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# Synthesis and applications of protein/peptide-polymer conjugates

Paul Wilson*

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Dr Paul Wilson, University of Warwick, Department of Chemistry, Coventry, Library Rd, CV4 7AL, UK.
E-mail: P.Wilson.1@warwick.ac.uk

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Recent advances in synthetic methodologies have brought us closer than ever to the precision conferred by nature. For example, the control possible in reversible deactivation radical polymerization enables us to design and synthesize macromolecules with unprecedented control over not only the polymer chain ends, but also the side chain functionality. Furthermore, this functionality can be exploited to afford chemical modification of peptides and proteins, with ever-improving site-specificity, yielding a range of well-defined protein/peptide-hybrid materials. Such materials benefit from the amalgamation of the properties of proteins/peptides with those of the synthetic (macro)molecules in question. Here, the latest developments in the synthesis of functional polymers and their use for preparation of well-defined protein/peptide-polymer conjugates will be discussed, with particular attention focused on modulating the stability, efficacy and/or administration of therapeutic peptides.

## 1. Introduction

Nature exploits complex intracellular machinery to control macromolecular precision and functionality. From mRNA transcription in the nucleus to translation in the ribosome, the primary sequence of amino acids is exquisitely and reproducibly controlled throughout the lifetime of the cell. Enzymatically-mediated, post-translational modifications can also take place which can expand the structural and functional diversity of proteins through precise modification of amino acid residues.[1]

A great deal of work has been conducted towards replicating the precision of nature in the laboratory. The development of synthetic methods that enable the production of macromolecules of equal precision and chain-length homology has become the nexus of some synthetic polymer scientists.[2] In step-growth polymerization efforts have been made to prepare monomers capable of undergoing high yielding, chemo- and regiospecific coupling reactions in the presence of orthogonal and/or protected functionality that can support further chain growth.[3, 4] In this way, discrete oligomeric molecules have been realized, but the multistep, stepwise nature of the syntheses is disadvantageous for the preparation of higher molecular weight species and higher order structures. To address this a number of ionic and radical chain-growth polymerizations have been developed with particular focus on optimizing the level of 'livingness' retained throughout the polymerization reaction. Reversible-deactivation radical polymerization (RDRP) is generally not as well controlled as ionic, particularly anionic, polymerizations, however it is much more robust and 'user-friendly' from a synthetic point view. Perfect sequence control requires sequential, iterative additions of single monomer units to every growing chain. To this end, single monomer addition has been demonstrated[5, 6] but radical reactivity precludes sequential single monomer addition, and therefore alternative approaches including kinetic control,[7, 8] solution[9, 10] and segregated templating[11] have also been investigated.

In the absence of nature's innate precision, specific chemical modification of proteins/peptides with (macro)molecules, referred to as bioconjugation,[12] can be challenging due the array of diverse functionality present in protein/peptide structures. Generally, modification is either achieved by targeting the intrinsic functionality presented by natural amino acid residues, or by extrinsic manipulation of the functionality present via (bio)chemical intervention, typically through mutation during protein expression or peptide synthesis, to introduced unnatural amino acid residues with specific and orthogonal functionality.[13] The latter confers enhanced specificity through the use of conjugating groups that contain the complimentary functionality, but can be time consuming, low yielding and compromise protein/peptide activity irrecoverably. Consequently, enhancing the site-specificity of chemical reactions that target natural amino acids is a much more attractive approach.

This talent article will offer a brief insight into the latest radical polymerization protocols developed for the synthesis of polymers with optimal control over the molecular weight distribution and functionality under biologically relevant conditions. Greater attention will be paid to the use of these polymers, and other (macro)molecules, for the modification of proteins and peptides. From well-established protocols, to novel methods that enhance site specificity, the influence on structure, function and activity will be considered with specific biological, medicinal or pharmaceutical applications in mind.

## 2. 'Precision' polymerisation using aqueous RDRP

The activation-deactivation equilibrium associated with RDRP protocols, such as Cu-mediated radical polymerizations; atom-transfer radical polymerization (ATRP),[14] single electron transfer living radical polymerization (SET-LRP);[15] reversible addition fragmentation chain-transfer polymerization (RAFT)[16] and nitroxide-mediated polymerization (NMP),[17] lends itself to manipulation to afford unprecedented control over the activation-deactivation equilibrium and therefore the radical concentration. Consequently, termination events can be

minimized resulting in exceptional control over the molecular weight, molecular weight distribution (dispersity, $Đ$) and chain-end functionality up to quantitative conversions. Re-initiation with sequential addition of alternative monomers can lead to the formation of multi-block copolymers with complex compositions in which the side chain functionality is not perfectly, but discretely distributed throughout each polymer chain.[18-21] The most recent advances in Cu-mediated RDRP have centred on retaining this control over polymerisation in aqueous and biologically relevent media and using external stimuli to control the activation-deactivation equilibrium (Figure 1).[22, 23]

Insert Figure 1 here

Historically, performing ATRP in aqueous solution has proved challenging owing to an increase in the activation-deactivation equilibrium constant ($K_{ATRP}$), enhanced rates of propagation ($k_p$) and the susceptibility of the $Cu^IX$/L complex (X = Br, Cl; L = amine ligand) to undergo $Cu^I$-X bond dissociation. Moreover, $Cu^IX$/L complexes undergo rapid and complete disproportionation in pure aqueous solutions forming elemental $Cu^0$ and $Cu^{II}X_2$/L.

In 2013,[24] this was exploited for the development of aqueous SET-LRP which employs $Cu^0$ as the activating species and $Cu^{II}X_2$/L as the deactivating species. Using *N*-isopropylacrylamide (NIPAM) as a model monomer reaction conditions were optimized with respect to the [$Cu^IX$] : [L] ratio as well as the reaction temperature, concentration and time to access a broad range of molecular weights (Figure 2).

Insert Figure 2 here

Subsequently, a variety of hydrophilic (meth)acrylate and acrylamide monomers have been shown to be compatible with the polymerization system from acrylamide[25] to functional monomers including; acryloylmorpholine,[26] oligo(ethylene glycol) methyl ether (meth)acrylate (OEG[M]A),[24] unprotected glycomonomers,[27] 2-acrylamido-2-methylpropane sulfonic acid sodium salt (NaAMPS) and zwitterionic acryloyl

phosphatidylcholine (APC).[28] The polymerization is also robust to deviations in the aqueous solvent composition, with controlled polymerization possible in buffer solutions,[24] complex alcoholic solutions,[29] carbonated water[30] and most significantly, blood serum.[31]

One potential limitation of the aqueous system is hydrolysis of the $\omega$-Br chain end of the target poly(acrylates/acrylamides), which was identified as a deleterious side reaction prior to, and during, the initial investigation.[32] To circumvent this, kinetic and mechanistic investigations were conducted to establish optimal reaction conditions to enable the formation of complex (multi)block copolymers via sequential monomer addition in pure aqueous media.[33, 34] Structural diversity and complexity can also be introduced through changes in the target polymer architecture. As well as simple linear polymers, telechelic homopolymers and (multi) block polymers have also been prepared.[35-37] Furthermore, Qiao and Becer have independently adopted the rapid, robust and benign conditions of the aqueous SET-LRP system for the synthesis of star polymers, which are opportunistic for use as unimolecular nanoparticles in biomedical applications, via arm-first and core-first approaches respectively.[38, 39] The work of Becer *et al* has expanded the complexity further through the synthesis of a 3-arm star pentablock copolymer with excellent control over the molecular weight distribution ($Đ < 1.14$).

Double hydrophilic block copolymers composed of short glycomonomer[27] or phosphatidylcholine[28] blocks, chain extended *in situ* with a longer thermoresponsive monomer blocks have also been prepared by aqueous SET-LRP. Upon heating above the lower critical solution temperature (LCST), the diblock copolymers self-assemble into polymer nanoparticles with good polydispersity indices, within the micellar size range (~ 30 nm, PDI < 0.12) (Figure 3).

Insert figure 3 here

## 3. Synthetic approaches to covalently-linked protein/peptide-polymer conjugates

Bio(macro)molecules such as proteins, peptides, nucleic acids, *etc*. are inherently bioactive, serving as therapeutics, specific ligands for cellular receptors and sources of genetic information. However, they often exhibit poor bioavailablity, can be highly immunogenic and rapidly cleared by the reticuloendethial system (RES) resulting in short plasma half-lives in their native forms. A popular approach to address this is the attachment of synthetic polymers to form bio(macro)molecule-polymer conjugates that synergistically combine the properties of the individual components to suit a particular application.[40] This was originally achieved using poly(ethylene glycol) *i.e.* PEGylation,[41] which upon conjugation to therapeutic proteins has been shown to increase bioavailablity and plasma half-life, whilst reducing immunogenicity and proteolysis. A number of peglyated protein therapeutics have been granted FDA approval and made it to the market, with several others demonstrating clinic potential.[42]

In light of the advances made by the latest ring opening polymerization ([C]ROP, ROMP) and RDRP (ATRP, SET-LRP, RAFT, NMP) protocols, such polymers are ideal candidates for post-polymerization modification and protein/peptide modification via 'grafting-to,' 'grafting-from,' and 'grafting-through' techniques which can be acheieved via covalent or non-covalent intereactions. ROP of *N*-carboxyanhydrides has been developed whereby functionality for site-specific protein ligation can be topologically installed *in situ*.[43] Cationic ROP of oxazolines can be terminated using functional nucleophilies, and the resulting polymers have been 'grafted-to' proteins.[44] Furthermore, side chain functionality, incorporated either in the oxazoline monomer or through post-polymerization has been exploited for the 'grafting-through' modification of polymer chains[45] and particles.[46] The latest generation of the Grubb's catalyst has also led to the use of ROMP in the preparation of protein-polymer conjugates via both 'grafting-to' and 'grafting-from' strategies.[47, 48] The resulting polymers

composed of norbornene-based monomers exhibit markedly different properties compared to the conventional (meth)acrylate derived polymers traditionally employed.

The modification of proteins/peptides with polymers prepared by RDRP is perhaps the most well studied and understood approach. Futhermore the precision conferred by RDRP enables the composition of synthetic (co)polymers to be manipulated to ellicit additional properties to bestow of target biomolecules such as stimulated responsivity,[49] usually via phase transition to fluctuations in temperature, pH and redox, all of which are associated with the microenvironment of stressed, diseased or malignant tissues and cells.

### 3.1 Covalent modification

The (ir)reversible, covalent attachment of polymers to protein/peptides is the most common approach to modification. Chain-end and side-chain functionality can be specifically incorporated to compliment the functionality presented by amino acid residues leading to conjugation via 'grafting-to' and 'grafting-through' methods respectively. Alternatively, proteins/peptides can be modified to include functional groups capable of (re-)initiating radical polymerization such as $\alpha$-halo esters for Cu-mediated polymerization, or thiocarbonylthio groups for RAFT in a 'grafting-from' approach.

#### 3.1.1. 'Grafting-to'

The grafting of polymer chains to a protein/peptide is a stepwise process wherein the polymer is initially synthesized with high $\alpha$- and $\omega$-chain end fidelity, before conjugation through one, or both, chains ends to target proteins/peptides through reaction at functional amino acid residues. Nucleophilic amines present at the *N*-terminus and in the side chain of lysine residues are attractive targets due their relatively high natural abundance and exposure at protein surfaces.[50, 51] They are reactive towards functional groups such as; carboxylic acids,[52] through carbodiimide coupling; activated esters (*e.g.* NHS-ester,[53] pentafluorophenol ester,[54] *etc.*) or thiazoline-2-thione groups,[55] through nucleophilic substitution; isocyanates,[56] by

nucleophilic addition; and aldehydes,[57] via reversible imine formation which can be subsequently reduced to yield secondary amines. More recently, polymers synthesized using squaric acid ester amide functional initiators[58] or CTAs[59] have been employed for amine-targeted bioconjugation, proving to be less prone to hydrolysis than activated ester analogues, leading to greater grafting efficiencies. NMP has been applied less frequently compared to RAFT, ATRP and SET-LRP. However functional SG1 alkoxyamines have been developed to install suitable chain-end functionality and enable conjugation to both proteins[60] and drugs.[61]

The relatively high natural abundance of amine-functional groups can also be disadvantageous, leading to heterogeneous products due to the addition of multiple polymer chains to target proteins/peptides, which itself is implicated in reduction or complete loss of bioactivity. Consequently, less abundant residues such as tyrosine[62] and tryptophan[63] have been targeted for modification through their electron-rich aromatic side chains. However these residues are often located in poorly accessible hydrophobic domains of larger proteins and require the use of elaborate functional linkers.

The pendent thiol group of cysteine residues is a much more attractive target for site-specific modification of proteins/peptides owing to its relatively low natural abundance and soft nucleophilic character. Thiol groups readily undergo disulfide bond metathesis with pyridyl disulfide functional polymers to form reductively labile protein/peptide conjugates.[64] Alternatively, nucleophilic substitution ($\alpha$-halocarbonyls) or addition ($\alpha,\beta$-unsaturated esters, maleimides, vinyl sulfones)[65] reactions proceed with good selectivity to furnish less labile thio-ether linked conjugates. Free thiol groups are prone to oxidation, and often exist as inter- or intramolecular disulfides, the stability of which is naturally controlled by the local redox environment. Chemically, reduction can be afforded using reducing agents such as phosphines, hydrazine and zinc, to yield free thiols, or thiolate anions that readily react with the reagents listed above. However, the existence of disulfide bonds between proximal cysteine residues

contributes to the tertiary and/or quaternary structure of proteins. Therefore, depending on their location, they are often crucial for maintaining structure or activity, both of which can be compromised upon disulfide bond reduction. As such reagents capable of re-bridging accessible disulfides are opportunistic for enhancing site-specificity with minimal disruption to the native protein structure and activity (Figure 4).

Insert figure 4 here

Weil *et al*.[66] have comprehensively reviewed and compared the known methods for disulfide bond modification as a route to forming protein, peptide and antibody-hybrid materials. The pioneering work of Brocchini *et al.,*[67] who employed *bis*-sulfone functional PEG to modify immunotherapeutic protein interferon, was followed by contributions from the groups and Caddick and Baker, who introduced mono-substituted (MSM)[68] and di-substituted maleimide (DSM)[69] derivatives as irreversible and reversible linkers respectively. More recently, Caddick *et al.* have also employed dibromopyridazinediones[70] which have two additional sites for functionalization, conferring homo- or hetero-bifunctional modification, and alkyne reagents[71] have been employed for radical thiol-yne chemistry at reduced disulfides. Haddleton, Davis and Wilson, have combined the efficiency of the DSM chemistry with the precision of RDRP and also introduced organic arsenicals as benign alternatives to DSM's, exhibiting comparable conjugation efficiency, reversibility and enhanced specificity.

Having demonstrated the potential of DSMs for protein modification, Caddick and Baker were the first to exploit their reactivity for PEGylation.[69] DSM-PEG was prepared and conjugated to somatostatin within 30 minutes and a cell based assay demonstrated that the conjugates retained the agonist activity of native somatostatin against a somatostatin receptor (SSTR2). This was then expanded to afford simultaneous PEGylation and fluorescent labelling of peptides using salmon calcitonin (sCT)[72] as a model peptide with the DSM moiety acting as both the conjugation linker and the fluorophore in the process referred to as conjugation-

induced fluorescence (Figure 5). The fluorescent properties of the DSM-linked conjugates is an attractive alternative to conventional protein/polymer-labelling which often requires incorporation of bulky, planar fluorophores that can alter solution properties.

Insert figure 5 here

Polymeric DSMs were initially prepared by ATRP, first in a sequential reaction whereby dibromomaleimide (DBM) was added to the $\alpha$-terminus of POEGMA in a post-polymerisation modification.[73] This was necessary due to the DBM functional group severely retarding the rate of ATRP, which was circumvented by employing a dithiophenolmaleimide (DTM) initiator capable of directly synthesizing DTM-POEGMA for subsequent conjugation.[74] Conjugation was shown to proceed rapidly, within 15 minutes, and stoichiometrically which alleviates the need for large excesses of polymer and extensive purification following conjugation. RAFT,[75] ring opening polymerization (ROP)[72] and aqueous SET-LRP[76] have also exhibited compatibility for the DSM functional group during the synthesis of DSM-functional polymers.

Arsenic (As) exists as a dichotomous enigma between toxicity and therapy in (bio)chemistry and medicine. It can exist in a number of oxidation states with $As^{III}$ and $As^{V}$ considered as 'biologically relevant.' The reactivity of arsenic towards thiols in each oxidation state is discrete and distinctive. In the presence of thiols, $As^{V}$ readily undergoes two electron reduction to $As^{III}$ which dominates the therapeutic and toxic potential of arsenic due to its high affinity for thiols, readily forming As-S covalent bonds, which is not possible for $As^{V}$. Mono-thiol adducts are prone to hydrolysis and subsequent re-oxidation (to $As^{V}$), however the affinity and stability is markedly enhanced for chelating vicinal or neighbours-through-space (proximal) dithiols, such as those presented by naturally occurring disulfide bonds.[77] This was exploited using a reduced derivative of *p*-arsanilic acid, which was initially shown to rapidly and stoichiometrically re-bridge the disulfide of sCT ($Cys^{1}$-$Cys^{7}$).[78] To explore the specificity for re-bridging disulfide bonds, native and reduced bovine serum albumin (BSA/rBSA) were employed as model

proteins containing a single thiol group in the absence and presence of an accessible, reduced disulfide respectively. Using Ellman's assay, $As^{III}$ was qualitatively shown to react with the proximal dithiols derived from the reduced disulfide but did not react with the remaining mono-thiol group ($Cys^{34}$) (Figure 6). Alternative thiol and disulfide reactive reagents containing maleimide and DSM functional groups were shown to react indiscriminately with all available thiol groups.

Insert figure 6 here

Polymeric arsenicals were synthesized by $Cu^0$-mediated RDRP in aqueous solution (aqueous SET-LRP) using a novel $As^V$-functional initiator to ensure the presence of a single $As^V$-group at each $\alpha$-chain end. $As^V$-poly(oligo[ethylene glycol methyl ether acrylate]) ($As^V$-POEGA) was shown to be non-toxic to a variety of cell lines, relative to non-functional controls, and was readily converted to $As^{III}$-POEGA, upon reduction with an excess of the glutathione. Conjugation to sCT (Figure 6) was achieved quantitatively (w.r.t. peptide) and confirmed by RP-HPLC, verified by quantitative release of native and reduced sCT from the conjugate upon addition of stronger chelating dithiols such as ethanedithiol or reduced lipoic acid.

### 3.1.2. 'Grafting-through'

Where the use of functional initiators and CTAs installs appropriate functionality at the polymer chain ends, the incorporation of functional monomers into the polymer composition can facilitate the attachment of multiple bioactive molecules in a 'grafting-through' process. This is an attractive method for increasing the grafting density and concentration of small bioactive molecules such as mono- and oligosaccharides,[79] peptides[80] and nucleic acids,[81] and has also been applied to larger antibody fragment proteins.[82] The level of control associated with RDRP allows mono or multifunctional scaffolds to be prepared that can be modified post-polymerization to afford conjugation. A plethora of monomers have been synthesized containing desirable functionality including; alkyne, epoxide, aldehyde/ketone, azlactone

hydrazyl, oxime or activated ester side chains, all capable of facile 'click'-like modifications.[83-88]

More recently, an acrylate monomer containing a thiol reactive DSM pendant group was prepared and copolymerized (0 – 10 mol%) with thermoresponsive monomer triethylene glycol methyl ether acrylate (TEGA) by $Cu^0$-wire mediated polymerization in DMSO ($M_n \approx 10000$ g.mol$^{-1}$, $Đ < 1.25$).[89] Having previously exploited the reactivity of DSMs at polymer chain ends for disulfide bond conjugation, monothiol reagents were employed in this case to quantitatively and reversibly modify the polymers. Tripeptide GSH and thio-glucose were employed as peptide and monosaccharide models. Successful conjugation was confirmed by DOSY NMR, SEC, changes in the cloud point temperature and, in the case of thio-glucose, an unexpected and as yet unexplained change in the fluorescence properties of the polymer, whereby fluorescence is quenched upon conjugation of the sugar moiety.

### 3.1.3. 'Grafting-from'

Historically, large stoichiometric excesses of functional polymers were required to achieve acceptable conjugation yields in the 'grafting-to' approach, presumably due to the steric hindrance associated with bringing together large synthetic and biological macromolecules. This in turn, imposes the need for an exhaustive purification step in any conjugation protocol in order to ultimately obtain pure protein/peptide-polymer conjugate material. The efficiency of the latest 'grafting-to' technologies, and systematic studies on the effect of linker length on conjugation yields go some way to addressing some of these limitations. As an alternative approach to protein/peptide conjugation, 'grafting-from' methods require the modification of the protein/peptide with a group capable of mediating radical polymerization *e.g.* initiators,[90] CTAs,[91] prior to polymerization, which can then proceed directly from the protein/peptide. This can markedly improve product homogeneity and alleviate the need for extensive purification as large conjugates are routinely separable from any remaining smaller molecules

such as unreacted monomers. On the other hand, the use of protein/peptide macroinitiators engenders the need for mild reaction conditions, particularly with respect to solvent and temperature which can compromise bioactivity following periods of prolonged exposure. Furthermore, due to limitations in the quantities of macroinitiator the polymerization should also be controlled and efficient at low concentrations and the protein/peptide should be compatible with chosen catalyst system.

To meet these requirements, the aqueous SET-LRP protocol described above was adventitiously discovered whilst optimizing reaction conditions that could facilitate Cu-mediated polymerization from biological macroinitiators. This technique was then adapted and employed for polymerization from a variety of proteins/peptides ranging from small therapeutic peptides (sCT, bovine insulin), to larger enzymes (lysozyme) and globular proteins (BSA, bovine haemoglobin, Hb), all of which were readily transformed into macroinitiators using simple NHS-ester and NHS-carbonate functional initiators (Figure 7).[92] The NHS-ester initiator reacts with the *N*-terminus and $\omega$-$NH_2$ of accessible lysine residues to form irreversible amide initiators whereas the NHS-carbonate can react with the same residues to form carbamate linkages, which could be subsequently cleaved under relatively mild conditions. The integrity of the polymerization taking place from the protein/peptides was then determined by standard solution characterization of the cleaved, 'grafted-from' polymer chains using NMR, SEC, IR, MALDI-ToF-MS.

Insert figure 7 here

Interestingly, upon modification to form the macroinitiators, the proteins/peptides exhibited variable solution properties. Soluble BSA macroinitiator, containing an average of 29 initiating groups, according to MALDI-ToF-MS, was used for the polymerization of dimethylacrylamide (DMA), NIPAM and OEGA to form conjugates which were characterized by SDS-PAGE and SEC. Cleavage of the polymer chains from proteins revealed that controlled polymerization

took place from each initiating site furnishing polymers with relatively narrow dispersities ($Đ$ < 1.30). Polymerization from the Hb macroinitiator (~12 initiating sites) was less controlled upon analysis of the cleaved polymers ($Đ$ = 1.68, NIPAM; $Đ$ = 1.62, OEGA). This was attributed to the presence of the heme groups, which themselves have been shown to mediate AGET-ATRP,[93] which can interact with the $Cu^{II}$[94] present in the aqueous SET-LRP system, sequestering it from solution leading to poorer control and broader dispersities.

Lysozyme and sCT were found to be insoluble in water upon conversion to their macroinitiator analogues containing ~5 and ~2 initiating sites respectively. However they were both readily dispersed in 0.5% aqueous solutions of sodium dodecyl sulfate (SDS). Under standard aqueous SET-LRP conditions, polymerization of NIPAM and OEGA from these dispersed macroinitiators was not well controlled ($Đ$ > 1.99). Poor control over polymerization had previously been reported during ATRP in emulsion using anionic surfactants which were implicated in the loss of halide ions from the solutions.[95] This causes an unfavourable imbalance in the activation-deactivation equilibrium which can be countered by the addition of NaBr to the surfactant solution. In the presence of an excess of NaBr, control over the polymerization of NIPAM was shown to be much improved from both lysozyme and sCT macroinitiators ($Đ$ < 1.25).

Insulin derived macroinitiators (1-3 initiating groups) were found to form stable colloidal solutions of polydisperse, irregular shaped particles (30-200 nm, TEM) at neutral pH. Surface initiated SET-LRP has previously be demonstrated using dispersed latex particles, functionalized with initiating groups, via $Cu^0$-wire mediated polymerization in water.[96] With this in mind, it was hypothesized that the aqueous SET-LRP system could mediated polymerization from the colloidal insulin particles dispersed in water. Polymerization of NIPAM yielded a conjugate with a relatively narrow dispersity ($M_n$ = 42900 g.mol$^{-1}$, $Đ$ < 1.28).

Cleavage of the polymer chains revealed well controlled PNIPAM with narrow dispersities ($M_n$ = 14500 g.mol$^{-1}$, $Đ$ < 1.16).

### 3.2 Non-covalent modification

In the field of dynamic, tunable and responsive polymers/materials, non-covalent interactions play an ever increasing role. With respect to non-covalent protein/peptide-polymer conjugations, modification is possible via high avidity interactions between proteins and natural and/or synthetic ligands incorporated into polymer compositions. Examples of natural ligands[97] include cofactors for enzyme reconstitution,[98] sugars[99] and biotin[100] that have high affinity interactions with lectins and streptavidin respectively. Alternatively, synthetic ligands have been designed that exploit electrostatic interactions,[101] metal-ligand affinity[102] and host guest interactions[103] to couple proteins/peptides and polymers. Finally, proteins/peptides can be incorporated into polymer gel matrices, either as non-covalent cross-linking groups (*e.g.* antibody-antigen interactions)[104] or as encapsulants,[105] both which have great potential as biological-stimuli responsive materials.

Glycopolymers can serve at synthetic analogues for natural oligo/polymersaccharides and complex glycoproteins that have naturally high affinity for specific lectin proteins. Recently, non-covalent PEGylation was demonstrated using hexavalent lectin RSL, derived from *Ralstonia solanacearum* and fucose-capped PEG as the complementary monosaccharide ligand.[106] The affinity of sugar-lectin interactions is often enhanced by multivalent sugar ligands that can complement the valency of the target lectin.[107] The synthesis of simple oligosaccharides is time and resource consuming, and is limited to small scales. Conversely, RDRP facilitates the synthesis of glycopolymers and the composition and high-order architecture or morphology can be intricately controlled. The composition can be varied using either synthetic vinylic glycomonomers or by using functional monomers that can be subsequently modified, post-polymerisation by functional glycosides. Homopolymers and

sequence controlled (multi)block copolymers have been prepared by SET-LRP and their binding to human C-type lectin (CTL) DC-SIGN has been reported.[108] The architecture has been varied by the synthesis of star polymers[109] or through preparation of glycopolymer nanoparticles; either polymeric nanoparticles[27] from self-assembly in solution or inorganic-polymer hybrid particles from the grafting of glycopolymers to, or from, the surface of inorganic nanoparticles such as iron oxide.[110, 111] In both cases non-covalent interactions with plant lectin ConA have been exemplified in polymer-lectin clustering experiments and turbidimetry.

The use of non-covalent interactions is becoming a viable alternative to covalent protein/peptide modification owing to the potential for temporal control over the conjugate integrity. The ideal system would benefit from high enough affinity to form a stable protein-polymer conjugate, but obviate any detrimental effects on bioactivity by facilitating release of the native protein in the presence of an appropriate competitive ligands.

## 4. Applications of protein/peptide polymer conjugates

### 4.1. Improving the 'shelf-life' of Oxytocin

Oxytocin is a versatile therapeutic neuropeptide, administered to treat a variety of physiological and psychological conditions. The World Health Organisation (WHO) list oxytocin as an essential medicine, primarily for its use as an agonist for uterine contraction to prevent, or treat, postpartum haemorrhaging (PPH) after delivery. Maternal mortality rates are generally low and continue to improve in developed nations. In stark contrast, mortality is unacceptably high in developing nations, in particular sub-Saharan Africa and South Asia, wherein 99% of worldwide maternal deaths will occur in 2016.[112] In these, often remote areas, deficiencies in simple medical resources and infrastructure such as cold chain transportation and storage can exacerbate complications that arise during pregnancy, delivery and recovery. This is because

oxytocin is chemically unstable in aqueous solution especially in hot and humid conditions.[113] Degradation severely diminishes therapeutic efficacy, to a point where it is quite common for levels of oxytocin, in samples labelled oxytocin, to contain zero active peptide in many countries where cold chain storage is not available.

A number of strategies for improving aqueous stability or precluding the need for aqueous solutions of oxytocin are being explored, including; the use of excipients in aqueous formulation,[114] disulfide bond engineering[115] and peptide-polymer conjugation.[76]

The disulfide bond ($Cys^1$-$Cys^6$) present is complicit in the instability of oxytocin due to its propensity to undergo dimerization, $\beta$-elimination, oxidation and polysulfide formation.[113] It is also an attractive site for modification with polymers containing disulfide reactive end-groups such DSM or arsenic. Using a DTM functional initiator, the conditions of aqueous SET-LRP were adapted to furnish DTM-functional POEGA of increasing molecular weight ($DP_n$ = 20, 50, 100; $M_n$ = 12, 31, 56 kDa; $Đ$ < 1.36). Upon addition of the crude polymer to reduced oxytocin, direct from the reaction mixture, conjugates of increasing molecular weight and size were formed. The effect of polymer conjugation on the stability of oxytocin was assessed via an accelerated stability assay, performed at 50 °C. The native oxytocin was shown to completely degrade over a period of 28 days, whereas the oxytocin-POEGA conjugates retained > 90% integrity over the same time period (Figure 8).[76]

Insert figure 8 here

Alternatively, the *N*-terminus was targeted for modification with linear PEG and comb polymers of OEG(M)A and oligo(2-ethyl-2-oxazoline) methacrylate (OEtOxMA) through NHS-ester and aldehyde (CHO) functional end-groups from which both irreversible and reversible conjugates could be formed.[116, 117] Comb polymers were synthesized from functional initiators using either $Cu^0$-wire mediated polymerization (NHS-POEGA, CHO-POEGA) or aqueous SET-LRP (CHO-POEGMA, CHO-P(OEtOxMA). Accelerated stability assays again demonstrated enhanced stability for oxytocin-polymer conjugates compared to

native oxytocin and oxytocin in the presence of unconjugated polymers, which proved that the stability enhancement was an effect of covalent conjugation as opposed to an excipient effect (Figure 8). Comb polymers were found to improve stability relative to linear analogues, however linear polymers granted improved release of the oxytocin from the conjugate as a function of pH, which could prove crucial from a bioactivity point of view.

The preparation of oxytocin-polymer conjugates has been shown to markedly improve the stability of the peptide in aqueous solution. Furthermore, the simple and dynamic chemistry employed is opportunistic for retaining bioactivity, which, along with tuning the local environment of the conjugate-linker, is currently being investigated.

### 4.2. Topical delivery of Polymyxins

Prophylactic and responsive administration of antibiotics is routine medical/surgical practice for the prevention and treatment of bacterial infections. Despite this, sepsis, the body's inflammatory response to infection by bacterial pathogens, is a major killer worldwide. Infections by multidrug-resistant (MDR) bacterial pathogens, such as Gram-negative 'superbugs' *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* are a major global healthcare burden. This is augmented by the lack of any novel antibiotic development over the last 30 years, and an increasing number of reports regarding new cases of resistance against the polymyxin family of antibiotics. Thus, there is an urgent need for new antimicrobials and/or improved antimicrobial therapies.

Open wounds such as those presented by severe burns, are particularly susceptible to 'superbug' infections due to the disruption of the epidermal barrier, the systemic apoptotic response and immunosuppression that disrupts self-defense mechanisms to fight infection.[118] Therefore, the use of topical antimicrobial chemotherapy has been fundamental and helped to improve the management and survival of patients with major burns by minimising the incidence of life-threatening burn wound sepsis.[119]

Polymyxins B and Polymyxin E (colistin)[120] are administered clinically for treatment of MDR gram-negative bacterial infections. The lipopeptide can bind to lipopolysaccharide (LPS) in the bacterial outer membrane, altering the permeability leading to cell death.[121] As a small and highly charged lipopeptide, colistin is subject to rapid renal clearance and incidence of nephrotoxicity[122] under systemic administrations. Considering this, and its high degree of functionality (5 amines from 2,4-diamino butyric acid residues) it was identified as a candidate for hydrogel-based topical delivery, using a simple, dynamic imine-based hydrogel system composed of amine-functional chitosan glycol and a *bis*-aldehyde functional linear PEG cross-linker (Figure 9).[123]

Insert figure 9 here

Although colistin contains five amine groups, alone it was not capable of forming a gel with the PEG crosslinker. However, in the presence of chitosan glycol, dynamic gels were formed, the integrity of which was unaffected by the presence of the colistin at concentrations between 25-250 $mg.L^{-1}$. Successful encapsulation was first demonstrated by washing the colistin-loaded hydrogels with water, samples of which were shown to contain no colistin. Release assays were performed in both PBS and cation-adjusted Mueller–Hinton (CAMHB) bacterial growth media, in which colistin was steadily released over a period of 24 hrs. Furthermore, the activity of the colistin-loaded hydrogels was comparable to equivalent concentrations of colistin in solution, in the absence of the gel matrix, and correlated well with the release kinetics in both *in vitro* assays (disk diffusion, time-kill) and an *in vivo* animal burn model.[124] Implications of this on viable dose regimens and nephrotoxicity in this system and in systemic delivery systems currently being developed are under investigation.

## 5. Conclusion

The ability to control molecular composition and architecture using RDRP has brought us closer than ever to the sophistication and complexity of nature and naturally occurring macromolecules. The development of aqueous SET-LRP has expanded the monomer

compatibility of Cu-mediated polymerization and enables polymerization to take place not only in pure aqueous media but also in biologically relevant media such as PBS and blood serum. Careful manipulation of the reaction conditions can also facilitate the formation of (multi)block copolymers which, in the case of responsive double hydrophilic block copolymers, can form higher-order structures under stimulus. This technique, and others, has been applied to the synthesis of polymers with high $\alpha$-end group fidelity, the functionality of which is predisposed for reaction with proteins/peptides. The functionality presented by proteins/peptides offers wealth of opportunity for chemical modification, often at the expense of site-selectivity and specificity. Using DSM and arsenical-functional polymers, conjugation to peptides can now be achieved rapidly, and stoichiometrically by re-bridging reduced disulfide bonds. The relatively low natural abundance of the cysteine, and the accessibility of disulfides render this approach highly specific, which is enhanced further by the use of arsenicals that react with disulfides with unrivalled specificity, even in the presence of free cysteine residues. Using these techniques, and others, peptide-polymer conjugates of therapeutic relevance (oxytocin, colistin) have been prepared and exhibit advantageous properties such as enhanced stability and controlled release. There is great potential in the combination of bioactive (macro)molecules with polymers, either covalently through conjugation or non-covalently using hydrogels or higher-order self-assembled structures, to address challenging biomedical problems, which is the working vision of my fledgling laboratory.

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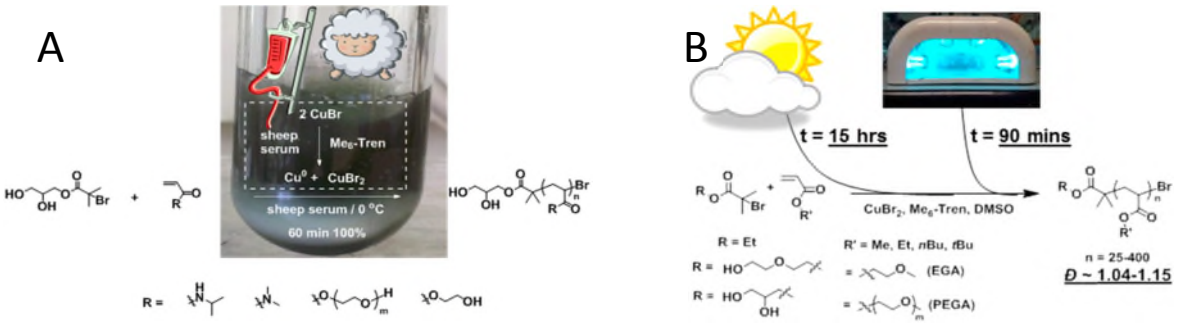


*Figure 1.* Recent advances in Cu-mediated polymerization allow polymerization to proceed in biological media[31] and under the influence external stimulus.[22]
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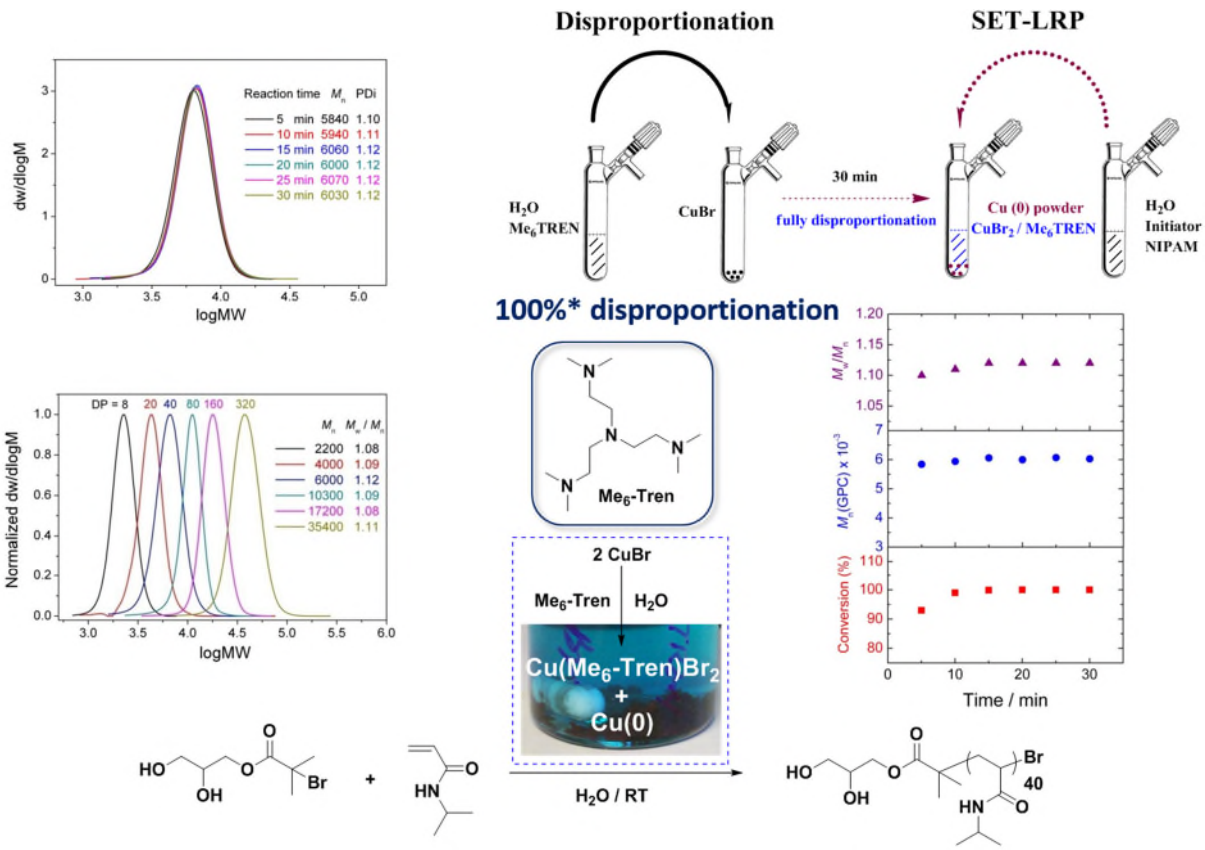


*Figure 2.* Rapid and complete disproportionation of $Cu^{I}Br/Me_6$-Tren exploited for the polymerization of NIPAM in pure aqueous media. The first example of aqueous SET-LRP.
Adapted with permission.[24] 2013, American Chemical Society.

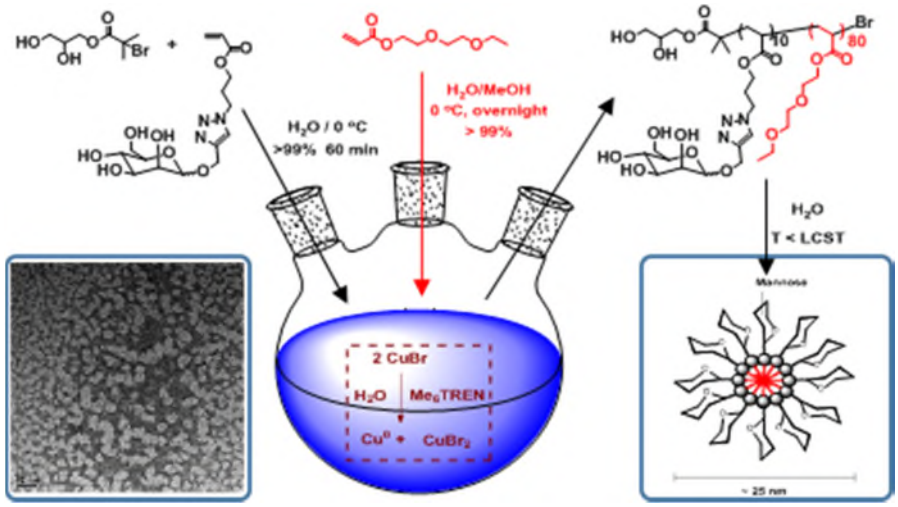


*Figure 3.* Synthesis and aggregation of double hydrophilic diblock copolymers prepared by aqueous SET-LRP via *in situ* sequential monomer addition.
Adapted with permission.[27] 2014, American Chemical Society.

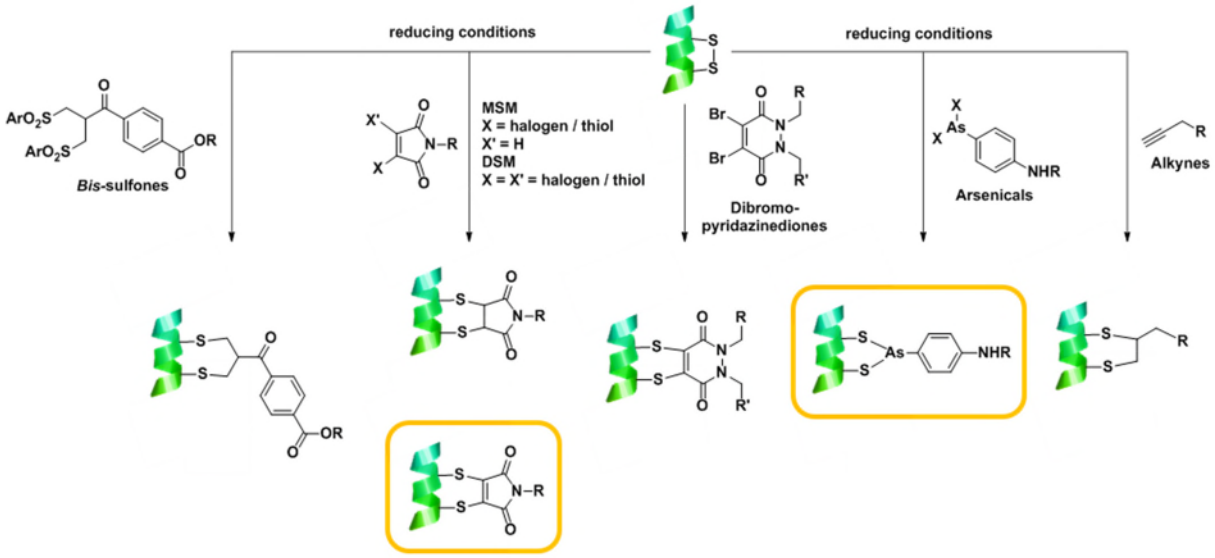


*Figure 4.* Site-specific protein/peptide conjugation targeting disulfides. The conjugation of DSM and arsenicals is highlighted for further discussion.

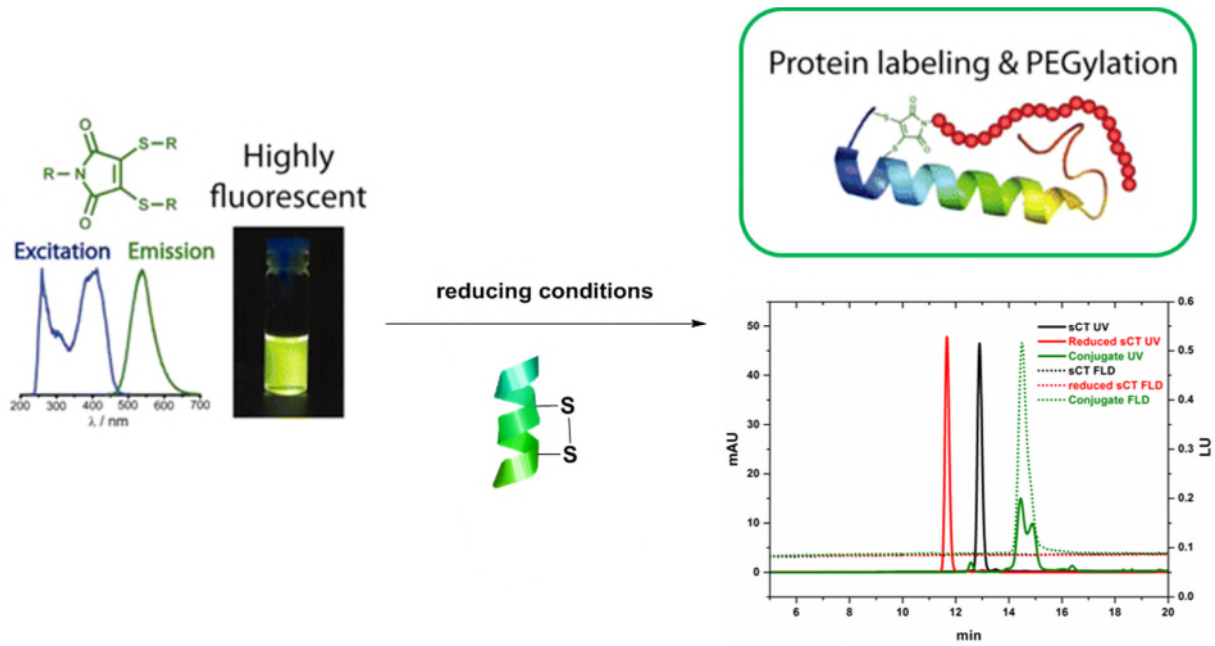


*Figure 5.* Conjugation-induced fluorescence occurs during the rapid and stoichiometric conjugation of DSM reagents to reduced disulfides.
Adapted with permission.[72] 2013, American Chemical Society.

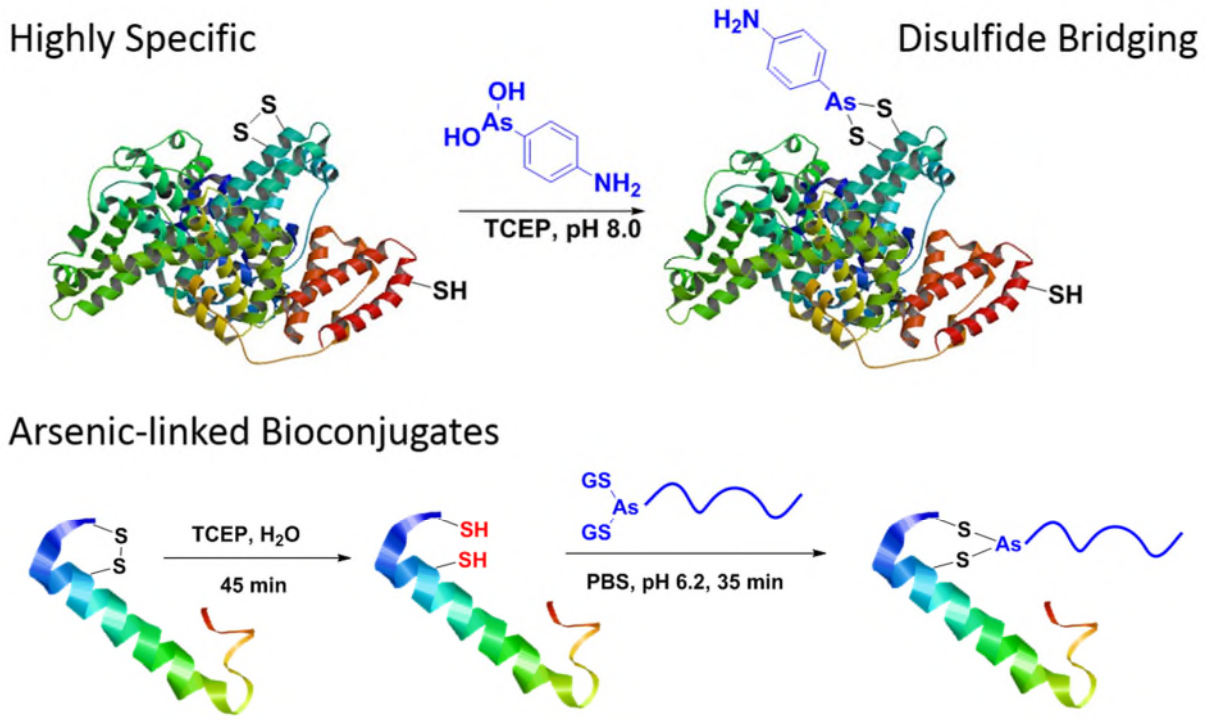


*Figure 6.* Organic arsenicals as highly efficient linkers in protein/polymer conjugation with unrivalled site-specificity for disulfide bond derived dithiols, in the presence of mono-thiols. Adapted with permission.[78] 2015, American Chemical Society.

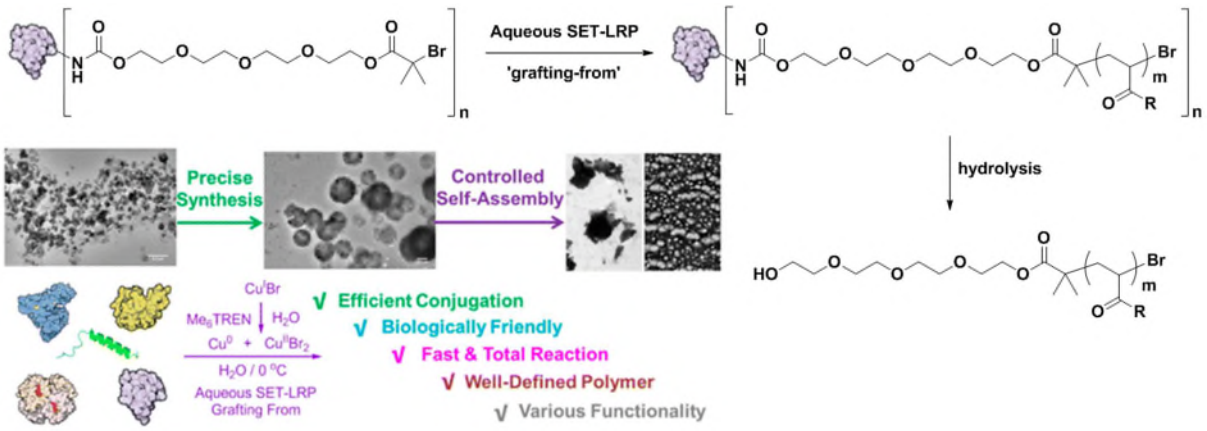


*Figure 7.* Organic arsenicals as highly efficient linkers in protein/polymer conjugation with unrivalled site-specificity for disulfide bond derived dithiols, in the presence of mono-thiols. Adapted with permission.[92] 2015, American Chemical Society.

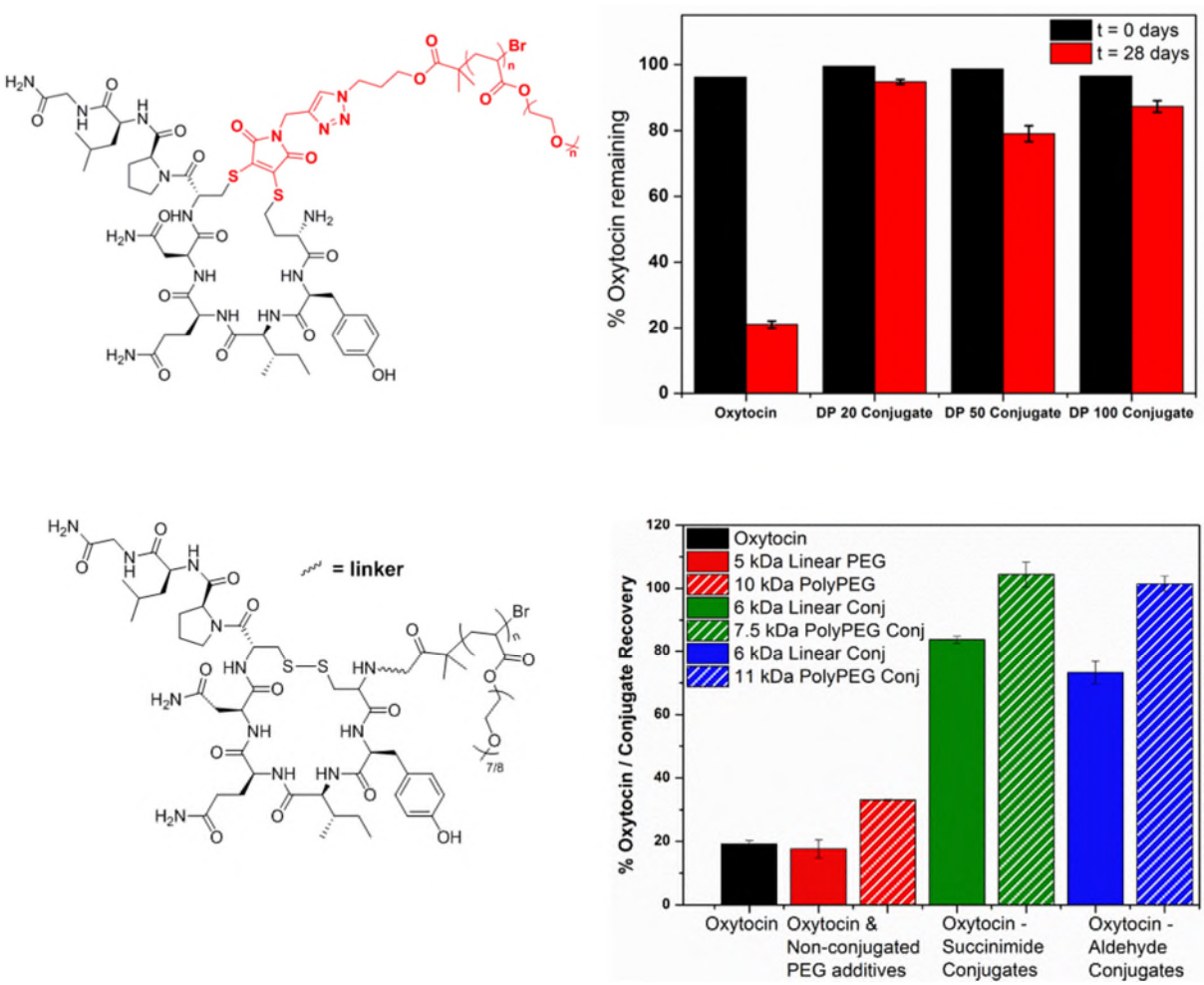


*Figure 8*. The stability of oxytocin is enhanced by polymer conjugation at both the disulfide bond and *N*-terminal amine.
Adapted with permission.[76, 116] 2015/2016, American Chemical Society.

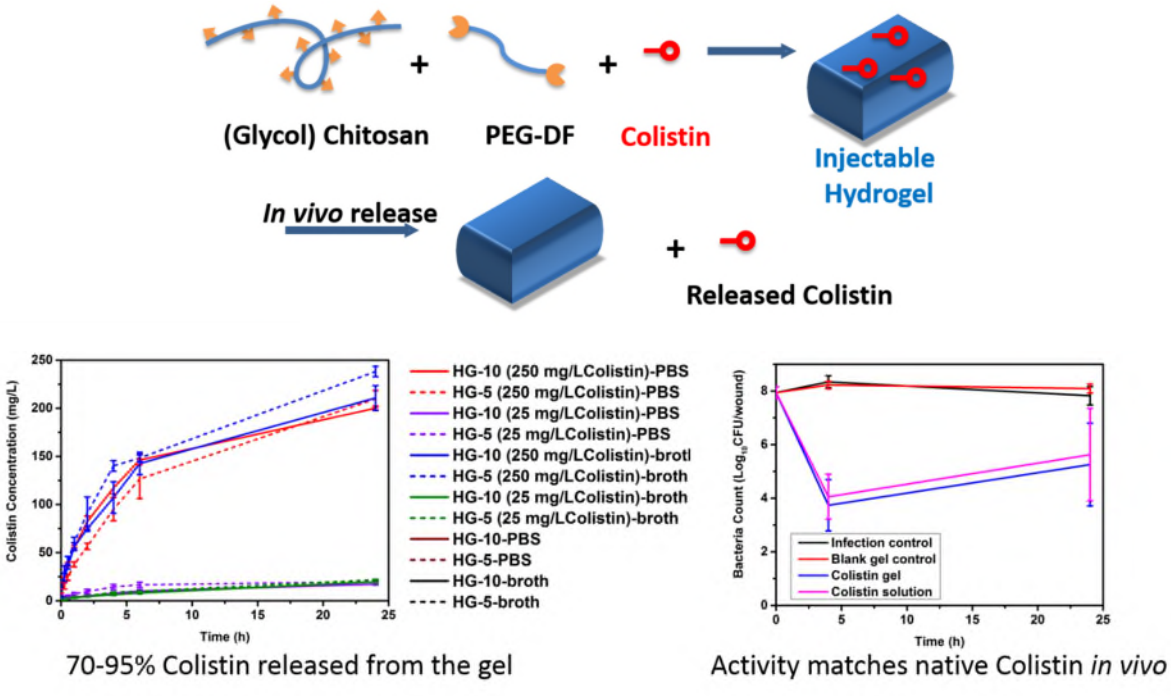

*Figure 9.* Schematic for the formation of colistin-loaded hydrogels which show efficient release and comparable activity to colistin in solution.
Adapted with permission.[124] 2016, Wiley VCH.

Paul Wilson studied chemistry (Bristol, MSci, 1st Class, 2006) before completing a PhD in organic chemistry (Warwick, 2010). As a post-doctoral researcher he initially worked in industry (Warwick Effect Polymers Ltd, 2010-2011), before returning to academia, becoming a senior research fellow for first, Prof Dave Haddleton (2011), and then Prof Tom Davis as part of the Monash – Warwick Alliance (2013). In 2016, he was awarded a prestigious Leverhulme Trust Early Career Fellowship, which he holds at the University of Warwick in the Department of Chemistry. He also holds an adjunct senior research fellow position at Monash Institute of Pharmaceutical Sciences. His research interests focus on developing and exploiting RDRP protocols to facilitate the preparation of complex, functional polymers with exemplary compositional and architectural precision, and the use of these polymers to prepare polymer-hybrid materials.

**State of the art RDRP and protein/peptide modification methods,** have brought synthetic polymer and materials science closer than ever to replicating the precision of nature. The development of aqueous SET-LRP and efficient, highly specific disulfide targeted modifications, using organic arsenicals, furnishes protein-polymer conjugates with unrivalled precision. Continued progress is crucial considering the clear benefits associated with attaching polymers to therapeutic peptides such as oxytocin and polymyxins.

Paul Wilson*

**Synthesis and applications of protein/peptide-polymer conjugates**