Polymeric dibromomaleimides as extremely efficient disulfide bridging bioconjugation and pegylation agents

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Abstract

A series of dibromomaleimides have been shown to be very efficacious at insertion into peptidic disulphide bonds. This conjugation proceeds with a stoichiometric balance of reagents in buffered solutions in less than 15 minutes to give discrete products whilst maintaining the disulphide bridge and thus peptide conformation. The insertion is initiated by disulfide reduction using a water soluble phosphine, tris(2-carboxyethyl)phosphine (TCEP) which allows for subsequent substitution of the two maleimide bromides by the generated thiols. Reaction of salmon calcitonin (sCT) with 2,3 dibromomaleimide (1.1 excess) in the presence of TCEP (1.1 equivalent) in aqueous solution at pH 6.2 gives complete production of a single conjugate which requires no work up. A linear methoxy poly(ethylene glycol) (PEG) was functionalized *via* a Mitsunobu reaction and used for the successful site specific and rapid pegylation of sCT. This reaction occurs in 15 minutes with a small stoichiometry excess of the pegylating agent to give insertion at the disulphide with HPLC showing a single product and MALDI-ToF confirming conjugation. Attempts to use the group in a functional ATRP polymerization initiator led to polymerization inhibition. Thus in order to prepare a range of functional polymers an indirect route was chosen *via* both azide and aniline functional initiators which were converted to 2,3 dibromomaleimides *via* appropriate reactions. For example, the azide functional polymer was reacted *via* a Huisgen CuAAC click reaction to an alkyne functional 2,3 dibromomaleimide. This new reagent allowed for the synthesis of conjugates of sCT with comb polymers derived from PEG methacrylic monomers which in addition gave appropriate cloud points. This reaction represents a highly efficient polymer conjugation method which circumvents problems of purification which normally arise from having to use large excesses of the conjugate. In addition the tertiary structure of the peptide is efficiently maintained.

Introduction

Conjugation of synthetic polymers to proteins and peptides is a well established method to enhance properties of native biomolecules and aid the introduction of polymer based therapeutics.[1-2] The covalent attachment of poly(ethylene glycol) (PEG) chains, *pegylation*, in particular have been well documented to yield conjugates often with improved solubility, stability and plasma half-lives.[3-19] The early, and probably still the majority of reports, describe the targeting of lysine residues due to their relative abundance, with a range of simple chemistries, including activated esters, which are now commonly employed for bioconjugation.[20-22] This approach often results in isolation of a statistical mixture of conjugated species. Targeting of cysteine residues has been explored as an alternative and a number of reagents have been developed to selectively target this thiol-containing residue. *N*-Substituted maleimide,[23-27] pyridyl disulfide,[28-30] and acrylic[31-32] functional polymers have been explored for conjugation at cysteine. However, free cysteine residues are relatively rare in proteins and other biomolecules, although they can be introduced by genetic engineering or by the reduction of disulfide bridges.[33-34] The latter approach has the inherent problem that if the reducible disulfides are essential for maintaining protein structure, shape, stability and bioactivity is lost. Brocchini *et al*. developed an elegant, but rather elaborate approach, to overcome this whereby reduced disulfides are re-bridged using a three-carbon linker.[35-36] This has the obvious advantage that the tertiary structure of the protein is largely maintained as well as the site-selective incorporation of functionality, such as PEG.[37]

Baker *et al*. recently reported the development of a range of different functional maleimides for cysteine modification and conjugation.[38-39] Dibromomaleimides were shown to be efficacious and fast reacting disulfide re-bridging agents, installing a rigid two carbon linker. In addition, dibromomaleimides have an added advantage that the formed linker is potentially cleavable in a reversible manner under intracellular-like conditions to rejuvenate the native polypeptide in its reduced state. This same team reported the development of new substituted maleimides, dithiomaleimides, which are less susceptible to side reactions with reducing agents, such as TCEP, and have been shown to have potential for *in situ* bridging protocols.[40] The development of controlled radical polymerization (CRP) techniques such as ATRP,[41-42] RAFT[43] and NMP[44] have enabled the ready synthesis of well-defined α-functional polymers for bioconjugation under undemanding experimental conditions. Herein, we report on the study of dibromomaleimide functional polymers for applications in protein disulfide bridging, which are synthesised by ATRP.

Results and discussion

We focussed on the modification of salmon calcitonin (sCT), a 32-amino acid hormone secreted by *C*-cells of the thyroid in mammals and by the ultimobranchial glands in submammals. This is currently used for the treatment of a range of bone conditions including post-menopausal osteoporosis, Paget's disease and hypercalcaemia.[45] This polypeptide contains a disulfide bridge (Cys^1-Cys^7) that can be reduced to give two sulfhydryl functionalities as targets for re-bridging conjugation with dibromomaleimides. Our initial experiments involved disulfide bridging using the commercially available 2,3-dibromomaleimide **(1)** (Figure 1).

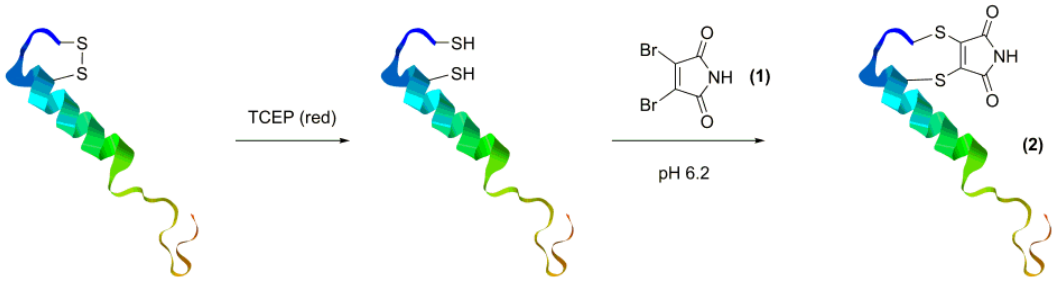

Figure 1. One-pot reduction of the disulfide bridge of sCT, followed by re-bridging using dibromomaleimide.

The disulfide bridge of sCT was reduced in the presence of tris(2-carboxyethyl)phosphine (TCEP) and the reaction monitored by RP-HPLC, ring opening is observed by a large shift in retention time due to the formation of two relatively polar thiols, giving a very convenient analytical handle (Figure 2). Following complete reduction of the disulfide bridge the reaction pH was adjusted to 6.2 and 2,3-dibromomaleimide (1.1 equivalents) was added. Immediately upon addition, the solution turned pale yellow, in accordance with previous observations for dithio-bridged maleimides[40] and after 10 minutes an aliquot was removed for RP-HPLC analysis.

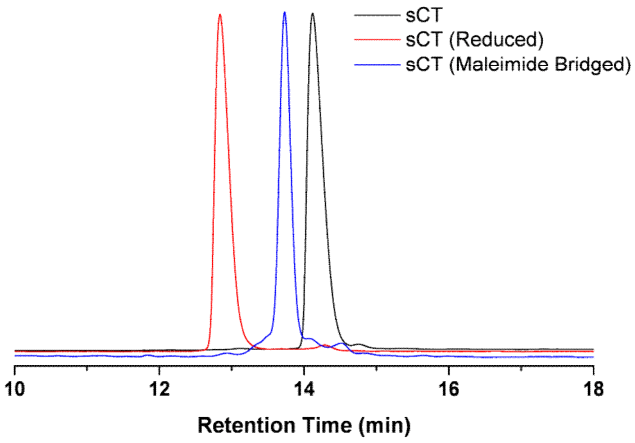

Figure 2. RP-HPLC analysis of sCT disulfide-bridging with 2,3-dibromomaleimide following reduction of the disulfide bridge of sCT.

Analysis of the reaction mixture revealed the complete consumption of the reduced sCT and the appearance of a new single peak (blue trace), ascribed as the bridged product, moving to longer retention time due to consumption of the two cysteine residues and conjugation of the maleimide. MALDI-ToF-MS showed the expected mass gain of 95.04 Da due to disulfide bridging of the maleimide (Figure 3). No traces of either native or reduced sCT were observed by MALDI-ToF-MS, in agreement with RP-HPLC, evidence of highly efficient bridging with no observable side-reactions. Trypsin digest of (**2**) and analysis by MALDI-ToF-MS revealed that the disulfide-containing fragment was the only modified species and circular dichroism revealed that the structural integrity of the peptide had been maintained following modification (see Supporting Information).

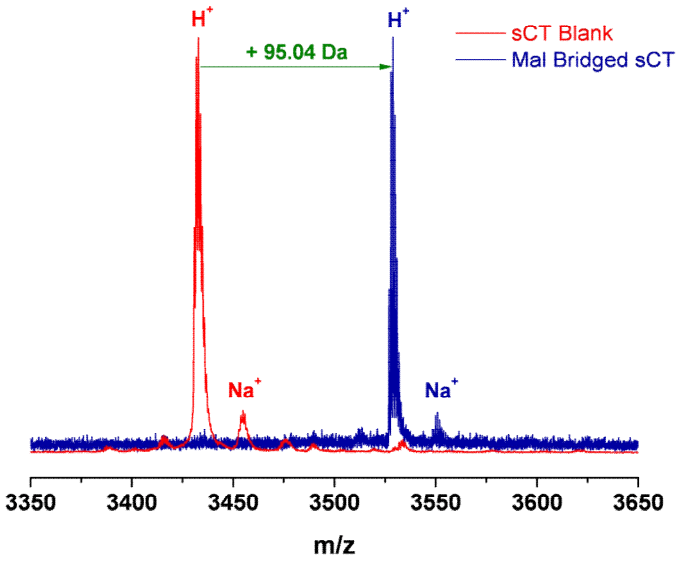

Figure 3. MALDI-ToF-MS analysis of native sCT (red trace) and maleimide-bridged sCT following sampling after 10 minutes.

In order to demonstrate the viability of this reaction for polymer bioconjugation, a linear PEG-chain with α-functional dibromomaleimide functionality was synthesised[40] using a modified Mitsunobu reaction.[46] This functionalised PEG_{5000} chain **(3)** was conjugated to reduced sCT under identical reaction conditions as with 2,3-dibromomaleimide **(1)**, (Figure 4).

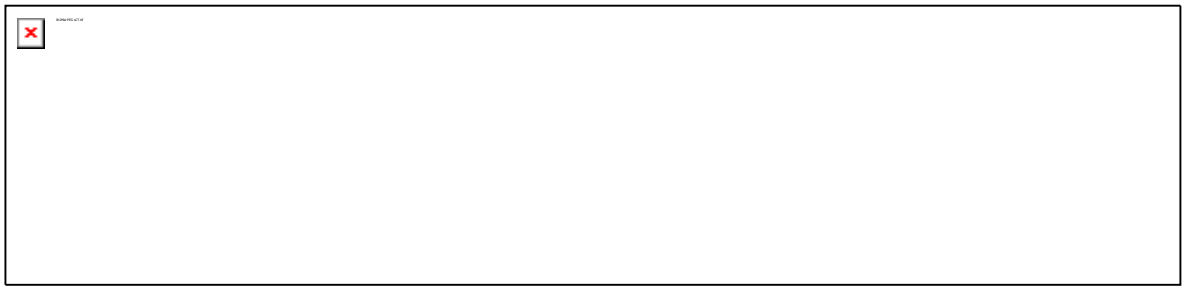

Figure 4. sCT disulfide re-bridging using the dibromomaleimide-functional linear PEG_{5000} chain.

As before addition of the dibromomaleimide-functional PEG_{5000} chain gives an immediate pale yellow colour, indicative of the bis-thio bridged maleimide. Analysis after 15 minutes by RP-HPLC revealed the complete disappearance of the reduced polypeptide as well as the formation of a new single species with retention time at approximately 18 minutes (Figure 5).

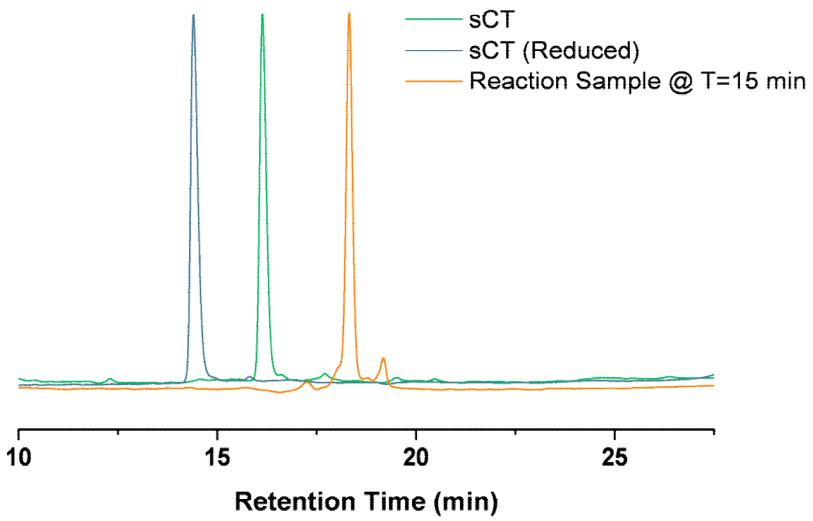

Figure 5. RP-HPLC analysis of the disulfide-bridging of sCT using a dibromomaleimide-functional PEG chain following reduction with TCEP.

MALDI-ToF-MS analysis showed a distribution of peaks around the expected mass (~ 8.5 kDa) upon conjugation of the PEG_{5000} chain to sCT (3431 Da) (Figure 6). A number of PEG-derived distributions were observed, with the predominant peaks corresponding to the sodiated conjugate and smaller peaks attributed to the protonated conjugate (Figure 6 and ESI).

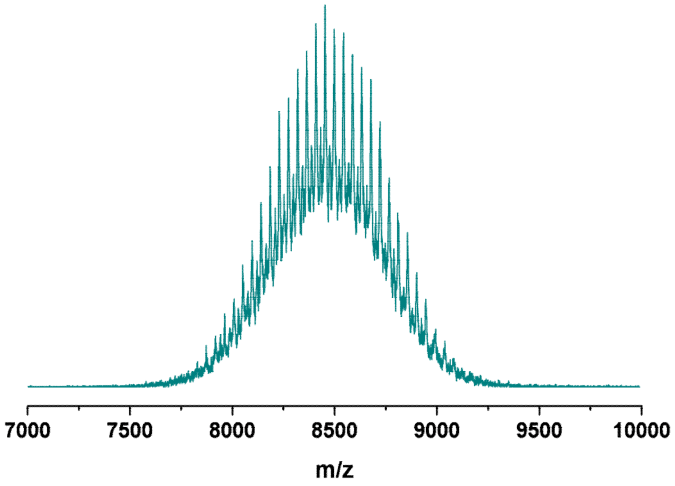

Figure 6. MALDI-ToF-MS of the reaction mixture after 15 minutes, showing the formation of the monoconjugated sCT-PEG_{5000} species, around 8.5 kDa as expected.

Despite successful functionalisation of PEG with dibromomaleimide using modified Mitsunobu conditions, both the efficiency and isolated yield were relatively low, with the requirement of column chromatography for purification of the final product. Thus, a strategy was devised to synthesise α-functional polymers by atom transfer radical polymerization (ATRP). Since each chain is grown from the initiator in this case, the synthesised polymer inherently contains the initiator-derived functionality. The dibromo-maleimide functionality was investigated as a novel end-group for the synthesis of maleimide-functional polymers. Since unfunctionalized

maleimides are well documented as highly reactive substrates in radical polymerizations,[47-49] the dibromo derivative was analysed as a viable functionality for the synthesis of functional polymers. Polymerization using standard ATRP conditions of PEG-methacrylate using ethyl 2-bromoisobutyrate was carried out in the presence of dibromomaleimide alongside a control reaction conducted as a reference (Figure 7).

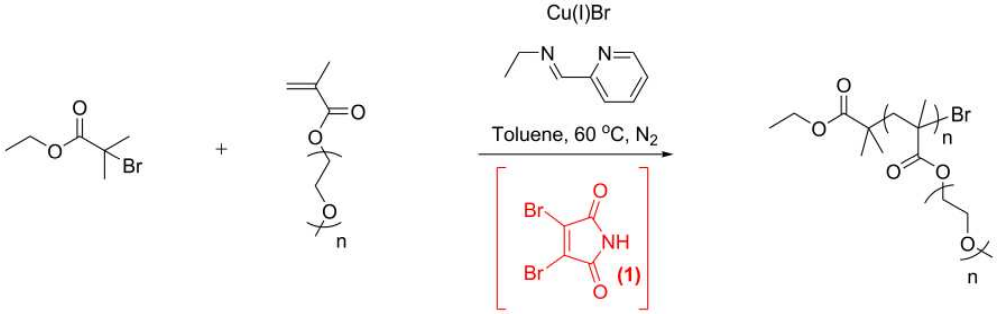

Figure 7. ATRP polymerization of PEGMA in the presence and absence of the dibromomaleimide functionality.

Large differences in the polymerization rates were observed (Figure 8). Polymerization in the absence of 3,4-dibromomaleimide proceeded as expected, with linear first order kinetics observed. However, polymerization in the presence of 3,4-dibromomaleimide was retarded in comparison with the blank polymerization.

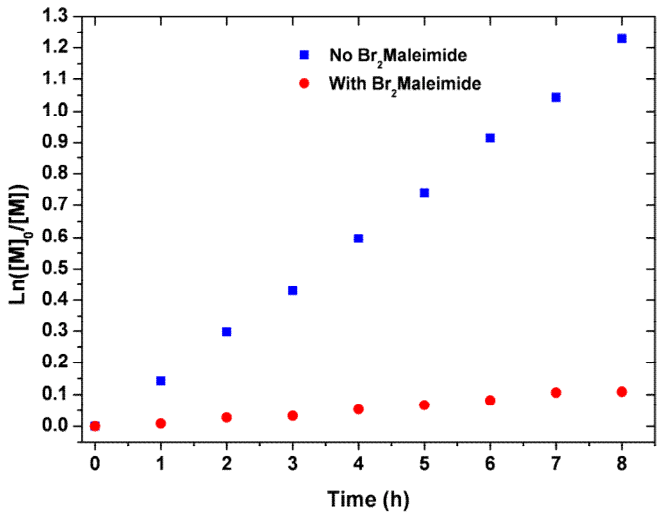

Figure 8. First order kinetic plot for the polymerizations of $PEGMA_{475}$ using [ethyl 2-bromoisobutyrate]:[Cu(I)Br]:[Lig]:[$PEGMA_{475}$] = 1:1:3:20, toluene/$PEGMA_{475}$ 2:1 (v/w), 60 °C in the absence (blue data) and presence of 3,4-dibromomaleimide (1) (1 eq. with respect to initiator) (red data).

Thus the direct polymerization using dibromomaleimide-functional initiators was abandoned as a viable route and alternative strategies investigated. Two post-polymerization modification[50] routes were investigated for the introduction of the dibromomaleimide functionality, firstly an azido-functional initiator **(5,** Figure 9**)** was synthesised for further transformation using CuAAC. A second strategy was devised whereby aniline-functional polymers were synthesised in order to introduce the maleimide functionality using a condensation reaction with dibromomaleic anhydride, followed by ring closure *via* acid-promoted dehydration (**6**, Figure 9).

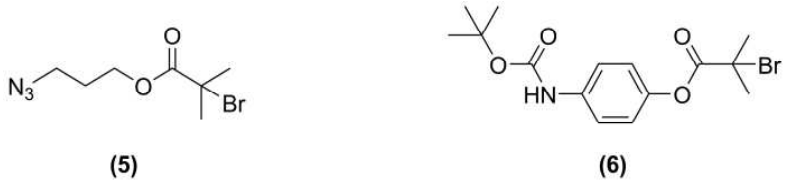

Figure 9. ATRP initiators employed in this study for the post-polymerization incorporation of dibromomaleimide functionality.

The azide-functional initiator **(5)** was synthesised as described previously[51] and was employed for the polymerization of oligo(ethylene glycol) methacrylates by ATRP. Polymers synthesised using this initiator have been shown to maintain an apparent 100 % of the azido chain end functionality,[51] an essential requirement for efficient post polymerization modification.

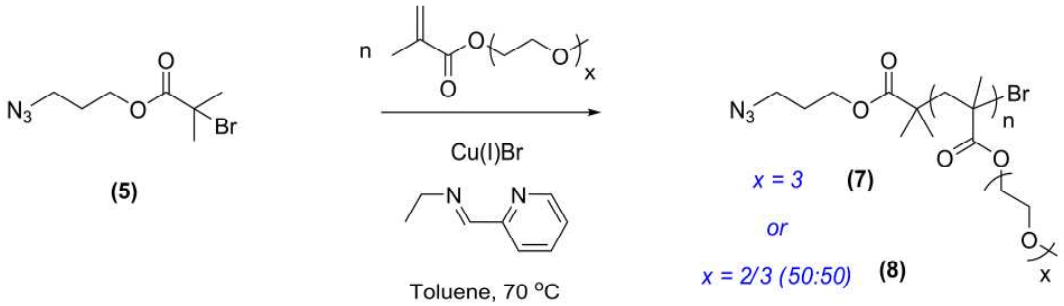

Figure 10. Polymerization of oligo(ethylene glycol) methacrylates using azide-functional initiator (7).

Polymerization of oligo(ethylene glycol) methacrylates has emerged as a popular route for the synthesis of thermoresponsive polymers[52-53] and protein conjugates.[32,54] Tri(ethylene glycol) methacrylate (TEGMEMA) was homopolymerized and copolymerized in a 1:1 molar ratio with di(ethylene glycol) methacrylate (DEGMEMA) using initiator **(5)** by ATRP (Figure 10) to synthesise azide-functional polymers **(7)** and **(8)**. Following purification, the polymers were isolated as colourless oils and the presence of the α-azido functionality confirmed by IR-spectroscopy (ESI). A dibromomaleimide-functional terminal alkyne was synthesised and the functionality installed to the polymers by CuAAC (Figure 11) and the presence of the new end-group confirmed by ^{13}C NMR, along with the loss of the characteristic signal from the azide observed by IR-spectroscopy. The thermoresponsive nature of polymers **(7)** and **(8)** were investigated (ESI), with the cloud points of showing little or no change following end-group modification.[55]

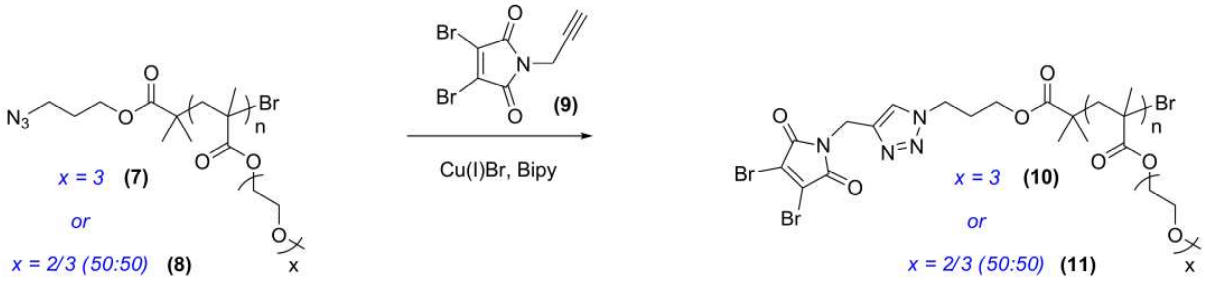

Figure 11. Installation of dibromomaleimide functionality to α-azido functional polymers using CuAAC.

A second strategy was also investigated as a route to dibromomaleimide functional polymers using *boc*-protected aniline initiator **(6)**. Oligo(ethylene glycol) methacrylate was homopolymerized and copolymerized with DEGMEMA by ATRP using the protected aniline initiator **(6)** in order to synthesise α-functional polymers **(16)** and **(17)**.

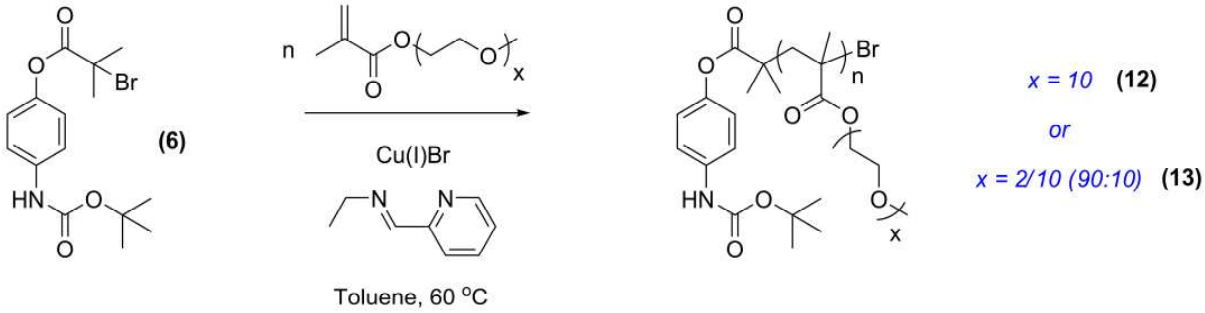

Figure 12. Polymerization of oligo(ethylene glycol) methacrylates using initiator (10) as a route to α-Boc-protected polymers.

Following purification and isolation, the *boc*-protecting group was removed by treatment with TFA and the obtained aniline converted to a dibromomaleimide-functional polymer following heating in the presence of dibromomaleic anhydride (Figure 13). Each of the post-polymerization modification steps were monitored by ^{1}H and ^{13}C NMR, with deprotection of the *boc*-group and functionalisation with dibromomaleic anhydride monitored by the shift of the aromatic protons and compared to synthesised model compounds (ESI).

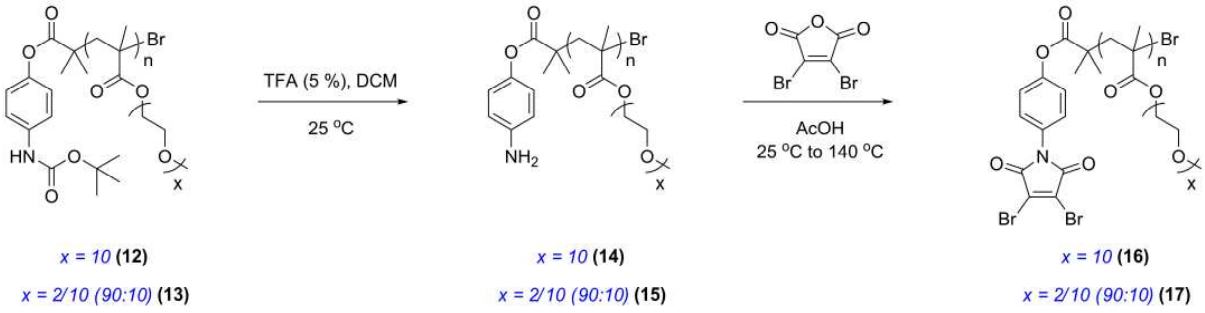

Figure 13. Post-polymerization modification of poly(oligo(ethylene glycol)) methacrylate by deprotection of the *boc*-protecting group to yield an α-aniline functionality, followed by treatment with dibromomaleic anhydride to furnish the dibromomaleimide end-group.

Following successful incorporation of the dibromomaleimide functionalities to the aniline- and azide-functional pre-polymers, conjugation to sCT was investigated. As previously, the disulfide bridge of sCT was reduced in the presence of TCEP and monitored by RP-HPLC. Following complete reduction of the disulfide, polymer **(10)** was added in a buffered solution at pH 6.2.

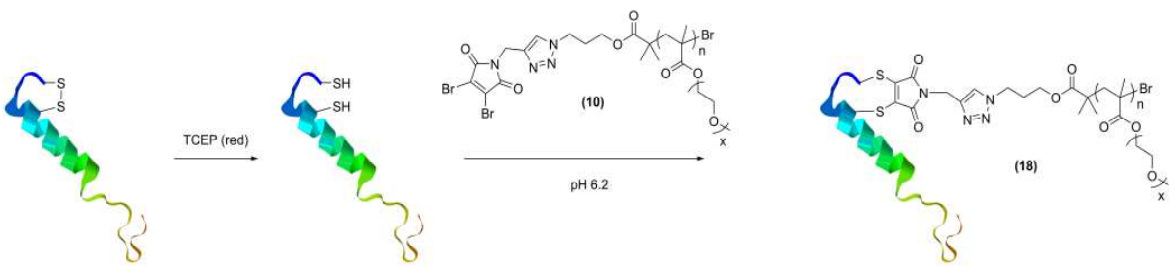

Figure 14. sCT disulfide re-bridging using dibromomaleimide functional polymer (10).

As previously, an immediate change in solution colour to pale yellow was observed[40] and analysis by RP-HPLC 20 minutes following addition of the polymer showed a complete loss of the signal corresponding to reduced sCT, along with the formation of a broad peak from the conjugate.

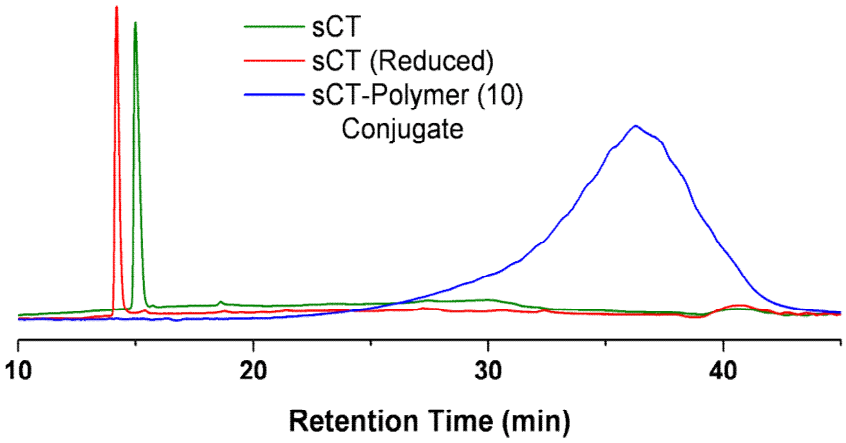

Figure 15. RP-HPLC analysis of the disulfide-bridging of sCT using polymer (10) following reduction of the disulfide bridge with TCEP.

Similarly, the conjugation of the dibromomaleimide-functional polymer synthesised *via* the aniline route was investigated. Following reduction of sCT using TCEP, polymer **(16)** was added in a buffered solution at pH 6.2 and the characteristic yellow colour was observed immediately. RP-HPLC analysis of the reaction mixture showed the loss of the signal corresponding to the reduced polypeptide, as well as the formation of the broad conjugate peak (ESI). These bioconjugation reactions demonstrate the power of this approach for the re-bridging of generated cysteine residues derived from reduced disulfides. The ability to rapidly (<30 minutes) and quantitatively modify polypeptides with equimolar amounts of dibromomaleimide functional polymers holds great promise for the development of a new class of bioconjugates. Timescales for conjugation have been dramatically reduced relative to commonly employed protocols and purification of products have been simplified due to a reduction in the required amounts of conjugating polymer.

Summary

In this study, we report disulfide re-bridging of salmon calcitonin using dibromomaleimide functional polymers. Direct incorporation of dibromomaleimide into synthetic polymers using ATRP was not possible due to an interfering effect of the functionality. As a consequence, two post-polymerization modification approaches were explored and successfully employed in order to install the dibromomaleimide group to the α-terminus of OEG-based polymers. In both cases, the polymers were successfully conjugated to salmon calcitonin *via* the re-bridging of the disulfide in a one pot system with equimolar equivalents of the peptide and polymer in a matter of minutes. Both routes serve as viable approaches to disulfide-bridging agents for polypeptides and proteins, although the *N*-phenyl maleimide derivatives have further advantages as both stable and cleavable linkers following controlled maleimide hydrolysis.[56] This approach has clear benefits over currently employed techniques since little or no purification of macromolecular species is required due to the stoichiometric nature of the reaction.

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Supporting Information Available

Experimental details, ^{1}H and ^{13}C NMR, IR, MALDI-ToF-MS and RP-HPLC data are available in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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