Direct Peptide Bioconjugation/Pegylation at Tyrosine with Linear and Branched Polymeric Diazonium Salts

Mathew W. Jones,[α] Giuseppe Mantovani*,[β] Claudia A. Blindauer ,[α] Sinead M. Ryan,[χ] Xuexuan Wang,[χ] David J. Brayden[χ] and David M. Haddleton.*[α]

[α] Department of Chemistry, University of Warwick, Coventry, UK, CV4 7AL. E-mail: D.M.Haddleton@warwick.ac.uk; Fax: +44 (0)24 7652 4112; Tel: +44 (0)24 7652 3256.

[β] School of Pharmacy, University of Nottingham, Nottingham, UK, NG7 2RD.

[χ] UCD School of Agriculture, Food Science and Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.

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Supporting Information Placeholder

ABSTRACT: Direct polymer conjugation at peptide tyrosine residues is described. In this study Tyr residues of both Leucine enkephalin and salmon calcitonin (sCT) were targeted using appropriate diazonium salt terminated linear mPEGs and poly(mPEG) methacrylate prepared by ATRP. Judicious choice of the reaction conditions – pH, stoichiometry and chemical structure of diazonium salt – led to high degree of site-specificity in the conjugation reaction even in the presence of competitive peptide aminoacid targets such as histidine, lysines and *N*-terminal amine. *In vitro* studies showed that conjugation of $mPEG_{2000}$ to sCT did not affect the peptide ability to increase of intracellular cAMP induced in T47D human breast cancer cells bearing sCT receptors. Preliminary *in vivo* investigation showed preserved ability to reduce $[Ca^{2+}]$ plasma levels by $mPEG_{2000}$-sCT conjugate in rat animal models.

Conjugation of poly(ethylene glycol), PEGylation, of biologically relevant peptide and protein drugs is a strategy that prolongs therapeutic half-life and reduces immunogenicity.[1-4] A number of PEG-protein therapeutics are currently prescribed for the treatment of a range of prevalent conditions including Hepatitis C, chemotherapy-associated neutropenia and leukemia and many more are currently in clinical and preclinical development.[2] PEGylation generally occurs by targeting nucleophilic aminoacids, such as the primary amine at *N*-termini, hystidine, lysine and at thiols at free cysteine residues with end-functional PEGs.[5, 6] In order for PEGylated proteins and peptides to retain an acceptable proportion of their original activity, conjugation must not occur at, or at the immediate vicinity of, crucial points in the polypeptide sequence. These would include at the binding sites of hormones or at the catalytic sites of enzymes. It is therefore key that polymer conjugation occurs preferentially, when not exclusively, at specific aminoacids known for not being part of (poly)peptide active sites. PEGylated protein therapeutics consisting of a mixture of positional isomers (PEGamers) – i.e. PEGylated IFN alfa-2a and 2b currently prescribed as first line treatment for hepatitis C and some forms of melanoma –have received in the past regulatory approval. This is due to largely improved pharmacokinetic profile and clinical efficacy compared to the native protein therapeutics. However, upon polymer conjugationsome of these therapeutics retain only a fraction of the activity of the original protein (as low as 7% for PEGylated IFN-alfa-2a). Although in vitro activity is only one of the many component responsible for the overall therapeutic efficacy of these biohybrid materials,the development of site specific conjugation strategies which are able to circumvent this problem is now considered a priority in PEGylation and conjugation science. To this end, in recent years considerable research effort has been spent in identifying and developing alternative and complementary PEGylation strategies to those being clinically used. One breakthrough was the work by Brocchini and coworkers, who first described the site-specific PEGylation of disulfide bridges of a number of protein and peptides using novel α-β-unsaturated sulfone derivatives.[7, 8]

The growing demand for the synthesis of well-defined bioconjugates *via* site-specific coupling has increased interest in tyrosine as a target for protein modification. During the past decade, a number of reports have emerged whereby tyrosine residues have indeed been targeted. González *et al*. reported the selective mono-iodination of tyrosine residues using iodinating reagent IPy_2BF_4[9] and this has been exploited for the introduction of further functionality using Suzuki-Miyaura coupling.[10] Recently Barbas and co-workers have reported an elegant ene-type conjugation of cyclic azadicarboxylate small molecules to Tyr residues of model proteins.[11]

Francis and coworkers have reported a number of efficient strategies whereby tyrosine residues were targeted *via* a three-component Mannich-type reaction, as well as alkylation of the residue *via* the hydroxide group using Trost-allylation conditions,[12] and coupling with diazonium reagents. This has rejuvenated the field of diazonium coupling and have described several reports on the modification of the tyrosine residues on bacteriophage MS2 viral capsids[13] and tobacco mosaic virus (TMV).[14] Wang *et al*. have since reported a study into the

functionalisation of TMV using a combination of diazonium coupling at tyrosine and and this has then been adapted to modify the tyrosine residues of M13 bacteriophage.[15]

Of the three techniques developed by Francis, diazonium-mediated targeting of tyrosine residues appeared to us to be be the most attractive for direct polymer conjugation of (poly)peptides. The three-component Mannich-type approach is generally a slow reaction, which makes it less attractive for the coupling of macromolecules, a process that in itself is slower compared to coupling with small molecules. Moreover, this generally requires a large excess of coupling reagents,[16-18] which again is less than ideal for polymer conjugation of proteins and peptides. Pd-catalysed *O*-alkylation by Trost allylation is an efficient process, although the need for a potentially toxic transition metal catalyst (although at very low concentration) may make this less attractive for the direct preparation of protein and peptide derivatives of pharmaceutical interest. Conversely, diazonium couplingsare metal-free generally fast processes that have the potential for being applied to polymer conjugation targeting of medically relevant proteins and peptides. In addition, they have been employed to generate pharmaceutically relevant therapeutics, including the sulfonamidochrysoidine prodrug (Prontosil).

Despite these promising recent advances, to date, no report has described *direct* polymer conjugation, including PEGylation, to tyrosine residues of (poly)peptides. In this currentwork we have developed a novel and potentially general route to polymer-peptide biohybrid materials, *via* preferential targeting of peptide tyrosine residues with appropriate diazonium salt-terminated polymers with various macromolecular architectures.

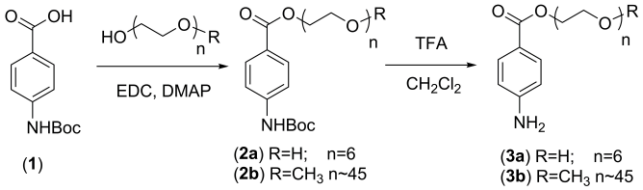
Results and Discussion

Design of polymer diazonium coupling agents.

Francis and coworkers highlighted the importance of utilizing highly reactive diazonium salts in order to achieve efficient Tyr targeting.[14] The reactivity of diazonium coupling agents can be increased by introducing electron-withdrawing groups in appropriate positions of their aromatic rings. In this regard, Francis *et al.* reported a decrease in the efficiency of diazonium decoration of the exterior protein shell of the tobacco mosaic virus (TMV), from >90 to ~30%, by replacing the diazonium salt of *p*-amino-nitrobenzene with the one obtained from *p*-amino benzamide, i.e. replacement of a NO_2 group to a less electron withdrawing $C(O)NH_2$.

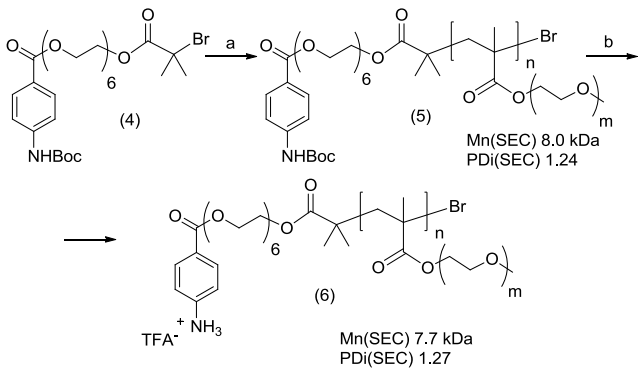
Diazonium reagents are generally prepared by reacting aromatic amines with *in situ* generated HNO_2 or nitrous esters.[19] In this work a range aniline-terminate-polymers were generated and converted to the required diazonium derivatives *in situ* during the peptide conjugation experiments. An ester moiety was chosen as the *para* group as, in addition to its electron withdrawing properties, it allowed for facile linking of the synthetic polymers employed with the aniline diazonium precursor moieties. In particular, two aniline-terminated poly(ethylene glycol)s (**3a**) and (**3b**) with FW = 401.5 Da and M_n= 2.0 kDa respectively, were prepared (Scheme 1). Briefly, Boc-protected 4-aminobenzoic acid (**1**) was reacted with an excess of hexa(ethylene glycol), for (**2a**), or a monomethoxy poly(ethylene glycol) (mPEG, 1.9 kDa) for (**2b**), in the presence of EDC and DMAP. Removal of the Boc protecting group with trifluoroacetic acid in dichloromethane afforded the desired aniline-terminated diazonium precursors (**3a**) and (**3b**) featuring electron withdrawing ester moieties *para*- to the aromatic amino group. For **(3b)** overall yields were high (79% from $mPEG_{2000}$ starting material) with extremely high purity confirmed by MALDI ToF analysis (see supporting information).

The range of polymers for use in diazonium conjugation of peptides was further expanded by preparing a grafted PEG-based polymer with different macromolecular architecture, p(mPEG MA), bearing the same α-functionality. Conjugating polymers with this structure have emerged in recent years as key macromolecular intermediates for the preparation of polymer-(poly)peptide conjugates [20-24] and others.[25-28] In this study a relatively low molecular weight comb polymer was prepared, in order to facilitate the purification and characterization of the peptide conjugates.



Scheme 1. Synthesis of poly(ethylene glycol)-functional anilines as precursors for diazonium-terminated conjugating PEGs

Polymerization[29-31] of mPEG(475) MA in the presence of Boc-protected-p-aminobenzoic ester initiator (**4**) and Cu(I)Br/pyridinylimine ligand afforded the polymer intermediate (**5**), M_n(NMR) = 8.0 kDa and PDi=1.24. Removal of the Boc-protecting group afforded the final aniline-terminated poly(mPEG(475) MA) polymer (**6**), which was used later as a diazonium-terminated polymer precursor.



Scheme 2. *Reagents and conditions:* a. Cu(I)Br/*N*-(ethyl)-2-pyridylmethanimine, $mPEG_{475}MA$, toluene, 50 °C; [(4)]:[$mPEG_{475}MA$]:[Cu]:[ligand] = 1:10:1:3; b. CF_3COOH, dichloromethane, 25 °C.

Conjugation to Tyrosine-containing peptides. Coupling of diazonium derivatives to (poly)peptides is a technique that has been exploited for more than 100 years.[32] The high reactivity of these substrates makes them ideal for protein labeling although one of the major drawbacks in using this approach lies in the very poor selectivity often observed in these conjugation reactions.[33-35] Diazonium derivatives have been used for non-specific targeting of a number of proteins for a range of applications spanning from the introduction of haptens onto

protein conjugates[36] to the supporting of proteins onto PVA polymer matrices.[37]

Tryptophan has been reported to react efficiently with diazonium salts such as 3-diazonium-1,2,4-triazole (3-DT) at very low pH (<3) with reactivity decreasing with an increase in pH.[38] All of the other amino acid targets, *N*-nucleophiles such as Lysine, (poly)peptide *N*-termini and Arginine, or *C*-nucleophiles Tyrosine and Histidine, and the *S*-nucleophiles free Cysteine, present acid/base moieties which affect their reactivity towards diazonium coupling reagents.[33] A general trend for the *N*-nucleophiles (Lys, Arg and *N*-terminal amine) is that the lower the pH, the higher proportion of the nucleophilic nitrogen centers will be in their protonated form, which is non-reactive towards electrophilic reagents.

Histidine and Tyrosine follow the same trend,[37] albeit for different reasons. Protonation of the His basic nitrogen removes electronic density from its aromatic ring, which can reduce – or even eliminate, depending on the pH - its nucleophilic character. Tyrosine residues can, in principle, react with nucleophiles both in their neutral Tyr and deprotonated tyrosinate forms. The latter, is more reactive than the former and an increase in the rate of diazo-coupling with an increase in the pH has been observed. For naphthols it has been reported that the reactivity of the naphtholate ion, is appoximately 10^{10} times higher than that of the undissociated naphthol, implying that it always reacts with diazonium salts in its $TyrO^-$ form unless at pH < 1-2, where the rate coupling reaction is almost negligible. Selective modification of protein Tyr residues so far has been restricted either to proteins lacking exposed competitive conjugation sites (i.e. Lys, Cys and His)[14] or to less reactive diazonium salts bearing a ligand, uridine 2'(3')-phosphate, able to direct them in the immediate vicinity of a specific Tyr residue in an enzyme binding site (Tyr73 of Ribonuclease A).[39]

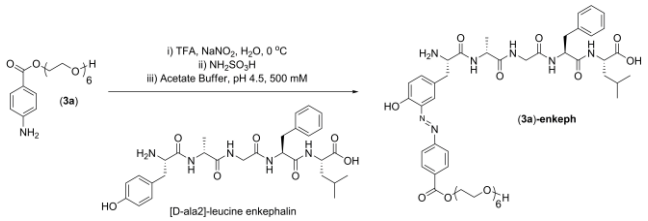
Herein we reasoned that preferential (or ideally exclusive) selectivity in targeting tyrosine residues could have been obtained by utilising appropriate diazonium reagents, by judiciously choosing the experimental conditions at which the coupling reactions are performed. The pKa of protonisable aminoacids can vary dramatically depending on where these fragments are within a (poly)peptide sequence. Remarkable differences from expected values are generally observed in non-solvent exposed aminoacids in which the presence of hydrophobic/hydrophilic local environments in addition to the presence of hydrogen bonds can alter remarkably their acid/basic behavior.

Very broadly speaking, protein *N*-nucleophiles all feature a relatively high pKa in aqueous environments, where the guanidine fragment of Arg has pKa ~12, ε-amino groups of Lys residues pKa ~10, *N*-terminal amino acid residues pKa ~8.[40] In their protonated form these fragments are not reactive towards electrophilic agents (i.e. conjugating polymers). Selective PEGylation of (poly)peptide *N*-terminal aminoacids can be achieved by reductive amination with α-aldehyde PEGs at pH 5-6, were a higher proprortion of more basic protein reactive sites, i.e. ε-amino groups of Lys residues, are all in their non reactive protonated form.

The pKa's of C-nucleophiles can be rather diverse, Tyr typically having pKa ~10 and His pKa ~6, although for the latter an extremely wide range of values, from 2.3 to 9.2, which correlate with burial within proteins, have been observed.[41] Due to the high reactivity of tyrosinate/tyrosine residues towards diazonium reagents, the coupling has been reported to occur at pH as low as 4.[37] In the same study the authors reported that over the 4.0-9.0 interval of pH investigated, Tyr analogues had higher reactivity than the corresponding His derivatives.

In this present work we aimed at reducing the pH at which the conjugation reaction was conducted to a point in which all nitrogen-containing amino acid would be in their protonated and non-reactive form, whilst utilizing reactive diazonium coupling agents till able to react with Tyr residues under these conditions. Tracey and Shuker have shown that the even at higher pH of 8.8, EWG-activated diazonium salts have the tendency to react with Tyr and His, even in the presence of unprotected Trp, Lys and terminal amino group.[42]

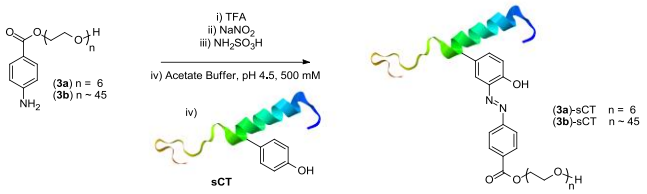
For the first conjugation experiment a model peptide, [D-ala2]-leucine enkephalin, an opioid pentapeptide containing a single tyrosine residue, was chosen (Scheme 3). In addition to the substituted phenol, Tyr1 presents an *N*-terminal amino group that could represent a competitive attachment site for polymer conjugation. Hexa(ethylene glycol) precursor (**3a**) was used as the conjugating agent, due to his intrinsically monodisperse nature and small size, which facilitated the study of the coupling reaction and the characterization of the final adduct(s). The conjugation reaction was carried out at pH 4.5, where all the amino groups were expected to be mainly in their protonated and non-reactive form. The process was monitored by ^{1}H NMR and was found to be extremely neat, with rapid and quantitative synthesis of diazonium derivatives from the parent anilines (see supporting information). The required diazonium salts was generated *in situ* by sequential addition of trifluoroacetic acid and $NaNO_2$, at 0 °C. After 30 min sulfamic acid was added, so as to destroy excess nitrous acid generated in the previous step. The pH was adjusted to 4.5 with 500 mM acetate buffer and [D-ala2]-leucine enkephalin was finally added. The reaction was performed at 4 °C for 24 h, during which the colour of the reaction solution gradually turned from colorless to orange due to formation of the azobenzene linker. After quenching of the excess diazonium coupling agent with *p*-cresol and purification by SEC chromatography, MALDI-ToF-MS analysis revealed the presence of a single product with the expected m/z = 982 Da $[M+H]^+$ for the monoadduct (**3a**)-enkeph (see supporting information). It is important to note that (**3a**)-enkeph monoadduct was only product observed, even though a 10:1 excess of diazonium conjugating agent was employed in the coupling reaction.



Scheme 3. Conjugation of diazo-functional model-PEG (**3a**) to Tyr1 residue of [D-ala2]-leucine enkephalin.

Following this successful modification of [D-ala^2]-leucine enkephalin, the versatility of the conjugation strategy was tested with a more complex peptide, Salmon Calcitonin (sCT). sCT is a 32-aminoacid hormone currently prescribed for the treatment of bone-related disorders including osteoporosis, Paget's disease and hypercalcemia. sCT's ipocalcemic proper-

tities have been ascribed to a number of concurrent phenomena, including its ability to inhibit the activity of osteoclasts in bones, and to promote Ca^{2+} renal excretion.[43] Most importantly for the scope of this study, sCT is a very well characterised peptide which presents only one Tyrosine residue (Tyr22) for polymer conjugation, in addition to a number of other potential conjugating sites, Cys1 (*N*-terminal amine), Lys11 and Lys18, His17, and Arg24, which made sCT an ideal substrate for testing the selectivity of the diazonium conjugation process. Moreover, Tyr22 is included in a short portion of the primary structure of sCT - from Leu19 to Tyr22 - that can be modified, or even completely removed, with no loss of bioactivity.[44] This made Tyr22 an ideal targeting site, as its modification was expected to have minimal impact on the bioactivity of sCT.



Scheme 4. Conjugation of mPEG diazonium precursors (**3a**) and (**3b**) to salmon calcitonin (sCT).

Initial investigations were again conducted using the hexa(ethylene glycol) precursor (**3a**) under conditions analogous to those previously optimized for [D-ala^2]-leucine enkephalin. Upon addition of sCT, no immediate colour change was observed but over a period of time, the solution became orange, due to the formation of the azo-linker. Samples were taken periodically and analysed by MALDI-ToF-MS. Analysis of the reaction mixture taken after 8 h confirmed the presence of unreacted sCT, along with a single peak corresponding to the mass of (**3a**)-sCT conjugate (Figure 1). It should be noted that MALDI-ToF-MS spectrometry is remarkably more sensitive to unmodified sCT than to (**3a**)-sCT, with the latter found to be the major product at the end of the diazonium coupling reaction. Neither for the synthesis of (**3a**)-enkeph not for (**3a**)-sCT, peak corresponding to the loss of 17 mass units from the conjugates, commonly observed in diazonium coupling with proteins and peptides,[14] was observed.

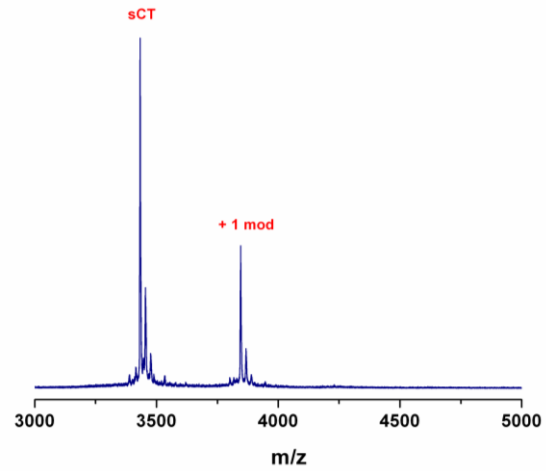


Figure 1. MALDI-ToF-MS spectrum of the diazonium-coupling reaction of sCT with (**3a**) at pH 4.5, at $[sCT]_0$:($[(\mathbf{3a})]_0$ =1:20.

Although the exact nature of such elimination product has never been established, one explanation for this could be a formal elimination of ammonia, possibly *via* reaction of the diazonium derivative with either the *N*-terminal amine or a Lys residue to generate a monoalkykl-triazene, followed by its β-elimination, a common decomposition route for these intermediates[45, 46] (although other degradation processes are also possible).[47]

The influence of the pH on the selectivity of the coupling process was then investigated by performing the conjugation reaction at pH 7.0 (500 mM). Upon incubation of sCT, an immediate colour change was observed with the solution turning dark purple. RP-HPLC analysis indicated that sCT had been immediately consumed and MALDI-ToF-MS analysis of the reaction solution showed a mixture of products, ranging from a small peak corresponding to native sCT to the polypeptide conjugated to as many as five molecules of hexa(ethylene glycol) diazonium (**3a**) (Figure 2). The presence of up to five molecules of conjugating molecules per sCT is may be ascribed to conjugation to Tyr22, His17, the *N*-terminal amine, Lys11 and Lys18, whilst the pKa of the Arginine (Arg24) may be too high to ensure efficient conjugation of this residue at pH 7.0.

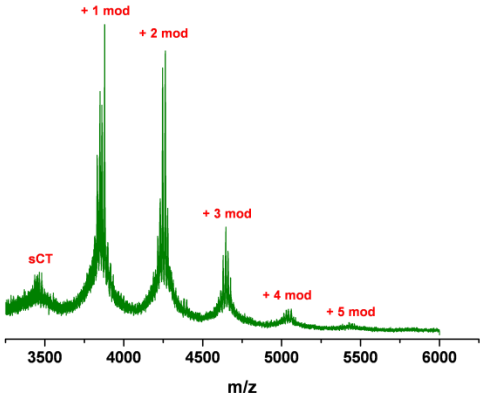


Figure 2. Hyperfine MALDI-ToF-MS spectrum of the diazonium-coupling reaction of sCT with hexa(ethylene glycol) diazonium (**3a**) performed at pH 7.0, at ($[(\mathbf{3a})]_0$:$[sCT]_0$ = 10:1.

The coupling reaction conducted at intermediate pH values, namely 5.4 and 6.2, led to multiple conjugation similar to that observed at pH 7.0, although the observed rate of conjugation was significantly slower (see supporting information).

Conjugation with $mPEG_{2000}$ (**3b**) at pH 4.5 was performed at 2.5, 10 and 20 **(3b)**:sCT molar ratios in order to optimise the rate and efficiency of the conjugation reaction. The reaction was monitored by RP-HPLCequipped with a C_{18} column. Conversion was found to be 21, 54, and 78%, respectively, after 56 h..

Pure (**3b**)-sCT was isolated, with relatively low purification yield (24%), after ion exchange –FPLC. MALDI-ToF-MS analysis (Figure 3) showed a single distribution of peaks with the observed mass in the range expected for (**3b**)-sCT. No conjugates with higher molecular mass were detected, neither by RP-HPLC nor MALDI-ToF-MS, suggesting again that under the conditions employed a 1:1 polymer:sCT was obtained.

Finally, the same coupling reaction was performed utilizing poly(mPEG(475) methacrylate) (**6**) as the diazonium precursor. The coupling process proved to be as efficient as the ones previously performed with the structurally simpler linear polymers (**3a**) and (**3b**), reaching 90% after 72 h at 4°C. Again, the conjugate was purified by ion-exchange chromatography (FPLC) affording pure conjugate. Biohybrid materials consisting of protein or peptide conjugates with comb mPEG (meth)acrylates are extremely difficult to characterize by MALDI-ToF mass spectrometry[48] with the only known example reported to date being restricted to conjugates with low M_n.[49] The conjugate sCT-(**6**) was characterized by cleavage of the diazo linker followed byanalysis of the resulting modified peptide and its tryptic digests (*vide infra*). All polymer conjugations were repeated several times (3 to 5 depending on the conjugate) and results were found to be reproducible.

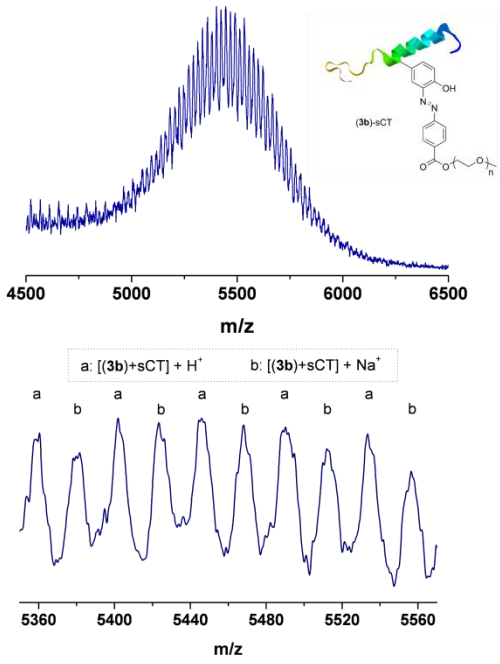


Figure 3. (top) MALDI-ToF-MS analysis of purified sCT-(**3b**) (PEG_{2000}). (bottom) Detail of sodiated and protonated (3b)-sCT. (**3b**)-sCT. A more detailed account is reported in the supporting information section.

Characterization of sCT-polymer conjugates: attachment site.

Following observations that under specific conditions, sCT-polymer monoconjugates could be synthesized, it was necessary to confirm the conjugation site. An immediately evident feature of all the conjugation reactions is the change of colour from colourless to orange, indicative of the formation of diazo adducts. In addition, the colour seemed to assume a more intense orange coloration with increasing pH. UV-vis analysis of isolated (**3b**)-sCT showed a maximum at λ = 471 nm, and upon lowering gradually the pH to 5.0, a disappearance of this was observed, with the formation of a band at λ = 372 nm (Figure 3). For comparison purposes, the diazonium salt of (**3b**) was conjugated with water-soluble model histidine (**8**) and tyrosine (**9**) mimics. UV-vis spectra of the isolated conjugates (**3b**)-(**8**) prepared from a His mimic showed very little, if any, dependence on the pH. Conversely, analysis of (**3b**)-(**9**) bearing a Tyr mimic changed markedly with the pH, with a band at approximately 500 nm with intensity that increased with the pH, a behavior which parallel that observed for the calcitonin conjugate (**3b**)-sCT. This is in agreement with literature data for other diaza-Tyr conjugates, in which the appearance of a band at approximately 500 nm was ascribed to a chromophore formed by deprotonation of a diaza-phenolic moiety.[39] Further titration studies allowed to estimate a pKa~11 for both the model diaza-Tyr conjugate (**3b**)-(**9**) and (**3b**)-sCT, again suggesting a strong involvement of Tyr22 in the diazonium polymer conjugation reaction.

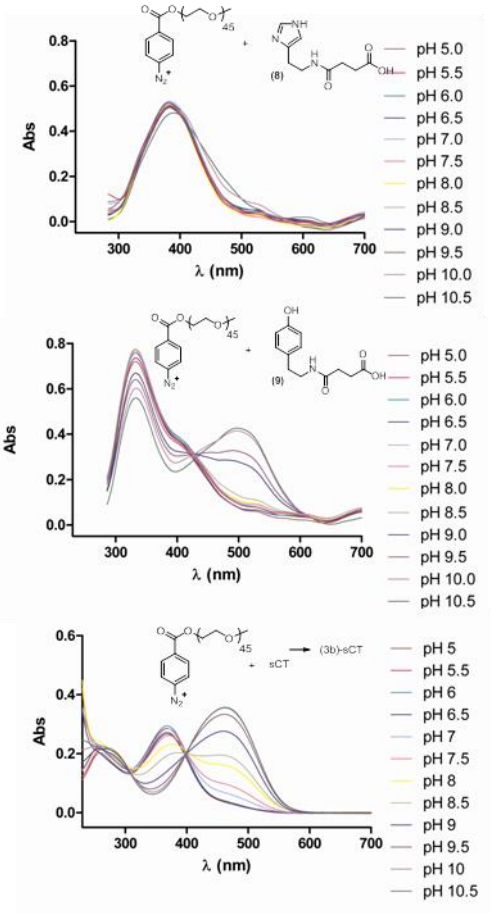


Figure 4. UV-Vis spectra of (**3b**)-sCT and purified conjugates of (**3b**) with histidine (**8**), and tyrosine (**9**) mimics, at various pH.

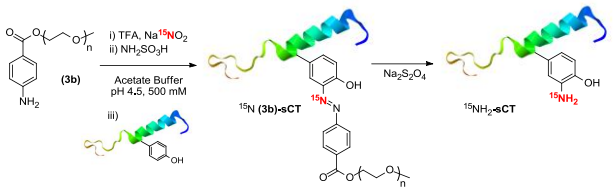
Further investigations involved the isotopic labeling of the peptide conjugates using $Na^{15}NO_2$ for the preparation of diazonium salts used for the coupling reactions. This allowed for the investigation of the peptides amino acid attachment site, by NMR, and also providid a means to discriminate the nitrogen atom introduced in the peptides from the nitrogen's already present, facilitating the characterization of the conjugates peptides by mass spectrometry.

Conjugation reaction of (**3a**) to [D-ala^2]-leucine enkephalin, and aniline-functional linear (**3b**) and comb (**6**) polymers to sCT was repeated as described previously, this time using $Na^{15}NO_2$. TOCSY NMR of (**3a**)-[D-ala^2]-leucine enkephalin confirmed the conjugation of (**3a**) to the model pentapeptide. Although the presence of conformational isomers makes the analysis of the conjugate rather complicated, spin systems corresponding to a 1,3,4-tri-substituted benzene ring further

provide strong evidence of conjugation to Tyr1 (see supporting information).

An additional potential advantage offered by peptide conjugates bearing a diazo linker is the possibility to selective cleavage in the presence of specific external reducing stimuli, i.e. colon azoreductase enzymes. Several currently prescribed therapeutics, i.e. Ipsalazide, Balsalazide and Sulfasalazine, are administered as prodrugs containing diazo linkers that can be cleaved by colon azoreductases releasing the required active drugs.[50, 51] Several drug delivery systems based on polymer containing diazo linkers have also been extensively investigated.[51-53] Reversible PEGylation of peptides with and colon release are beyond the scope of the present work.

The polymers were cleaved from the conjugated peptides using sodium dithionite. The reaction was optimized and investigated using a model tyrosine conjugated prepared by reacting the diazonium derivative obtained from mPEG aniline (**3b**) and *p*-cresol. This was then used in conjugation reactions as a means to quench the excess of diazonium polymers. Conjugates with *p*-cresol were therefore produced at the end of every conjugation run, and appeared to be ideal simple substrates for the optimization of the reductive cleavage of diaza linkages. Upon addition of 1 eq. of sodium dithionite reducing agent an immediate loss of the characteristic orange colour of the model conjugated was observed, indicating rapid and quantitative cleavage of the azobenzene-linker, yielding the parent aniline functional polymers and 2-amino-4-methyl phenol, as confirmed by ^{1}H NMR, UV-Vis and MALDI-ToF-MS analysis (see supporting information).



Scheme 5. ^{15}N isotopic labelling of the azobenzene linker in the diazonium coupling of aniline mPEG (**3b**) to sCT, followed by release of $^{15}NH_2$-sCT by reductive cleavage with $Na_2S_2O_4$.

The experiment was then repeated with ^{15}N labeled (**3b**)-sCT and (**6**)-sCT which resulted in the release of sCT with an additional $^{15}NH_2$ group (Scheme 5 for (**3b**)-sCT).

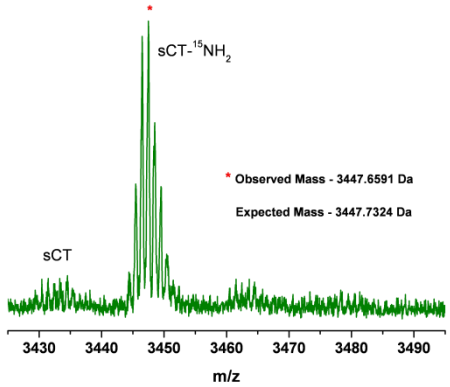


Figure 5. MALDI-TOF spectrum of sCT-$^{15}NH_2$ adduct obtained by cleavage of the (**6**)-sCT azo-linker using sodium dithionite.

Again, upon addition of sodium dithionite to a solution of all sCT conjugates at pH 7.2, an immediate loss of the characteristic orange colour of the linker was observed and the reduction confirmed by UV-Vis spectroscopy.

MALDI-ToF-MS analysis of the cleaved conjugates (Figure 5 for (**6**)-sCT) confirmed the presence of the amino-modified sCT, with the expected mass gain of 16.0 Da observed due to introduction of a -$^{15}NH_2$ group to the polypeptide. Small signals corresponding to unmodified sCT and traces higher mass species were also observed, which may be derived from higher conjugated species. Qualitative analysis of MALDI-ToF-MS spectra showed that smaller amounts of these minor impurities were present when poly(mPEG$_{(475)}$ MA) (**6**) was employed, suggesting that its branched structure may provide better protection towards further polymer conjugation.

In order to determine the modification site of the amino-modified polypeptides sCT-$^{15}NH_2$ obtained by reductive cleavage of sCT-polymer conjugates were subjected to trypsin digestion, which cleaved them at the *C*-terminus of their Lys and Arg residues. Under these conditions unmodified sCT yields four fragments, Cys1-Lys11, Leu12-Lys18, Leu19-Arg24, and Thr25-Pro(NH_2)32. This approach to determine the attachment site in sCT-polymer conjugates was conducted by analogy to that previously described by us[49] and Lee *et al.*[54] His17 and Tyr22 residues reside on separate fragments, Leu12-Lys18 and Leu19-Arg24 respectively, and therefore the selectivity of the conjugation process could be determined by MALDI-ToF-MS analysis of the digest fragments. For both ^{15}N (**3b**)-sCT and (**6**)-sCT analysis of the region corresponding to the Leu19-Arg24 tyrosine-containing fragment revealed a distribution corresponding to a $^{15}NH_2$ labeled fragment (addition of 16 Da) as well as the analogous residue from the digestion of the traces of unconjugated sCT present as an impurity in the isolated (**6**)-sCT biohybrid material, and some secondary distributions (Figure 6).

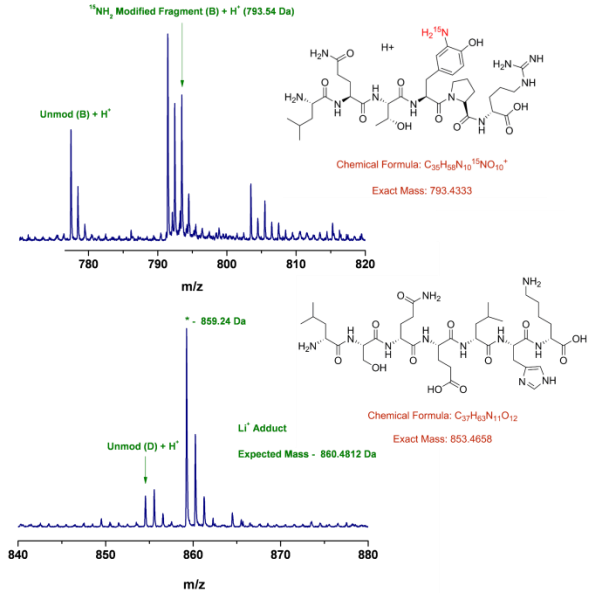


Figure 6. Trypsin digestion of (**6**)-sCT conjugate: MALDI ToF MS analysis of Leu12-Lys18 and Leu19-Arg24.

All of the other trypsin digest fragments were identical to those of native sCT, which is consistent of little, if any, conju-

gation of the diazonium polymer at any other residues but Tyr22.

***In vitro* and *in vivo* preliminary studies on the bioactivity of sCT-polymer conjugates.** In order to assess the biological activity of the conjugates prepared in this study, i*n vitro* tests were carried out by monitoring the ability of the (**3b**)-sCT conjugate to recognize and activate the calcitonin receptor in T47D human breast cancer cells.

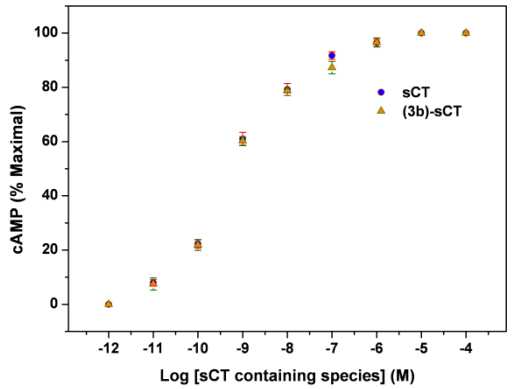


Figure 7. ELISA measurement of the increase of intracellular cAMP induced in T47D human breast cancer cells bearing sCT receptors by (**3b**)-sCT and native sCT.

In vitro results revealed that (**3b**)-sCT was able to significantly elevate intracellular cAMP levels (Figure 7) with no loss in bioactivity compared to native sCT. In these experiments two negative controls, PBS alone and and polymer **(3b)** in PBS were employed. Both negative controls showed no cAMP was produced. A positive control; forskolin was also included to ensure that the cell model was working correctly.

Following the very promising *in vitro* behavior of this conjugate, *in vivo* tests aimed at investigating the ability of (**3b**)-sCT to lower [Ca^{2+}] plasma levels in mammals were conducted using male Wistar rats. Blood samples were taken periodically up to 240 minutes after injections into the tail vein and the plasma total calcium analysed. The (**3b**)-sCT conjugate was shown to have a comparable ability in lowering plasma calcium compared to unmodified sCT (Figure 8). Negative control data using i.v. injection of PBS were without effect on plasma calcium levels.[22] Homeostatic events mean that plasma calcium levels do not move from 2.5 mM in untreated conditions.

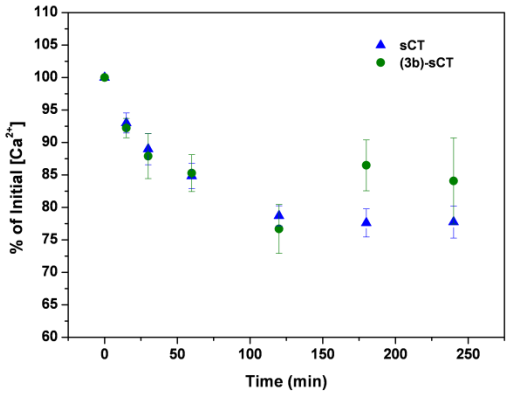


Figure 8. Plasma total calcium profiles of male Wistar rats following intravenous injections of sCT (blue triangles) and (**3b**)-sCT conjugate (green circles).

In summary, this work describes novel general approach to direct polymer conjugation to peptides, based on diazonium-terminated materials with various macromolecular architecture. A short oligoethylene glycol, a well-defined linear PEG and poly($mPEG_{(475)}$ methacrylate) prepared by ATRP were synthesized and employed for the targetting of Tyr residues of [D-ala2]-leucine enkephalinamide and salmon calcitonin model peptides. Experimental conditions, especially in terms of the pH used for the peptide conjugation reactions, were optimized for the specific targeting of tyrosine residues. UV-vis and NMR experiments, in conjunction with mass spectrometry analysis of the fragments obtained from trypsin digestion of reduced polymer-peptide conjugates indicated good selectivity for Tyr conjugation. Preliminary *in vitro* experiments aiming at monitoring the increase of intracellular cAMP induced in T47D human breast cancer cells bearing sCT receptors by (**3b**)-sCT revealed a virtually identical potency for the diazo protein-peptide conjugate compared to native sCT. This PEGylation approach is reversible and parent aniline polymers can be recovered from their peptide conjugates in the presence of appropriate reducing agents, including sodium dithionite, and, potentially, azoreductase enzymes like those found in the gastrointestinal tract of mammals.[55,56, 57] which may open the way for the use of our approach for the development of colon specific drug delivery systems. *In vivo* preliminary experiments revealed that our diazo sCT-PEG_{2000} conjugate displayed a comparable ability in lowering the plasma [Ca^{2+}] of male Wistar rats.

The approach for polymer-peptide conjugation presented in this work could complement well the set of polymer conjugation techniques – i.e. *N*-terminal amine conjugation with aldehyde-functional polymers - that already allow to preferentially (or sometimes exclusively) target specific peptide amino acids in the presence of other possible peptide conjugation sites. Direct polymer conjugation to tyrosine residues appears to be a rather versatile approach and could potentially be applied to conjugates which include a plethora of different polymeric materials other than PEG or PEG-based polymers. In addition, this strategy could be expanded to a number of other biologically relevant peptides (and possibly proteins), as long as the conjugation conditions are optimized to take into account the specific characteristics (i.e. exposition and pKa of relevant amino acid residues) of the conjugated peptide. This work showed that for the specific case of sCT Tyr22 conjugation with linear PEG_{2000} occurred with no loss of bioactivity *in vitro*, as measured by the increase of intracellular cAMP induced in T47D human breast cancer cells bearing sCT receptors. Preliminary experiments *in vivo* showed that the same conjugates had ability to lower plasma total [Ca^{++}] in Wistar Rats comparable to that of sCT, further confirming that polymer conjugation did not interfere with the bioactivity of sCT. Tyr22 is included in a portion of the sCT peptide sequence that can be modified or even removed with no loss of bioactivity. This in itself highlights the importance of having an ample toolkit of conjugation techniques that allow site-specific conjugation at sequence domains that are not involved in the active sites of proteins and peptides. The present study aimed at complementing the existing polymer conjugation techniques with what to the best of our knowledge is the first example of site-specific direct polymer conjugation at Tyr residues.

Supporting Information. Experimental section, including synthetic procedures, characterization of polymers and all intermediates, bioconjugation experiments, in vitro and in vivo test procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*D.M.Haddleton@warwick.ac.uk

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