspecific binding. Utilization of this method to graft from NcysHFBI enabled facile synthesis of these surfaces.[88]

3.7 Photo-mediated ATRP (photo-ATRP)

Photo-ATRP processes have also gained considerable interest due to the high degree of temporal control provided by using light as an external stimulus for the generation of active Cu(I) species. The need for external radical initiators or reducing agents is replaced with the requirement of additional ligands in the system which also serve as reductants under light irradiation.[89] The Matyjaszewski group has recently utilized visible-light (Blue LED) mediated ATRP to graft oligo(ethylene oxide) methyl ether methacrylate (OEOMA) monomers from the surface of BSA and DNA in order to develop BSA- and DNA-polymer conjugates.[90] Notably, polymerization was carried out in aqueous conditions and in the presence of low ppm of Cu (50-1000 ppm). The deployment of glucose, glucose oxidase, and sodium pyruvate (Glu, GOx, SP) was used to deoxygenate the polymerization mixture, thus precluding the need for N2 sparging or freeze-pump-thaw procedures.[90] The complete elimination of metal catalysts in order to synthesize protein-polymer conjugates would be a welcome development, especially given the recent progress in metal-free photo-ATRP methodology.[91-94]

3.8 Electrochemical ATRP (eATRP)

Electrochemically mediated ATRP was first reported by the Matyjaszewski group in 2011 and has garnered attention due to its general tolerance of oxygen, low catalyst loading, removal of chemical reducing agents, and feasibility of temporal control.[95] In the case of eATRP, polymerization is performed in an electrochemical cell with regeneration of the reduced metal species controlled by an applied cathodic current. Application of an anodic current acts to oxidize the metal species thereby terminating the polymerization.[95-97] Many groups have taken advantage of eATRP for a variety of unique applications.[98-100] The Matyjaszewski group recently utilized eATRP for grafting-from initiator functionalized DNA substrates under aqueous conditions.[101] A screen printed electrode (SPE) was used to bypass the large reaction volumes required of electrochemical setups, which would render grafting-from using eATRP an unreasonable method for expensive biomolecules. SPE's are inexpensive substrates that can be smaller than 1 cm^2 and contain a working electrode, counter electrode, and a reference electrode typical of electrochemical setups.

Interestingly, the authors were able to decrease reaction volumes down to 75 μL using SPEs. This large decrease in reaction volume necessitated the use of an enzyme degassing system consisting of GOx, glucose, and SP rather than traditional degassing methods. Polymerization of 2-(methylsulfinyl)ethyl acrylate (MSEA) and OEGMA was carried out from the DNA-macroinitiator in the presence of $CuBr_2$ and TPMA under eATRP conditions and an extensive study was carried out to elucidate the effects that different catalyst and ligand loadings had on the reaction outcome. Notably, DNA-polymer conjugates larger than 25 kDa were able to be synthesized in <30 minutes, despite the low monomer conversion observed.[101]

4 Biomolecule-polymer conjugates accessed through RAFT polymerization

RAFT polymerization is a living and controlled radical polymerization technique that quickly garnered popularity after its initial discovery was reported in 1998.[102-104] Control during RAFT polymerization is obtained through a degenerative chain transfer process that is mediated by a chain transfer agent (CTA; also referred to as a RAFT agent).[11, 105, 106] The RAFT agent is a small molecule organic compound typically composed of a thiocarbonylthiol group, some common architectures include xanthates, trithiocarbonates, dithioesters, and dithiocarbamates.[107, 108] The RAFT agent structure is often conceptually split into two parts, the “R” group and the “Z” group (Figure 11).[105, 106] The R group reinitiates radical polymerization while the Z group is involved in both modulating the reactivity of the thiocarbonyl bond towards radical addition and also in stabilizing the subsequent radical formed on the thiocarbonyl carbon atom (Figure 11). A useful feature of RAFT polymerization is that both the R and Z groups are incorporated on the polymer chain ends upon termination, allowing for further functionality to be engendered at either end.[105, 106, 108]

The widely accepted mechanism for traditional RAFT polymerization involves initiation using a prototypical radical initiator such as the thermally activated initiator azobisisobutyronitrile (AIBN) (Figure 11).[11, 106, 109] Photochemical initiation is another popular form of initiation for RAFT polymerization and will be discussed later in the review. Initiation and radical addition to monomers produce propagating oligomeric radicals which can then enter into the RAFT equilibrium whereby the propagating radicals add into the thiocarbonyl bond of the RAFT agent (**I**) and produce a stabilized radical on the thiocarbonylthio carbon atom (**II**) (Figure 11). During this equilibrium process, (**II**) can fragment back to produce the oligomeric propagating radical and the intact RAFT agent, or homolytic cleavage of the R group can occur whereby the RAFT agent becomes appended to the end of the oligomer (**III**) and the R group radical can then re-initiate polymerization. After this initial equilibrium process, polymer chains propagate through the addition of monomers and either enter into the RAFT equilibrium process again with the thiocarbonylthio capped polymer chains or continue adding monomers. It is this rapid equilibrium process that affords control over the polymerization because the concentration of stabilized radical intermediates is greater than that of the propagating radicals, thus unproductive termination pathways are greatly limited (Figure 11). RAFT polymerization

is a popular method in the bioconjugation realm due to its wide monomer scope, solvent compatibility, and general avoidance of transition metals (though there are exceptions such as in photo-induced electron transfer-RAFT (PET-RAFT)).[110, 111] Although there exist many examples of generating polymers through RAFT with bioactive functionality either at the end of the polymer or as pendant groups, this section of the review will only cover peptide-, protein-, and nucleic acid-polymer conjugates by grafting-from using RAFT. [112-116]

4.1 Protein-polymer conjugates

The initial report of grafting-from a protein using RAFT polymerization was by Davis, Bulmus and co-workers where they developed a pyridyl disulfide functionalized trithiocarbonate RAFT agent which could undergo disulfide exchange with Cys-34 on BSA thereby forming a macroRAFT agent where BSA acted as the Z-group. This was planned due to Z-group retention on polymer chain ends (Figure 12, top).[117] Formation of the macroRAFT agent was monitored by UV-Vis spectroscopy to visualize the formation of the 2-pyridinethione byproduct that occurs after PDS exchange and absorbs in the 340-370 nm range. The authors opted to initiate polymerization of PEG-acrylate (PEGA) using γ-irradiation which offered the benefit of room temperature initiation in aqueous conditions suitable to the stability of BSA. Though γ-irradiation can potentially be detrimental to protein structure and function, the authors determined both BSA and glucose oxidase to retain up to 92% and 88% activity after 6 hours of γ-irradiation, respectively. Polymerization of PEGA was carried out in the presence of the macroRAFT agent in a mixture of water and DMF under γ-irradiation at room temperature thereby producing BSA-polymer conjugates which were characterized by GPC, MALDI, and non-denaturing PAGE. The BSA-polymer conjugate was treated with tris(2-carboxyethyl)phosphine (TCEP) in order to reduce the disulfide linkage thus facilitating disassembly of the polymer from the protein to allow for characterization of the polymer itself. However, the disulfide bond remained intact upon treatment with TCEP, only demonstrating partial reduction for low molecular weight conjugates which the authors attributed to the shielding of the disulfide bond by the polymer. Treatment of the BSA-poly(PEGA) conjugates in the presence of concentrated TCEP solutions completely denatured the protein but allowed for analysis of the polymers. Characterization of the polymers revealed a few drawbacks, though the molecular weight of the polymers increased linearly with increasing monomer conversion, the polymers exhibited dispersity values of up to 2.0 with increasing molecular weight. This observation was attributed to the steric hindrance imparted by BSA in addition to the growing polymer chains during the equilibrium process. Additionally, there was an observed two-hour inhibition period at the start of polymerization which was attributed to slow fragmentation of the macroRAFT agent. Despite these minor drawbacks, this elegant work paved the way for the expansion of grafting-from using RAFT to generate biomolecule-polymer conjugates (Figure 12, top).[103]

The Davis and Bulmus groups quickly expanded on their initial work by polymerizing NIPAAm and hydroxyethyl acrylate (HEA) in the presence of the BSA-macroRAFT agent using a room temperatu and water soluble radical initiator (VA-044).[119] An inhibition period was observed at the beginning stages of polymerization as in their

earlier work, however the polymerization exhibited a linear evolution of $Ln[M]_0/[M]$ versus time indicative of a steady concentration of radicals in solution over the course of polymerization. TCEP reduction of the protein-polymer conjugate was carried out and the polymers analyzed via GPC which again showed a linear increase in molecular weight with increasing monomer conversion. Interestingly the poly(NIPAAm) samples were relatively monodisperse exhibiting dispersity values up to 1.3 for the higher molecular weight polymers. This is in stark contrast to their above described grafting-from polymerization of PEGA which exhibited dispersity values up to 2.0. Activity studies were also carried out to determine the esterase-like activity of BSA to hydrolyze *p*-nitrophenyl acetate. The BSA-macro RAFT agent, BSA incubated with VA-044, and BSA-poly(NIPAAm) conjugates all remained active, while controls in which BSA was heated to 85 °C in buffer for 4 hours lost up to 80% activity. Additionally, BSA-poly(NIPAAm) conjugates displayed increased LCST values in comparison to poly(NIPAAm) alone, which increased as the polymer molecular weight of the conjugate decreased.[119] In an interesting follow-up work, the Davis group was able to employ similar polymerization methodology in the presence of a bifunctional PDS trithiocarbonate RAFT agent to access polymers harboring biomolecules at each end through a combination of grafting-to and grafting-from approaches.[120]

The thermoresponsive properties of BSA-poly(NIPAAm) conjugates were studied further by the Sumerlin group (Figure 12, bottom).[118] A BSA-macroRAFT agent was developed through attachment of a maleimide functionalized RAFT agent on Cys-34. Instead of attaching BSA to the Z-group portion of the RAFT agent similar to the Bulmus and Davis work mentioned above, Sumerlin and co-workers attached BSA on the R-group portion of the RAFT agent (Figure 12, bottom). This offers several unique advantages; the thiocarbonyl bond is more accessible for chain transfer thereby improving on the control over polymerization processes. Additionally, having the thiocarbonylthio group at the polymer chain end would allow for post-polymerization modification without interfering with the protein on the other end of the polymer. Polymerization of NIPAAm in the presence of the BSA-macroRAFT agent was carried out in pH 6.0 phosphate buffer using VA-044 as the radical initiator similar to earlier reports. Purification of the BSA-poly(NIPAAm) conjugates was carried out via thermal precipitation at 40 °C. The purified BSA-poly(NIPAAm) conjugates were treated with TCEP and the corresponding polymers characterized via GPC which showed an increase in polymer molecular weight with increasing monomer conversion and high molecular weight polymers (234 kDa) exhibited a dispersity of 1.38. The BSA-macroRAFT agent and the BSA-poly(NIPAAm) conjugate both retained their secondary structure as determined by circular dichroism and also retained esterase-like activity (>90% activity compared to native BSA). The activity of the protein could be modulated due to the thermally responsive nature of poly(NIPAAm) (Figure 13). Upon exposure to heating above the LCST of the BSA-poly(NIPAAm) conjugate (40 °C), the protein activity was reduced to 75%. Interestingly, the protein activity was regained upon cooling of the conjugate solution below the LCST and this process was cycled up to 5 times with no apparent loss in protein activity after each cycle (Figure 13).[118]

The Sumerlin group next explored the synthesis of more complex thermoresponsive protein-polymer conjugates through RAFT polymerization by implementing methodology which allowed for the formation of block copolymer architectures through grafting-from.[121,

122] In their initial work, the group attached a RAFT agent to Cys-34 on BSA through a maleimide linkage, again appending the protein to the R-group of the RAFT agent. [121] The BSA-poly(NIPAAm) conjugate was synthesized, purified, and subsequently characterized using SDS-PAGE. Retention of the trithiocarbonate end-group could be visualized using UV-Vis spectroscopy with the characteristic absorption appearing at ~310 nm. The BSA-poly(NIPAAm) conjugate was then employed as the macroRAFT agent in the subsequent polymerization of DMA using similar conditions to those mentioned above to produce the second block. UV-Vis spectroscopy indicated retention of the trithiocarbonate end-group even after addition of the DMA block. Additionally, the thermoresponsive properties were measured using DLS which showed an increase in the hydrodynamic diameter of the protein-polymer conjugate at elevated temperatures.[121] Similar work targeted the seven Lys amino acid residues on Lyz using a NHS-ester functionalized RAFT agent thereby forming a Lyz-macroRAFT agent with the protein on the R-group.[122] Lyz-poly(NIPAAm)-*b*-poly(DMA) conjugates were synthesized using similar polymerization conditions to their previous work and their thermoresponsive solution behavior analyzed. [122]

Konkolewicz, Page, Berberich, and co-workers used RAFT polymerization and a combination of grafting-to and grafting-from procedures to perform a thorough study detailing the effects of different types of polymers of varying lengths and structures on the stability and activity of Lyz conjugates.[123] The activity of Lyz was tested using a standard spectrophotometric technique that measures lysis of *Micrococcus lysodeikticus* in the presence of Lyz. As might be expected, the Lyz-polymer conjugate activity was reduced as the molecular weight of the polymer increased. Charged polymers attached to Lyz were also studied with negatively charged polymers resulting in reduced enzyme activity due to charge repulsion on the negatively charged *M. lysodeikticus* cell. Cationic polymers had the opposite effect, as might be expected. Additionally, the thermal stability of the Lyz-polymer conjugates was reduced. However, the chemical stability towards treatment with the protein denaturant guanidine HCl was increased with the highest molecular weight conjugates exhibiting the greatest stability, most likely due to a shielding effect imposed by polymers on the enzyme surface.[123]

The establishment of photochemically initiated grafting-from polymerization using RAFT proved to be a significant addition to the practitioners' toolbox which introduced an additional degree of control over the polymerization process. Chen and co-workers used a specially prepared *Escherichia coli* inorganic pyrophosphatase (PPase) protein in which they exchanged the Lys-148 amino acid residue with Cys through site-directed mutagenesis. [124] The Cys amino acid residue was used as the conjugation site for the attachment of a maleimide functionalized RAFT agent, thus generating the PPase-macroRAFT agent with the protein appended as the R-group. Polymerization of NIPAAm was performed in water and at room temperature under visible (420 nm) light irradiation in the presence of (2,4,6-trimethylbenzoyl)phenyl phosphonic acid sodium (TPO-Na) as the photoinitiator. The polymerization exhibited rapid kinetics with molecular weights reaching 150 kDa within 30 minutes. Notably, the authors were able to control polymerization by either turning the light source 'on' or 'off'. Polymerization was halted upon removal of the light source but continued when the light source was turned on. This process was cycled and the molecular

weight of the PPase-poly(NIPAAm) conjugate was followed by SDS-PAGE. Because the conjugation site of the RAFT agent and the conjugated polymer are near the active site of the protein, the PPase activity was greatly reduced. However, an interesting observation was made when it was determined that PPase-poly(NIPAAm) conjugates harboring higher molecular weight poly(NIPAAm) show an increased activity over the wild-type PPase above the LCST of the conjugate (45 °C).[124]

Boyer and co-workers reported on using PET-RAFT to graft-from BSA in aqueous conditions (Figure 14).[125] PDS exchange was used to append a trithiocarbonate-based chain transfer agent on BSA to generate the BSA-macroRAFT agent where BSA was appended as the R-group. Grafting-from polymerization of either DMA or OEGA was carried out in aqueous conditions in the presence of $Ru(bpy)_3Cl_2$, the BSA-macroRAFT agent, and blue LED irradiation and proceeded in a controlled manner to produce monodisperse protein-polymer conjugates. Additionally, the prepared BSA-polymer conjugates retained identical activity to native BSA as determined by an esterase activity assay.[125] In 2017, the Sumerlin group expanded on PET-RAFT methodology by employing an organophotocatalyst to polymerize an extended class of monomers under visible-light irradiation and also demonstrated the construction of block copolymers grafted-from Lyz.[126]

Beyond protein-polymer conjugation, the ability to engineer whole live cell surfaces with polymers of controlled molecular weights and architectures represents an important challenge.[127] Seminal work by Soh, Hawker, and coworkers used grafting-from techniques to assemble polymers on live cell surfaces (Figure 15).[128] Though there were a few previous reports of using grafting-to methods to obtain polymer-functionalized cells, the conjugation efficiencies were often minimal which necessitated the need for a large excess of synthesized polymer. Additionally, uncontrolled polymerization techniques to encapsulate cells resulted in the cells being difficult to access. Polymerization of PEG modified acrylamide monomers was carried out in the presence of live wild-type *Saccharomyces cerevisiae* (Baker's yeast) cells under PET-RAFT conditions with eosin Y as the photocatalyst and triethanolamine as the co-catalyst (Figure 15). In order to maximize cell viability, the PET-RAFT conditions were modified in order to quicken polymerization kinetics and monomer conversion was kept low in order to maximize end-group retention. Notably, the yeast cells remained intact and were able to undergo cell proliferation after being subjected to polymerization conditions. After these promising initial results, the authors introduced dibenzocyclooctyl (DBCO) functionality on the yeast cell surfaces through NHS-ester conjugation (Figure 15). This allowed for the efficient conjugation of azide-modified RAFT agents through strain-promoted 1,3-dipolar cycloaddition (SPAAC) thereby producing cell-based macro-RAFT agents. PEG-based acrylamide monomers were then polymerized using the modified PET-RAFT conditions employing the cell-based macro-RAFT agents and also a sacrificial RAFT agent in order to maintain control over polymerization. Additionally, the authors were able to incorporate azide functionality along the polymer chain for post-polymerization functionalization with an Alexa Fluor 647 strained alkyne analogue. Using confocal microscopy of the fluorescently labeled cell-polymer conjugates indicated that fluorescence was localized only on the cell surface. This experiment showcased that cell-surface polymers were able to undergo post-polymerization

modification using bioorthogonal methods and that polymerization only occurred on the cell surface without any observable polymer growth occurring inside the cell. Notably, the cell-polymer conjugates derived from grafting-from polymerization exhibited much greater grafting densities than those prepared using the traditional grafting-to technique. The authors were able to carry this technique forward to a mammalian cell line through the non-covalent modification of Jurkat cell membranes with a RAFT agent modified lipid. Cell viability assays performed after polymerization from the cell surface exhibited up to 90% viability and also retained their metabolic activity.[128]

4.2 Peptide-polymer conjugates

Some examples of grafting-from oligopeptide sequences using RAFT polymerization were reported by the Perrier and Börner groups.[129-131] Börner and coworkers' first manuscript of this kind describes the SPPS of an oligopeptide in which the N-terminus was modified with a dithiobenzoate-based RAFT agent via DCC coupling while on-resin (Figure 16).[129] Treatment of the resin-bound peptide with a TFA cleavage cocktail and subsequent precipitation resulted in the production of the oligopeptide-macroRAFT agent (**1**) with the oligopeptide positioned as the R-group. The authors noted the formation of a thioamide byproduct which is due to nucleophilic attack of the peptide amine terminus with the dithioester functionality during the DCC coupling step (Figure 16). This byproduct was observed via electrospray ionization mass spectrometry (ESI-MS) and is probable given the sensitivity of dithiobenzoate-based RAFT agents towards aminolysis. Despite this, the authors proceeded with polymerization of *n*BA using **1** as the thioamide byproduct would not interfere with polymerization conditions. However, the authors also developed a second approach to creating oligopeptide-macroRAFT agents by modifying an oligopeptide ATRP macroinitiator on resin. This was then reacted with a pyridinium salt of dithiobenzoic acid to produce **2** without formation of the previously observe thioamide byproduct (Figure 16). Polymerization of *n*BA employing either of the oligopeptide-macroRAFT agents generated monodisperse polymers and exhibited linear polymerization kinetics typical of a controlled RAFT process. However, it is important to note the significant inhibition observed at the start of polymerization which lasted between 4-8 hours depending on the oligopeptide-macroRAFT agent used.[129] In a later report, the authors developed a trithiocarbonate-based GGRGDS oligopeptide-macroRAFT agent (**3**) which was much more stable than the dithiobenzoate derivative published earlier.[130] Notably, polymerization of *n*BA using **3** did not exhibit an inhibition period at the start of the polymerization, though it is not clear if the inhibition period was due to differences in the oligopeptide structure or differences between the dithiobenzoate or trithiocarbonate structures. The authors employed **3** in the polymerization of NIPAAm to produce oligopeptide-poly(NIPAAm) conjugates, the ω-trithiocarbonate group was then reduced to afford a poly(NIPAAm) terminated with a free thiol which could be used for surface functionalization on a gold substrate. Cell adhesion of L929 mouse fibroblasts was examined on the GGRGDS-poly(NIPAAm) functionalized surface and was found to be faster than the corresponding poly(NIPAAm) functionalized surfaces.[130] The Börner group has also developed ABC-triblock co-polymers from oligopeptide-macroRAFT agents synthesized on-resin.[132]

An important additional consideration in the construction of oligopeptide-macroRAFT agents is hydrolysis of nitrile (CN) groups on the RAFT agent structure during cleavage of resin-bound peptides (Figure 17).[133] Because typical peptide cleavage cocktails make use of strongly acidic conditions (>95% TFA), RAFT agents appended to peptide structures during SPPS undergo hydrolysis thereby forming carboxamide functional groups in place of the nitrile substituent (Figure 17).[133-135] Despite this, Thang and co-workers were still able to perform controlled RAFT polymerization of DMAEMA, OEGMA, and *n*-butyl methacrylate (*n*BMA) monomers employing trithiocarbonate-based oligopeptide-macroRAFT agents in which the nitrile substituent had undergone hydrolysis to the corresponding carboxamide.[133] Though the earlier reports by the Börner group made use of nitrile modified dithiobenzoate RAFT agents in which no carboxamide formation was reported, they used a 2% TFA in DCM cleavage cocktail which cleaved the oligopeptide from the resin but did not remove the protecting groups present on the amino acid residue side-chains.[129]

4.3 Nucleic acid-polymer conjugates

Grafting-from DNA using RAFT was first employed by the He group for purposes of DNA biosensing.[136, 137] A few of their reports detailing their use of grafting-from for DNA detection made use of ATRP conditions (vide supra), however the transition metals employed for polymerization formed complexes with DNA molecules which complicated their intended application due to unwanted background noise. RAFT seemed to be a promising alternative due to the absence of transition metals.[136] Complementary oligonucleotide probes were functionalized on a gold surface and a separate complementary oligonucleotide sequences were synthesized with a trithiocarbonate-based RAFT agent attached to the amine terminus of the sequence via NHS-ester chemistry. The strands were allowed to hybridize and ligation was performed using a T4 ligase, thereby affixing the RAFT agent to the complementary strand placed on the gold substrate, forming surface immobilized DNA-macroRAFT agents. RAFT polymerization of OEGMA was performed from the modified surfaces in the presence of water at 30 °C using AIBN as the thermal initiator. Film characterization was performed using AFM, ellipsometry, and ATR-FTIR. Notably, the polymer film generated from the DNA-macroRAFT agents were much thicker than those generated by ATRP described in their previous reports and a large reduction in the background signal was observed, most likely due to the absence of transition metals during polymerization.[136] A thorough follow-up report was published soon after detailing the effects of variations in polymerization conditions (initiator concentration, temperature, reaction time, RAFT agent surface density) on film thickness of the DNA-polymer conjugates.[137]

RAFT polymerization from DNA-macro-RAFT agents have also been used for electrochemical target DNA (tDNA) biosensing applications.[138, 139] Recently reported methodology makes use of immobilized peptide nucleic acid (PNA)-DNA duplexes on gold electrode substrates that have been modified with dithiobenzoate-based RAFT agents using Zr^{4+} mediated coupling chemistry targeting the phosphate groups on the PNA-DNA duplexes. Polymerization of ferrocenylmethyl methacrylate (FcMMA) using VA-044 in the presence of the DNA-macroRAFT agent-modified surface was carried out and the

electrochemical response of the prepared polymer modified surfaces was tested. Only gold electrodes which contained the tDNA-polymer conjugates exhibited an oxidation peak on the square-wave voltammogram, with a peak potential of 0.3 V. Electrochemical analysis of gold substrates which served as controls (PNA probe, tDNA, Zr^{4+}, RAFT agent, VA-044, or FcMMA) did not exhibit any visible oxidation peaks on the square-wave voltammogram. Notably, oxidation peak currents of the tDNA-polymer substrates increased linearly as the surface concentration of tDNA increased and allowed for the detection of the tDNA at concentrations as low as 3.2 aM.[138, 139]

Recently, photo-RAFT processes have also been used to develop DNA-polymer conjugates via grafting-from.[45] Barner-Kowollik, Ng, Weil, and co-workers developed two ssDNA-macroRAFT agents based on either 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPADB) or 2-(butylthiocarbonothioyl)propionic acid (BTPA) with the ssDNA appended as the R-group of the RAFT agent. CPADB and BTPA were functionalized with either a PFP or NHS-ester group and coupled to the amine terminus of the purified ssDNA (NH2-ssDNA). RAFT polymerization employing the BTPA- or CPADB-DNA macroRAFT agents was performed using acrylamide, acrylate, or methacrylate based monomers in the presence of eosin Y and ascorbic acid under blue LED irradiation. DNA-polymer conjugates could be purified by membrane filtration and were characterized by GPC and native PAGE. While polymerization from the ssDNA-macroRAFT agent was operative, the polymers were not monodisperse owing to potential side reactions with the ssDNA which produced low molecular weight tailing in the GPC traces of the DNA-polymer conjugates. Additionally, high molecular weight shoulders were observed in the GPC spectra of ssDNA-poly(OEGMA) conjugates most likely due to transesterification or side reactions to the growing polymer backbone.[45, 140] Impressively, the purified ssDNA-polymer conjugates were able to undergo hybridization with a complementary ssDNA sequence which had been modified with a Rhodamine dye on the DNA terminus thereby showcasing that the prepared ssDNA-polymer conjugates maintain functionality.[45]

5 Biomolecule-polymer conjugates accessed through ROMP

Ring-opening metathesis polymerization (ROMP) has early roots stemming from the initial discovery of olefin metathesis in the 1950s, unlike the more recently developed ATRP and RAFT methodologies which were reported in the mid-1990s.[17, 46, 47, 141, 142] The introduction of well-defined single-component carbene complexes based on molybdenum or ruthenium, spearheaded by the Schrock and Grubbs groups, has paved way for significant advances in ROMP thereby enhancing the synthesis of well-defined and monodisperse polymers through the living polymerization of strained cyclic olefin monomers.[10, 17, 143, 144] In particular, the augmented functional group tolerance and increased stability in aqueous media of the ruthenium-based carbenes introduced by Grubbs and co-workers expanded the scope of chemical transformations and facilitated expansion of ROMP into the realm of biomolecule-polymer conjugation.[145-148] As such, ROMP bolsters the chemical toolbox available to chemical biologists and materials chemists alike through the expansion of available polymers with unique function, structures, and material properties.[10, 149]

The ROMP mechanism involves coordination of a transition-metal based carbene to a strained cyclic olefin and subsequent [2+2] cycloaddition to form a metallacyclobutane intermediate.[17, 150] The metallacyclobutane then undergoes [2+2] cycloreversion to yield a new olefin and transition-metal carbene which exists on the end of the propagating polymer chain. The transition-metal carbene catalyst will continue to react with other cyclic olefin monomers with the typical driving force being the release of ring strain from the strained cyclic olefin or an increase an entropy when polymerizing non-strained cyclic olefins. Ruthenium-based transition-metal carbene complexes are the most widely utilized in the context of biomolecule modification due to their stability and functional group tolerance.[17, 39]

5.1 Grafting-from proteins using ROMP

Isarov and Pokorski reported the only example of developing protein-polymer conjugates through grafting-from using ROMP (Figure 19).[151] Because the polymerization needed to be carried out in buffered aqueous solutions compatible with the protein, the authors synthesized a water-soluble Grubbs 3rd generation carbene analogue. Taking inspiration from the Emrick group who prepared PEG-functionalized ruthenium carbene derivatives, Isarov and Pokorski synthesized a PEGylated Grubbs 3rd generation derivative (**4**) through a ligand exchange reaction of to displace the original bromopyridyl groups with PEGylated pyridyl ligands to generate **4** (Figure 19).[151, 152] The catalyst was found to be stable in water for over 10 hours as confirmed by ^{1}H NMR monitoring of the alkylidene resonance in D_2O which remained unchanged over the course of the study. Test polymerizations to evaluate catalyst activity were carried out with PEGylated norbornene monomers in both DCM and PBS in order to compare reactivity in both organic and aqueous conditions. Ultimately, polymerization kinetics were found to be slower in aqueous solutions most likely due to a decreased rate of ligand dissociation.[151]

Norbornenyl groups were added to reactive Lys residues on the protein surface of Lyz. Treatment of the protein with *exo*-norbornene dicarboxylic anhydride resulted in the modification of up to 5-6 Lys residues per protein. Excess amounts of **4** were then reacted with the norbornenyl modified Lyz to yield the protein macroinitiator which was used for the in-situ growth of PEGylated norbornene monomers from the protein surface. Large monomer loadings (>200 equiv per protein) were ultimately needed to initiate polymerization due to the poor accessibility of the catalytic site on the protein. As a result, only high molecular weights were able to be targeted, since at low monomer concentrations no polymerization could be initiated.[151]

In a follow-up work from the Pokorski group, this challenge was resolved by using a grafting-to approach to instead attach the synthesized polymer to the protein.[153] This allowed for greater control during polymerization and enabled the synthesis of monodisperse polymers of varying molecular weights to be fully characterized and subsequently conjugated to the protein through the Lys residues. The grafting-to strategy is utilized to a greater extent for the conjugation of polymers prepared through ROMP.[12, 154, 155] Though grafting-from is under-utilized in the context of ROMP, the initial disclosure from

Isarov and Pokorski provides an important foundation with which to develop improved systems through careful tuning of catalyst systems.[151, 153]

5.2 Grafting-through approaches with ROMP

The bulk of examples for ROMP in the context of biomolecule modification use the grafting-through strategy. In grafting-through, a biomolecule or bioactive material is attached directly to a monomer (such as norbornene) thus generating a “macromonomer”. The prepared macromonomer can then be polymerized, resulting in a polymer chain with the pendant bioactive cargo attached. Several elegant examples make use of grafting-through using ROMP to prepare polymer materials with potential therapeutic utility.[156-159] The employment of grafting-through using ROMP in the context of biomolecule modification has been developed extensively for materials bearing nucleic acids or DNA and oligopeptides.

5.2.1 Oligopeptides—There exist many examples of grafting-through using ROMP to generate polymer architectures harboring pendant amino acids and oligopeptides. Grubbs and co-workers disclosed many of the initial reports detailing the modification of amino acids and peptides using ruthenium-based carbene complexes. Early manuscripts in the 1990’s detailing the extensive use of ring-closing metathesis (RCM) for the generation of cyclic peptides established a foundation for the use of ruthenium-based carbenes to invoke chemical transformations on densely functionalized substrates; interested readers are directed to these literature reports.[160-162] In an early example from Maynard and Grubbs transitioning into ROMP, template directed RCM was used to create cyclic crown ether monomers with a pendent phenylalanine (Phe) amino acid residue.[163] These cyclic monomers were subsequently polymerized through ROMP to yield polyethers with novel olefinic backbone structures and bioactive side chains. Olefin containing polyethers are of interest primarily due to their structural similarity with PEG; however the presence of olefins along the backbone of the polymer introduces a greater degree of synthetic utility making possible the introduction of complex functionality through post-polymerization modification techniques. Polymerization attempts using unprotected phenylalanine substrates were ultimately stymied due to solubility challenges which led to the employment of protected amino acids in earlier approaches.[164-166] Follow-up work focused on developing co-polymers with more complex functionality. Polymers composed of a norbornenyl backbone with pendant oligonucleotide sequences Gly-Arg-Gly-Asp-Ser (GRGDS) and Pro-His-Ser-Arg-Asn (PHSRN) were synthesized and their biological activity tested.[167, 168] RGD is a peptide sequence on extracellular proteins which mediates binding to cell surface integrins thereby influencing cell processes. The SRN sequence, physically near the near the RGD sequence in the extracellular matrix protein fibronectin, enhances binding to integrins. Norbornene monomers harboring the RGD or SRN oligopeptides were homo- or copolymerized using Grubbs 2nd generation catalyst. Using ROMP allowed for the fine-tuning of the final polymer to incorporate a controlled amount of RGD. The oligopeptide functionalized monomers remained protected over the course of polymerization in order to maintain solubility which necessitated a post-polymerization deprotection strategy resulting in water-soluble polymers to be used in biological assays. Cell adhesion inhibition studies were performed where fibronectin coated surfaces were incubated with human foreskin

fibroblast (HFF) cells in the presence of either oligopeptide homo- and co-polymers, the oligopeptides alone, or buffer. Interestingly, the co-polymers consisting of pendant GRGDS and PHSRN sequences inhibited HFF adhesion to fibronectin to a greater degree than homopolymers with pendant GRGDS and the GRGDS or PHSRN peptides alone, highlighting the synergistic effect of the two oligopeptide sequences and underscoring the utility of ROMP to prepare bioactive materials.

Several groups sought to expand or improve upon the use of ROMP to access pendant oligopeptide polymer architectures in order to better understand structure/function relationships of oligopeptide grafted polymers. The Muthukumar and Emrick groups used ROMP to generate polyelectrolytes through the polymerization of OEG or pentalysine oligopeptide functionalized cyclooctene monomers thereby accessing homo- or co-polymers which differed in charge density.[169] Solution behavior could be modulated based on the length of the OEG or Lys blocks and on the graft density of Lys blocks. Additionally, it was found that poly(cyclooctene)-graft-pentalysine polymers underwent complexation with DNA under acidic conditions but was disrupted under basic conditions. This characteristic can potentially be utilized in plasmid DNA transfection applications.[169] Conrad and Grubbs also utilized ROMP to develop polymers bearing the pendant VPGVG elastin oligopeptide attached to a norbornene monomer.[170] An OEG functionalized norbornene comonomer was also included to varying degrees along the polymer chain. The LCSTs of each polymer were then studied as a function of the polymer concentration in solution, degree of polymerization, or OEG comonomer feed. Ultimately it was found that the LCST depended greatly on the ratio of oligopeptide versus OEG monomers along the copolymer chain and on the concentration of the copolymer in solution, but to a lesser degree on the molecular weight of the copolymer.[170]

The Gianneschi group has greatly expanded on the development of oligopeptide containing homo- or block- (co)polymers prepared via grafting-through using ROMP. In their earliest examples, copolymerization of either hydrophilic or hydrophobic norbornene-based monomers functionalized with oligopeptides was achieved via ROMP which resulted in the formation of bioactive nanoparticles (Figure 20).[171] The Gianneschi group has also been successful in developing protecting-group free strategies towards accessing polymers harboring pendant oligopeptides using ROMP.[172] These initial works served as a foundation to understand proteolytic stability of peptide-based nanoparticles and established a set of synthetic principles to better develop polymers harboring pendant oligopeptide functionality. Expansion of this platform led to the development of polymer brushes harboring pendant cell-penetrating peptides (CPPs) which were resistant to proteolysis and also maintained their cell penetration activity.[173, 174] The elegant use of ROMP has also allowed for facile tuning of polymer architectures and grafting densities which has made possible unique nanoparticle materials for drug delivery, fluorescence imaging, and for either the evasion or uptake of macrophages.[175, 176] The ring-opening metathesis polymerization-induced self-assembly (ROMPISA) ushered in by the Gianneschi group has also proven to be an extremely useful method for the generation of well-defined nanostructures.[177, 178]

5.2.2 Nucleic acids—ROMP has been used to great extent in the polymerization of nucleic acid containing polymers. Initial reports by Williams and co-workers have utilized ROMP to develop polymers with pendant nucleic acid functionality via grafting-through of norbornene monomers substituted with either thymine, adenine, cytosine, guanine, or uracil.[179, 180] The monomers were poorly soluble, however, and polymerization of the monomers was only able to produce short oligomers due to precipitation over the course of the reaction. Bazzi and Sleiman were able to successfully synthesize homopolymers and copolymers containing adenine side chains via ROMP using a succinimide additive to solubilize the resulting polymer through hydrogen bonding interactions.[181] Work by Weck and co-workers has also developed template-directed strategies to carry out ROMP of nucleic acid based monomers.[182, 183]

Expanding further on this, Herrmann and co-workers recently reported a strategy to solubilize DNA in the organic phase by exchanging counter-ions on the backbone with quaternary ammonium surfactants.[184] These DNA-side chains are then modified with norbornene and polymerized via ROMP to yield DNA homopolymers of varying architectures. This strategy allowed for higher yields for DNA-based conjugates and overcame challenges of nucleic acid modification in organic solvents.[184]

Gianneschi and co-workers have shown recent progress in preparing nucleic acid brush polymers and co-polymers from PNA functionalized norbornene monomers.[185] Homopolymers made from the oligonucleotide substituted monomers or amphiphilic brush copolymers made up of a pendant oligonucleotide block and a benzyl-substituted poly(norbornene) block were synthesized through ROMP. The authors were able to develop spherical nanoparticles from the synthesized brush copolymers which harbored the oligonucleotide block exposed as the outer shell of the sphere. Notably the oligonucleotides present on the nanoparticle surface were able to undergo hybridization with complementary DNA oligonucleotide sequences thereby showcasing post-polymerization functionality.[185]

6 Summary and outlook

Utilizing controlled polymerization methods to graft-from biomolecules has enabled rapid access to biomolecule-polymer conjugates which combine the unique traits of both the biomolecule and the synthetic polymer attached to it. Over the course of the review, we have examined recent developments in the generation of biomolecule-polymer conjugates synthesized using grafting-from techniques tracing back from the initial discoveries employing ATRP, RAFT, or ROMP. There are many advantages to utilizing grafting-from techniques with purification and small molecule coupling efficiencies being the two most critical. Additionally, each controlled polymerization process holds inherent advantages and disadvantages in the context of grafting-from biomolecules. While an advantage of ATRP is the high degree of flexibility offered by the availability of many different variations based on the polymerization process, this also presents a challenge as the correct choice of ATRP method to use may not always be straightforward to the novice user. The deployment of transition-metal based reagents and the need for oxygen-free conditions also presents a challenge in the context of grafting-from. RAFT polymerization is an incredibly versatile method which allows for the polymerization of a wide range of monomers in a variety of

solvent conditions, and the absence of transition-metal based reagents is also a significant advantage of the technique. However, RAFT in the context of grafting-from suffers from relatively slow reaction kinetics or long inhibition periods which may be dependent on the structure of the biomolecule-macroCTA, the amount of oxygen in the reaction mixture, or on the reagent concentrations. On the other hand, ROMP typically exhibits fast polymerization kinetics and allows access to polymers made from monomers which may not be readily polymerized using controlled radical techniques. However, the need to employ transition-metal based reagents, especially from the biomolecule surface represents a significant challenge. Fortunately, all together these methods provide scientists with a plethora of opportunities to prepare protein-polymer conjugates of choice.

We anticipate renewed interest in grafting-from processes, given the recent resurgence and creativity by many groups to investigate new bioconjugation techniques which have expanded the scope of targetable amino acid residues and increased the conjugation yields.[6, 36-38, 41, 186-190] The relatively recent highly-efficient methods to label methionine and histidine residues on proteins provide additional, less abundant, amino acid residues with which to polymerize from, if reagents bearing ATRP initiators can be accessed.[36, 37, 191-193] Additionally, the rapid progress of metal-free and photo-induced controlled polymerization techniques paves way for the synthesis of polymers from more therapeutically relevant biomolecules that exhibit greater sensitivity to reaction conditions.[91-93, 126, 194, 195] It would be an exciting and much needed addition to identify water-soluble organic photo-reductants capable of promoting ATRP processes in purely aqueous solutions in the absence of transition metals.[196, 197] Oxygen tolerant polymerization techniques that can operate at low volumes also represent a significant breakthrough that may play a role in further development of grafting-from techniques.[198-200] Combined altogether, newly developed polymerization and conjugation techniques may soon allow for rapid access and high-throughput screening of constructs to develop new biomolecule-polymer conjugates with vastly unique properties that would give additional insights into structure-property relationships of complex bioconjugates.

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Abbreviations

AA	ascorbic acid
AFM	atomic force microscopy
AGET	activators generated by electron transfer
AIBN	azobisisobutyronitrile
ARGET	activators regenerated by electron transfer
ATRP	atom transfer radical polymerization

BSA	bovine serum albumin
BTPA	2-(butylthiocarbonothioyl)propionic acid
***c*GFP**	cyclic green fluorescent protein
CPADB	4-cyano-4-(phenylcarbonothioylthio)pentanoic acid
CPP	cell-penetrating peptide
CTA	chain-transfer agent
CuAAC	copper-catalyzed azide-alkyne cycloaddition
CuBr	copper(I) bromide
$CuBr_2$	copper(II) bromide
CuCl	copper(I) chloride
Cys	cysteine
DBCO	dibenzocyclooctyl
DCC	*N,N*-dicyclohexylcarbodiimide
DLS	dynamic light scattering
DCM	dichloromethane
DMA	*N,N*-dimethylacrylamide
DMAEMA	2-(dimethylamino)ethyl methacrylate
DMF	*N,N*-dimethylformamide
DNA	deoxyribonucleic acid
Dox	doxorubicin
DTT	dithiothreitol
eATRP	electrochemically-mediated ATRP
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization mass spectrometry
FcMMA	ferrocenyl methyl methacrylate
FPLC	fast protein liquid chromatography
Gd	gadolinium
GFP	green fluorescent protein

GMA	glycidyl methacrylate
GOx	glucose oxidase
GPC	gel-permeation chromatography
Hb	hemoglobin
HEA	hydroxyetheyl acrylate
HEMA	2-hydroxyethyl methacrylate
HRP	horseradish peroxidase
HSF	horse spleen apoferritin
ICAR	initiators for continuous activator regeneration
IFN-α	interferon-α
LC-MS/MS	tandem liquid chromatography mass spectrometry
LCST	lower critical solution temperature
Lys	lysine
Lyz	lysozyme
MALDI-ToF	matrix assisted laser desorption/ionization time-of-flight
Mb	myoglobin
MES	2-(*N*-morpholino)ethanesulfonic acid
MOEGMA	methoxy oligo(ethylene glycol) methacrylate
MPC	methacryloyloxyethyl phosphorylcholine
MPEGMA	monomethoxy poly(ethylene glycol)-methacrylate
MSEA	2-(methylsulfinyl)ethyl acrylate
NaBr	sodium bromide
***n*BA**	*n*-butyl acrylate
***n*BMA**	*n*-butyl methacrylate
NHS	*N*-hydroxysuccinimide
NIPPAm	*N*-isopropyl acrylamide
NMP	nitroxide-mediated polymerization
OEGA	oligo(ethylene glycol) acrylate
OEGMA	oligo(ethylene glycol) methacrylate

PBS	phosphate buffered saline
PDS	pyridyl disulfide
PEG	poly(ethylene glycol)
PEGA	poly(ethylene glycol) acrylate
PEGMA	poly(ethylene glycol) methacrylate
PET-RAFT	photo-induced electron transfer-RAFT
PFP	pentafluorophenyl
Phe	phenylalanine
Photo-ATRP	photo-mediated ATRP
PLP	pyridoxal-5-phosphate
PMDETA	*N,N,N′,N″,N‴*-pentamethyldiethylenetriamine
PNA	peptide nucleic acid
PPase	pyrophosphatase
RAFT	reversible addition-fragmentation chain transfer polymerization
RCM	ring-closing metathesis
RDRP	reversible deactivation radical polymerization
rh-GH	recombinant human growth factor
ROMP	ring opening metathesis polymerization
ROMPISA	ring-opening metathesis polymerization-induced self-assembly
SAv	streptavidin
sCT	salmon calcitonin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SET-LRP	single electron transfer living radical polymerization
siRNA	small interfering ribonucleic acid
SPAAC	strain-promoted 1,3-dipolar cycloaddition
SPE	screen printed electrode
SP	sodium pyruvate

SPPS	solid-phase peptide synthesis
SrtA	sortase A
ssDNA	single-stranded DNA
***t*BA**	*tert*-butyl acrylate
TBAF	tetra-n-butylammonium fluoride
TCEP	tris(2-carboxyethyl)phosphine
tDNA	transfer DNA
TEA-Br	tetraethylammonium bromide
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TIPS	triisopropylsilane
TL	*Thermomyces languginosa*
TPMA	tris(2-pyridylmethyl amine
TPO-Na	(2,4,6-trimethylbenzoyl)phenyl phosphonic acid sodium
VA-044	2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
VI	*N*-vinylimidazole
VLP	virus-like nanoparticle

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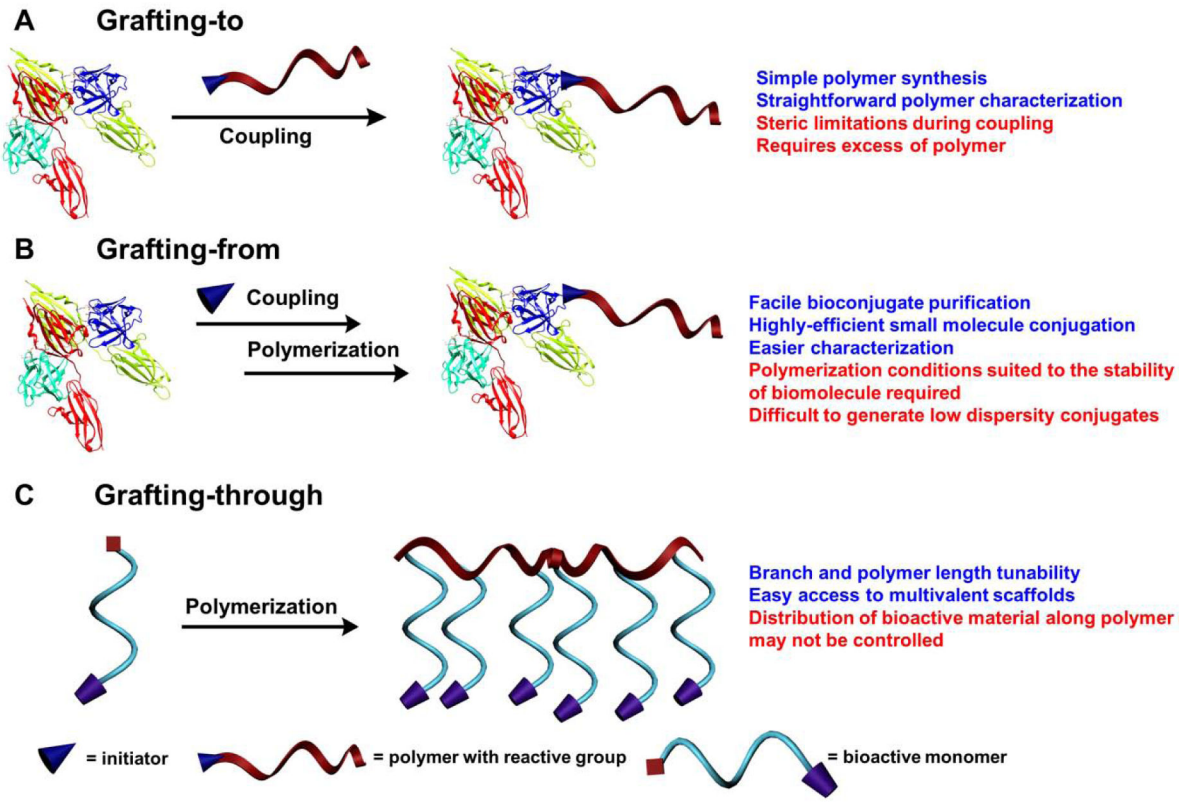

Figure 1.

Schematic representations depicting different methods of preparing biomolecule-polymer conjugates along with a list of key advantages or disadvantages.

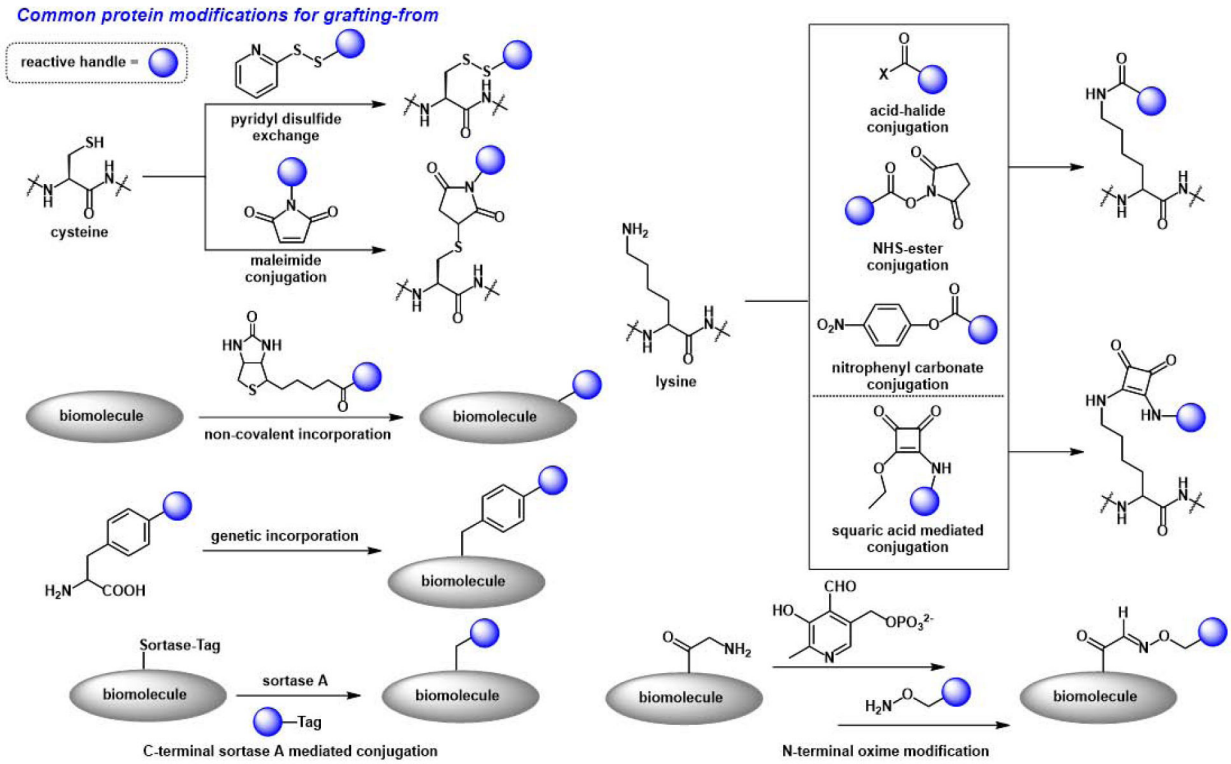

Figure 2.
Commonly used protein modification techniques for grafting-from.

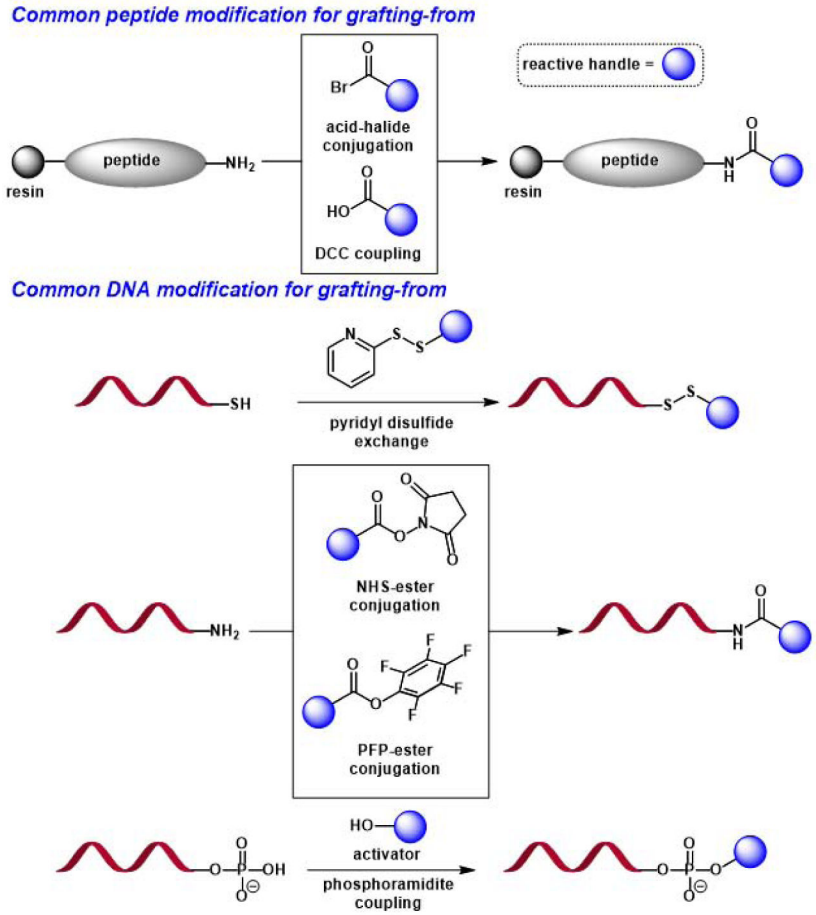

Figure 3.
Commonly used peptide and nucleic acid modification techniques for grafting-from.

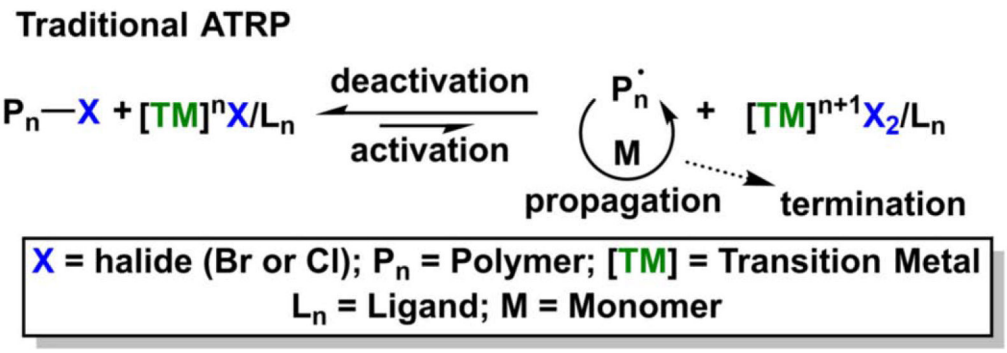

Figure 4.
Mechanism of the traditional ATRP process.

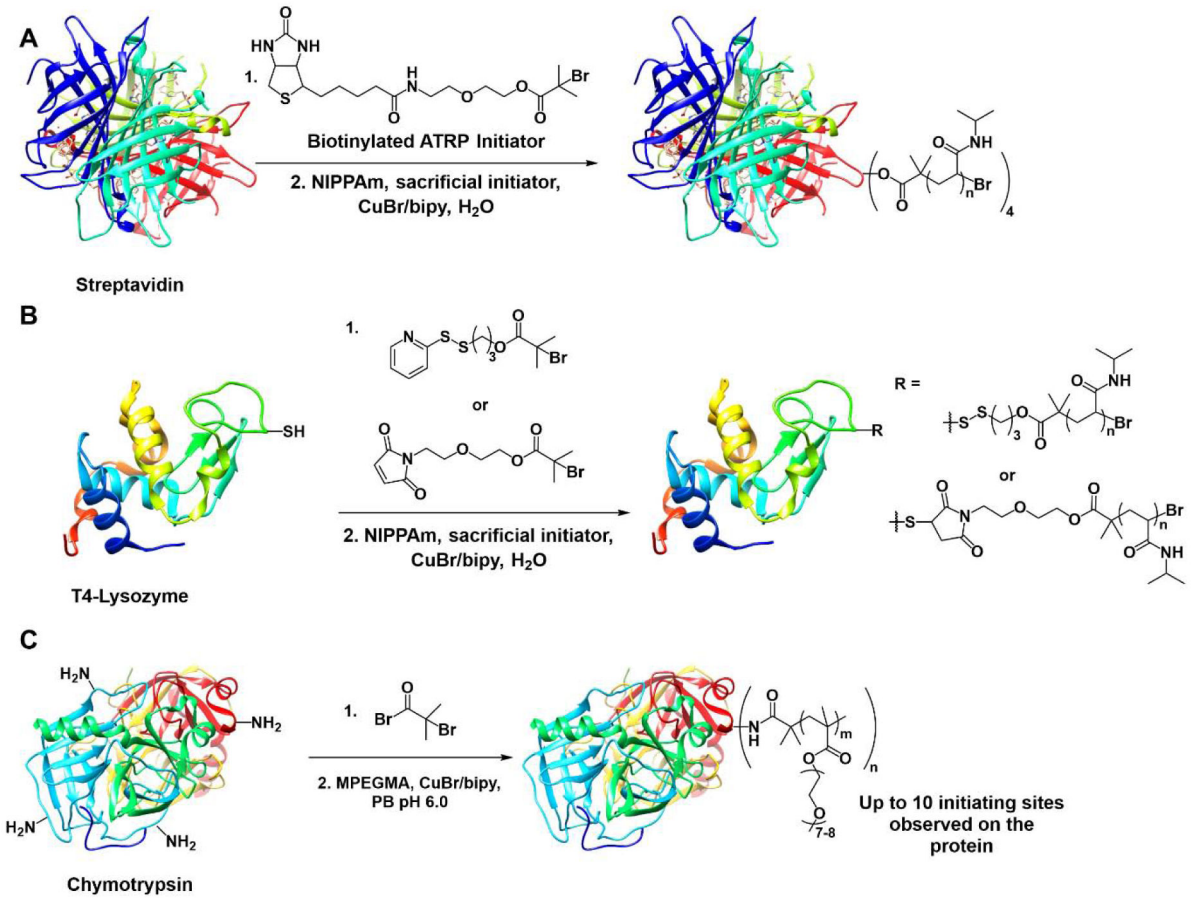

Figure 5.

Examples of grafting-from polymerization using ATRP. (A) Maynard and co-workers use of SAv-biotin interaction in order to develop a SAv-macroinitiator to be employed in the controlled polymerization of NIPAAm.[49] The Maynard group Cys targeted approaches towards polymer-protein conjugate generation via grafting-from.[50] (C) The Matyjaszewski and Russell groups work in developing a chymotrypsin macroinitiator through Lys conjugation.[52]

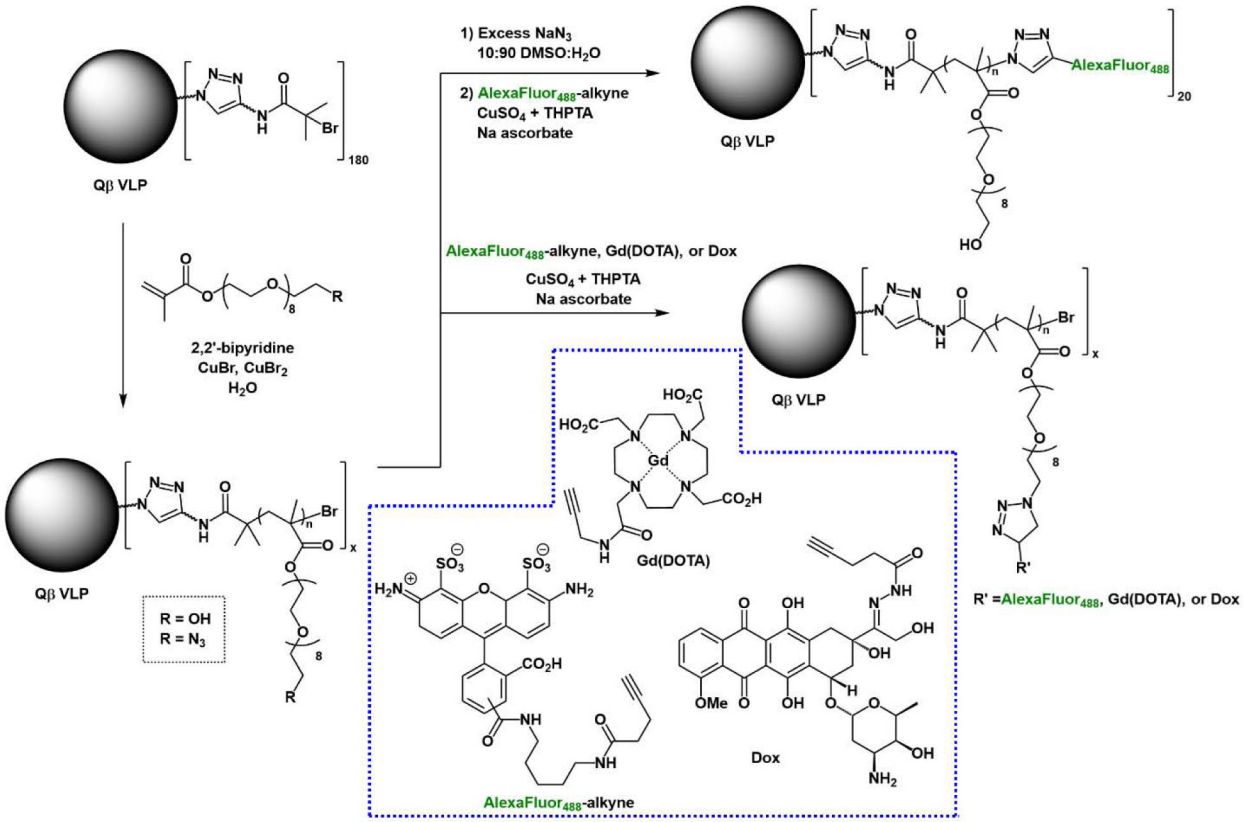

Figure 6.

Grafting-from example from the Finn group in which they modified QB VLP-macroinitiators to synthesize polymers which could undergo post-polymerization modification with an array of bioactive substrates.[62]

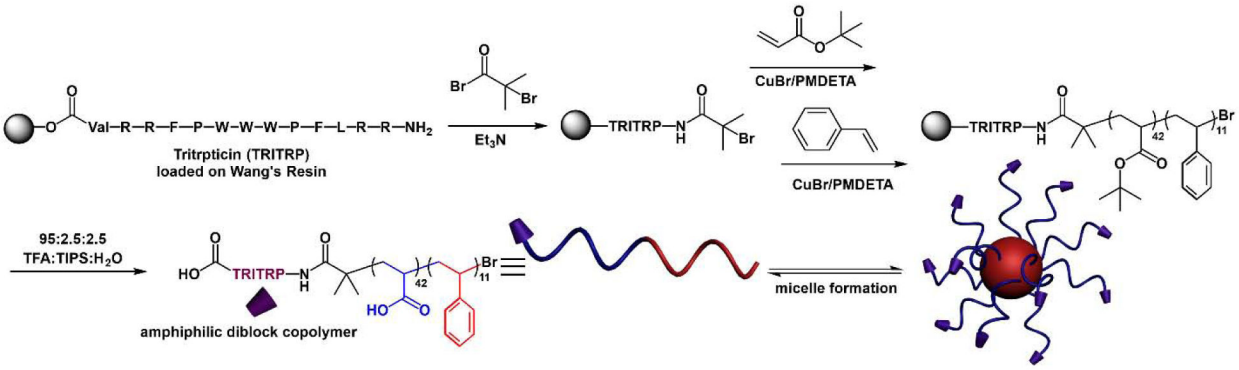

Figure 7.

Development of peptide containing amphiphilic block copolymers which assemble to form micelles exhibiting a high degree of anti-microbial activity.[42]

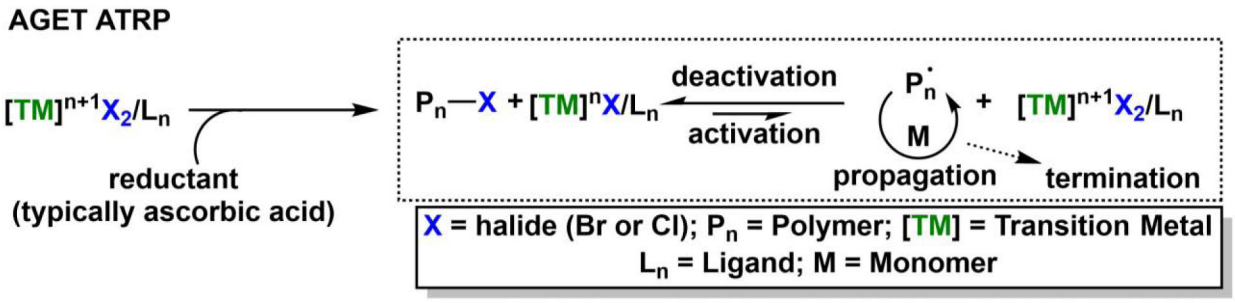

Figure 8.
Mechanism of AGET ATRP.

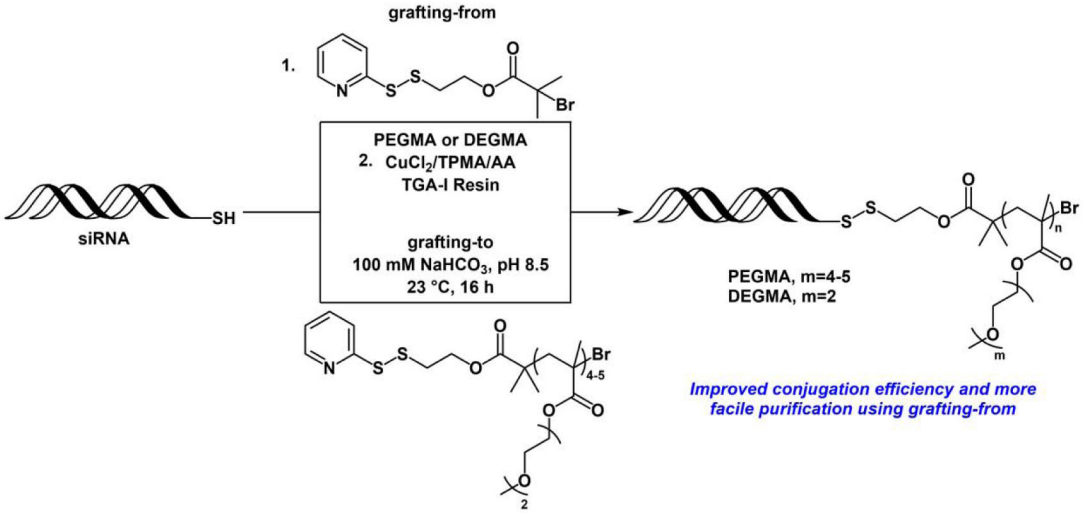

Figure 9.

Development of siRNA-polymer conjugates via grafting-to or grafting-from. Use of the grafting-from technique exhibited improved conjugation efficiency and easier purification than the grafting-to method.[74]

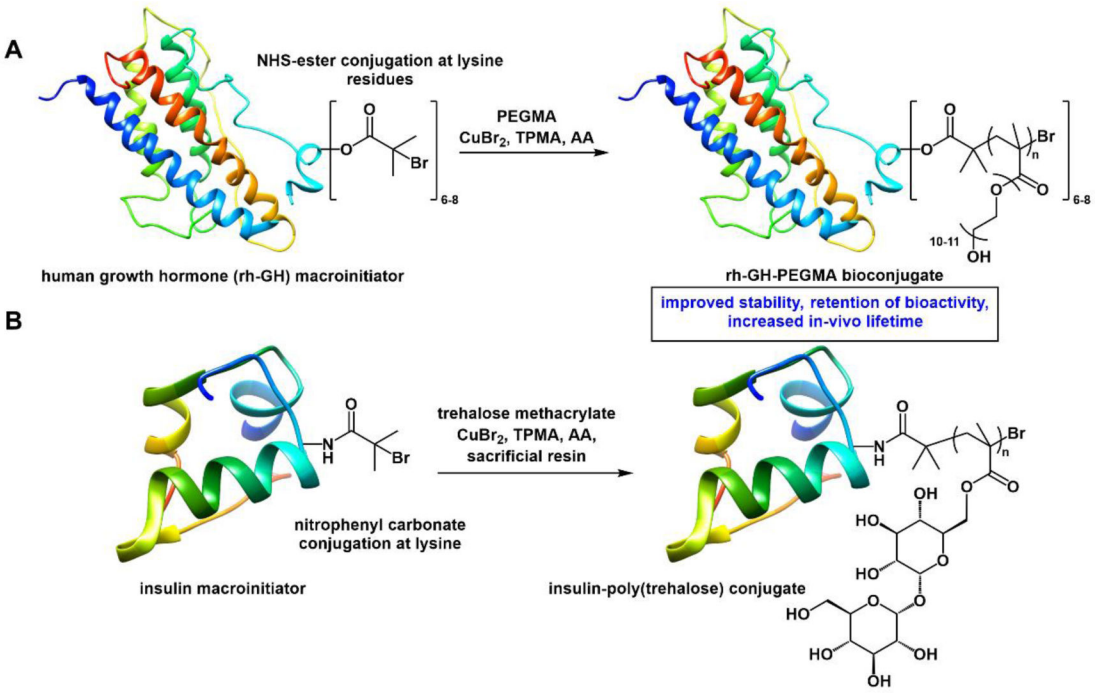

Figure 10.

(A) Use of grafting-from to develop rh-GH-PEGMA. (B) Generation of insulin-poly(trehalose) conjugates. Both conjugates retained bioactivity and exhibited an increased *in-vivo* lifetime in comparison to the native proteins.[78, 79]

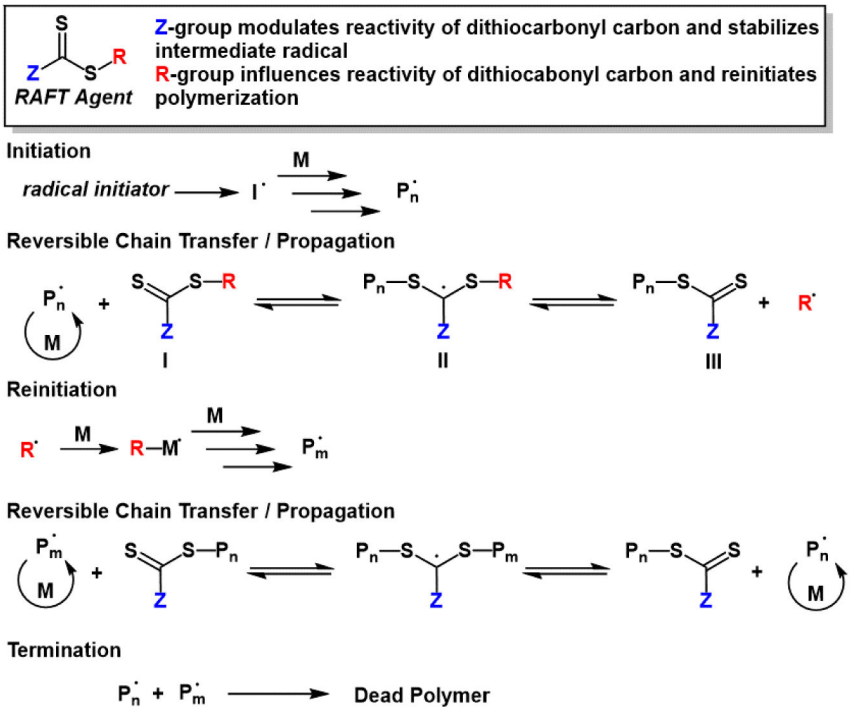

Figure 11.
RAFT polymerization mechanism.

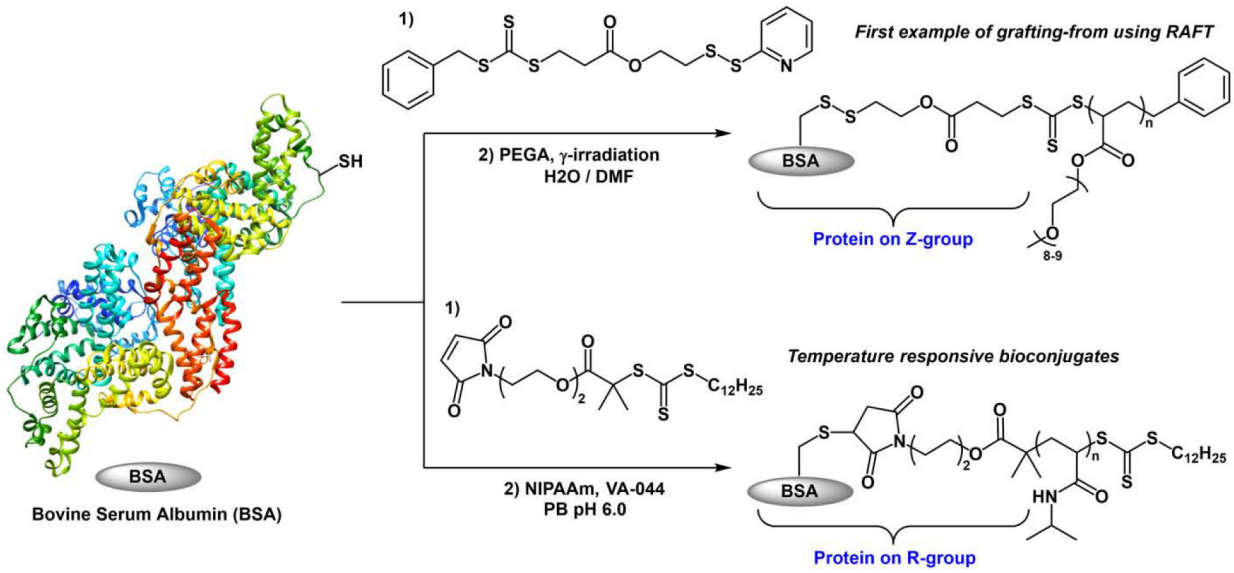

Figure 12.

(Top) The Davis and Bulmus groups work developing protein Z-group modified macroRAFT agents and polymerization from BSA.[117] (Bottom) Sumerlin group work on developing R-group modified macroRAFT agents to develop thermoresponsive protein-polymer conjugates.[118]

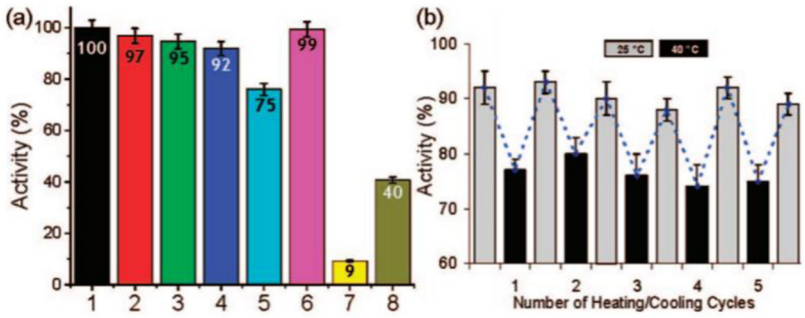

Figure 13.

"(a) Activity of (1) BSA, (2) BSA-macroCTA, (3) BSA-poly(NIPAAm) (free BSA present) with conjugated polymer of 234,000g/mol, (4)BSA-poly(NIPAAm) thermal precipitate, (5) BSA-poly(NIPAAm) thermal precipitate at 40 °C assay temperature (with respect to BSA at 40 °C), (6) BSA+poly(NIPAAm) physical mixture, (7) poly(NIPAAm), (8) BSA after incubation at 75 °C for 3 h. All assays were conducted with identical [BSA]. (b) Activity of BSA-poly(NIPAAm) thermal precipitate during thermal cycling between 25 and 40 °C." [118], Copyright 2008. Reproduced with permission from the American Chemical Society.

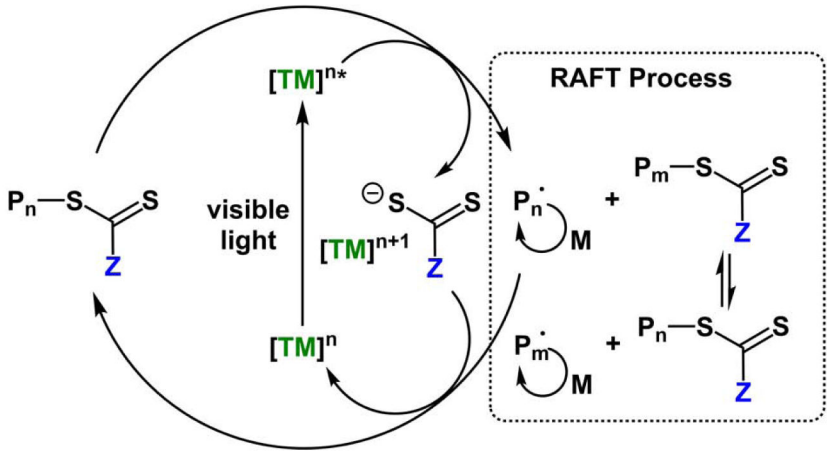

Figure 14.
Proposed mechanism of a prototypical PET-RAFT polymerization using a transition-metal based chromophore.

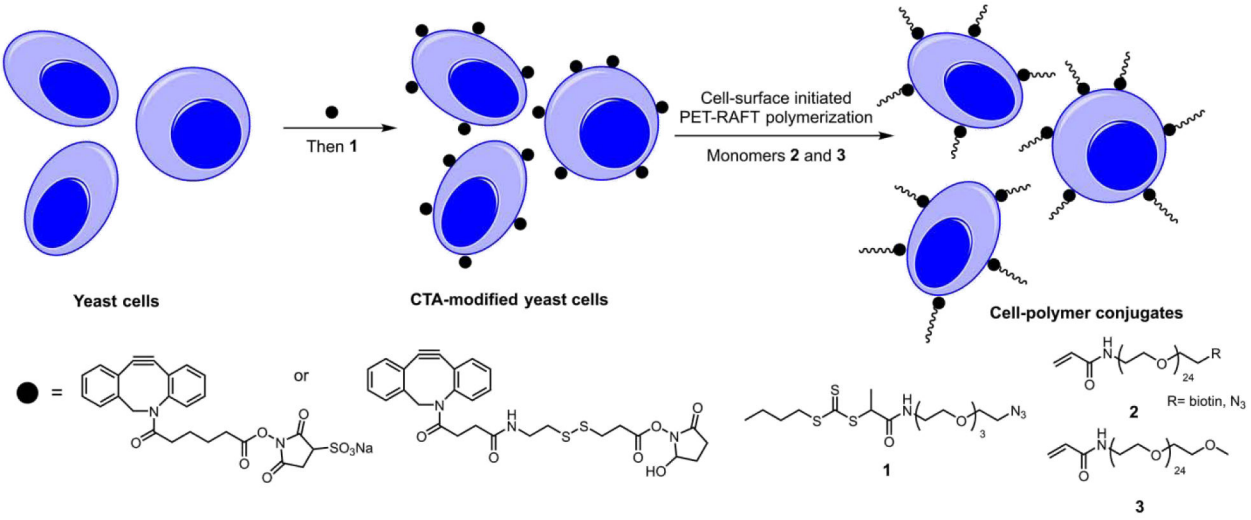

Figure 15.
Modification of yeast cells with RAFT agents and polymerization from yeast cell surfaces.

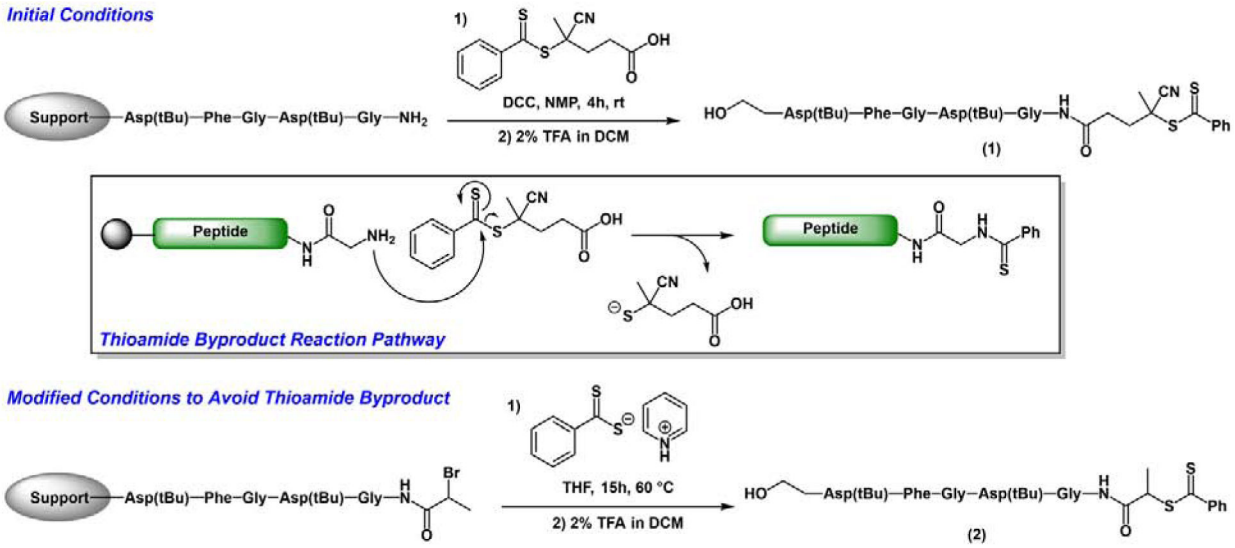

Figure 16.
Development of oligopeptide-macroRAFT agents.

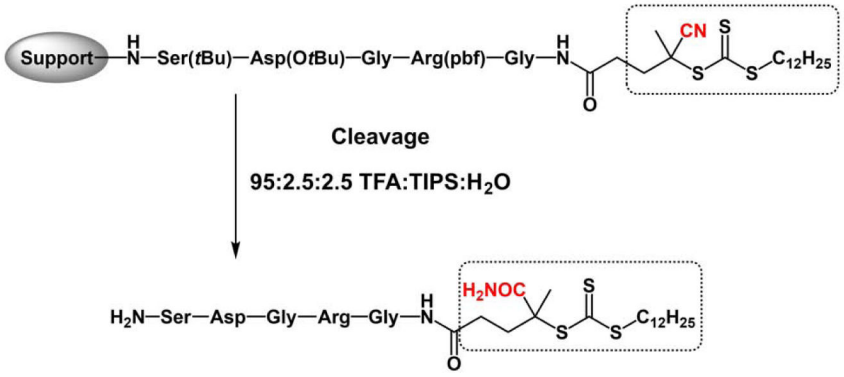

Figure 17.

Exhibited hydrolysis of nitrile substituted RAFT derivatives after treatment with standard peptide cleavage cocktails.[133]

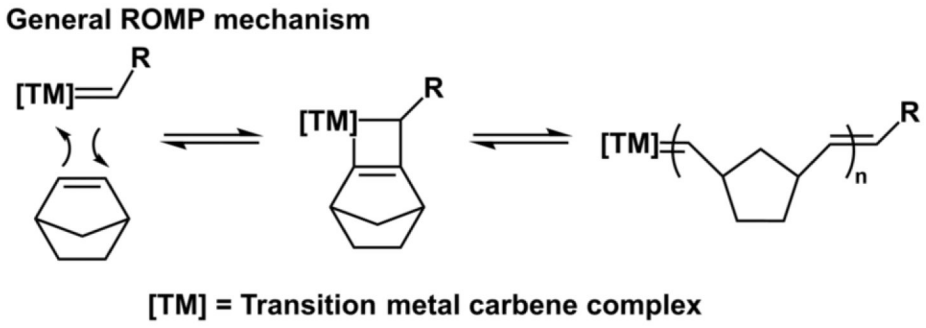

Figure 18.
General mechanism of ROMP polymerization.

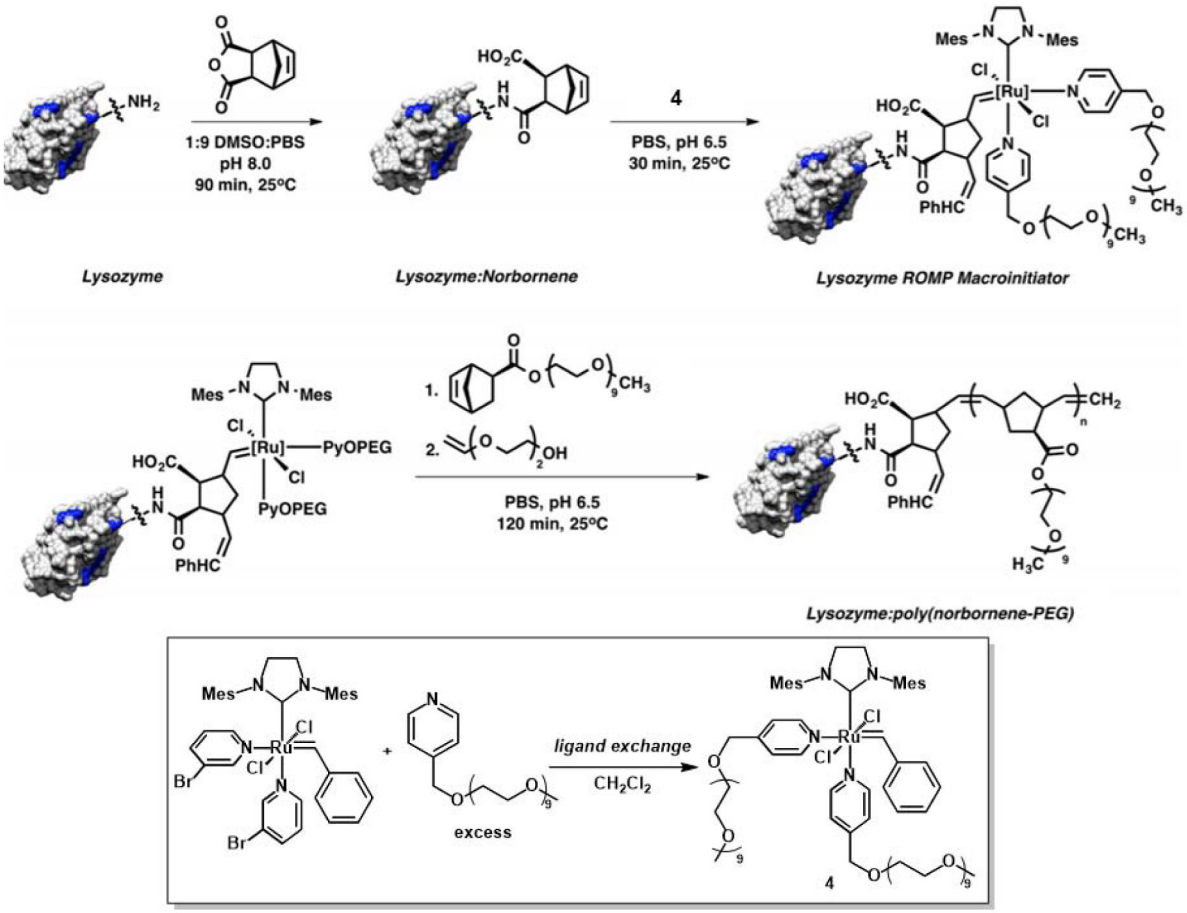

Figure 19.

ROMP from Lyz to develop Lyz-polymer conjugates using a water-soluble Ru-based carbine. [151], Copyright 2015. Adopted with permission from the American Chemical Society.

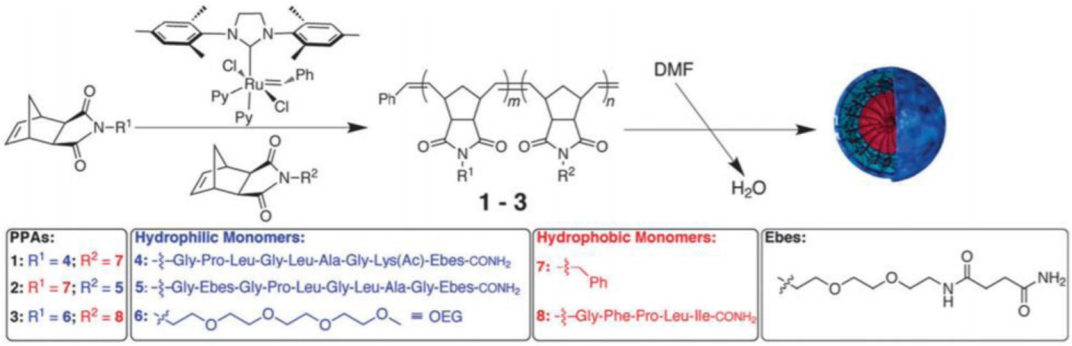

Figure 20.

Development of copolymers containing pendent oligopeptides via ROMP for the generation of peptide-containing nanoparticles. [171], Copyright 2013. Reproduced with permission from the Royal Society of Chemistry.