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

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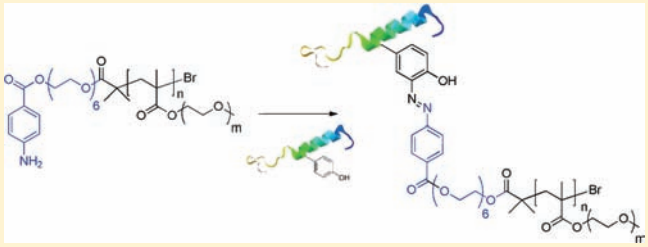
Supporting Information

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 monomethoxy poly(ethylene glycol)s (mPEGs) and poly(mPEG) methacrylate prepared by atom transfer radical polymerization. Judicious choice of the reaction conditions—pH, stoichiometry, and chemical structure of diazonium salt—led to a high degree of site-specificity in the conjugation reaction, even in the presence of competitive peptide amino acid 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's ability to increase 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), or 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 amino acids, such as the primary amine at *N*-termini in hystidine and lysine and 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 in the immediate vicinity of, crucial points in the polypeptide sequence. These would include the binding sites of hormones and the catalytic sites of enzymes. It is therefore key that polymer conjugation occurs preferentially, if not exclusively, at specific amino acids 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 profiles and clinical efficacy compared to the native protein therapeutics. However, upon polymer conjugation, some 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 components 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 PEGylation strategies alternative and complementary to those being used clinically. One breakthrough was the work by Brocchini and co-workers, who first described the site-specific PEGylation of disulfide bridges of a number of proteins 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 co-workers have reported a number of efficient strategies whereby tyrosine residues were targeted via a three-

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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 several reports have described 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 functionalization of TMV involving diazonium coupling at tyrosine, and this has 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 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-catalyzed *O*-alkylation by Trost allylation is an efficient process, although the need for a potentially toxic transition metal catalyst (albeit at very low concentration) may make this less attractive for the direct preparation of protein and peptide derivatives of pharmaceutical interest. Conversely, diazonium couplings are 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.

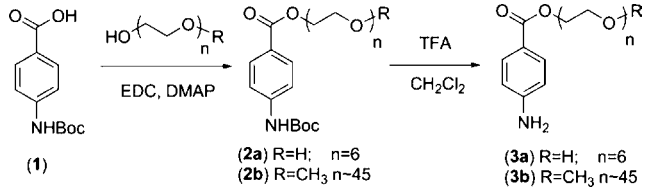
Despite these promising recent advances, to date, no report has described *direct* polymer conjugation, including PEGylation, to tyrosine residues of (poly)peptides. In this current work we have developed a novel and potentially general route to polymer−peptide biohybrid materials via preferential targeting 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 co-workers 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 (EWGs) 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 TMV, from >90 to ∼30%, by replacing the diazonium salt of *p*-amino-nitrobenzene with the one obtained from *p*-aminobenzamide, i.e., replacement of a NO_2 group with 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 of aniline-terminated 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,

Scheme 1. Synthesis of Poly(ethylene glycol)-Functional Anilines as Precursors for Diazonium-Terminated Conjugating PEGs



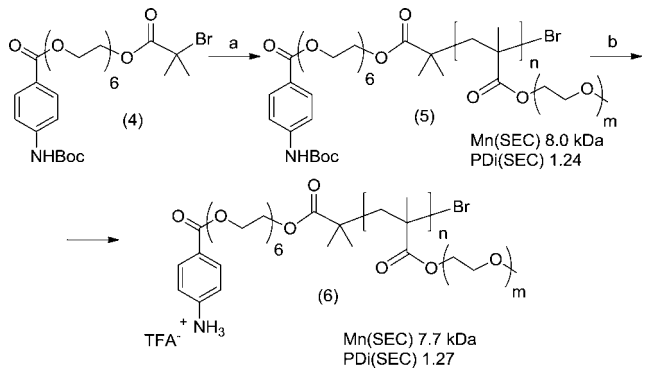
Boc-protected 4-aminobenzoic acid (**1**) was reacted with an excess of hexa(ethylene glycol) for **2a**, or a monomethoxy poly(ethylene glycol) (mPEG, 1.9 kDa) for **2b**, in the presence of EDC and DMAP. Removal of the Boc protecting group with trifluoroacetic acid in dichloromethane afforded the desired 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 available 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.

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**, Scheme 2), which was used later as a diazonium-terminated polymer precursor.

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

Scheme 2[a]



[a]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.

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these conjugation reactions.[33−35] Diazonium derivatives have been used for nonspecific targeting of a number of proteins for a range of applications spanning the introduction of haptens onto protein conjugates[36] to the support of proteins on 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, *C*-nucleophiles tyrosine and histidine, and *S*-nucleophile free cysteine, present acid/base moieties which affect their reactivity toward diazonium coupling reagents.[33] A general trend for the *N*-nucleophiles (Lys, Arg, and *N*-terminal amine) is that with lower pH, higher proportions of the nucleophilic nitrogen centers will be in their protonated form, which is nonreactive toward 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 in both 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 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 of the coupling reaction makes it 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 utilizing appropriate diazonium reagents, by judiciously choosing the experimental conditions at which the coupling reactions are performed. The pK_a of protonizable amino acids 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 amino acids, in which the presence of hydrophobic/hydrophilic local environments in addition to the presence of hydrogen bonds can alter remarkably their acid/basic behavior.

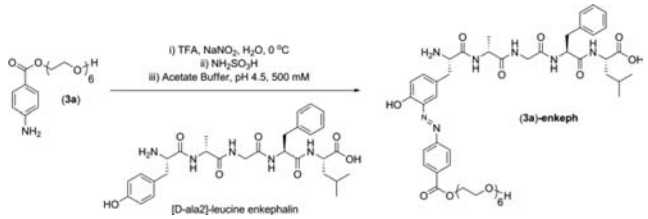
Very broadly speaking, protein *N*-nucleophiles all feature a relatively high pK_a in aqueous environments, where the guanidine fragment of Arg has pK_a ≈ 12, ε-amino groups of Lys residues pK_a ≈ 10, and *N*-terminal amino acid residues pK_a ≈ 8.[40] In their protonated form these fragments are not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

The pK_a values of C-nucleophiles can be rather diverse, Tyr typically having pK_a ≈ 10 and His pK_a ≈ 6, although for the latter an extremely wide range of values have been observed, from 2.3 to 9.2, which correlate with burial within proteins.[41] Due to the high reactivity of tyrosinate/tyrosine residues toward 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 at which all nitrogen-containing amino acids would be in their protonated and nonreactive form, while utilizing reactive diazonium coupling agents able to react with Tyr residues under these conditions. Tracey and Shuker have shown that even at the 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 a terminal amino group.[42]

For the first conjugation experiment, a model peptide was chosen, [D-ala2]-leucine enkephalin, an opioid pentapeptide containing a single tyrosine residue (Scheme 3). In addition to

Scheme 3. Conjugation of Diazo-Functional Model PEG 3a to Tyr1 Residue of [D-ala2]-Leucine Enkephalin



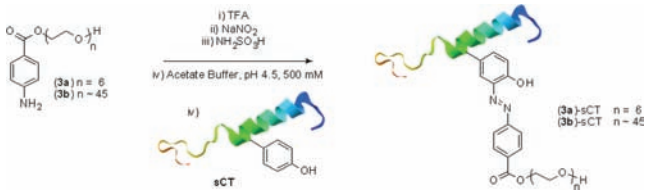
the substituted phenol, Tyr1 presents an *N*-terminal amino group that could represent a competitive attachment site for polymer conjugation. Hexa(ethylene glycol) precursor **3a** was used as the conjugating agent, due to its intrinsically monodisperse nature and small size, which facilitated the study 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 nonreactive 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 were 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 time 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 size exclusion 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 the only product observed, even though a 10:1 excess of diazonium conjugating agent was employed in the coupling reaction.

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-amino-acid hormone currently prescribed for the treat-

ment of bone-related disorders including osteoporosis, Paget's disease, and hypercalcemia. sCT's hypocalcemic propertities 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 characterized peptide which presents only one tyrosine residue (Tyr22) for polymer conjugation, in addition to a number of other potential conjugating sites, including Cys1 (*N*-terminal amine), Lys11 and Lys18, His17, and Arg24, which make 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 makes Tyr22 an ideal targeting site, as its modification is expected to have minimal impact on the bioactivity of 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 color change was observed, but over a period of time, the solution became orange, due to the formation of the azo-linker (Scheme 4).

Scheme 4. Conjugation of mPEG Diazonium Precursors 3a and 3b to Salmon Calcitonin (sCT)



Samples were taken periodically and analyzed by MALDI-TOF-MS. Analysis of the reaction mixture 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 nor for **3a**-sCT was the peak observed corresponding to the loss of 17 mass units from the conjugates, commonly observed in diazonium coupling with proteins and peptides.[14]

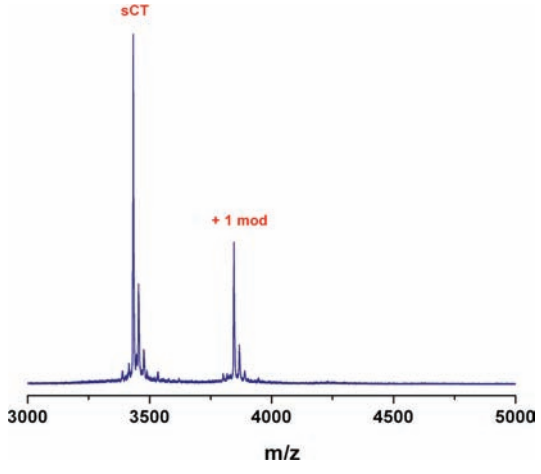


Figure 1. MALDI-TOF-MS spectrum of the diazonium-coupling reaction of sCT with **3a** at pH 4.5, at $[sCT]_0$:$[\mathbf{3a}]_0$ = 1:20.

Although the exact nature of such an 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 monoalkyl-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 color 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

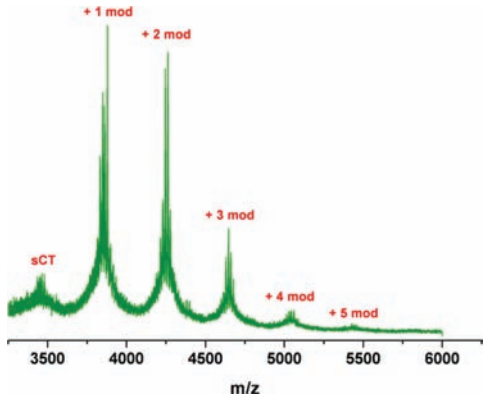


Figure 2. Hyperfine MALDI-TOF-MS spectrum of the diazonium coupling reaction of sCT with hexa(ethylene glycol) diazonium (**3a**) performed at pH 7.0, at $[\mathbf{3a}]_0$:$[sCT]_0$ = 10:1.

of up to five molecules of conjugating molecules per sCT may be ascribed to conjugation to Tyr22, His17, the *N*-terminal amine, Lys11, and Lys18, while the pK_a of Arg24 may be too high to ensure efficient conjugation of this residue at pH 7.0.

The coupling reaction conducted at intermediate pH values, namely 5.4 and 6.2, led to multiple conjugations 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 optimize 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

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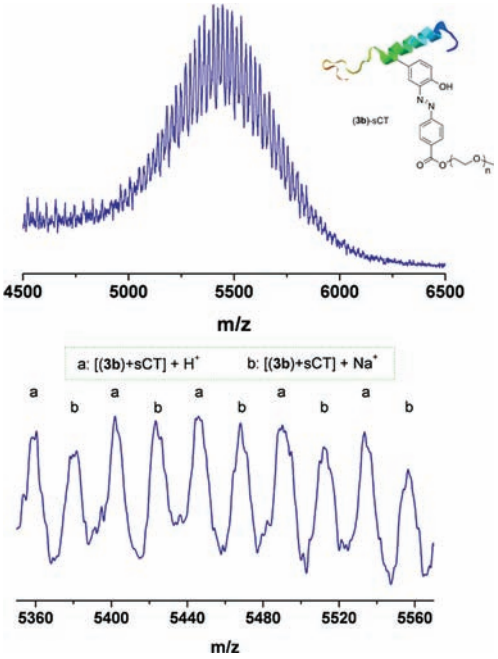

Figure 3. (Top) MALDI-TOF-MS analysis of purified sCT-**3b** (PEG_{2000}). (Bottom) Detail of sodiated and protonated **3b**-sCT. A more detailed account is reported in the Supporting Information.

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 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] 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 by analysis of the resulting modified peptide and its tryptic digests (*vide infra*). All polymer conjugations were repeated several times (3−5, depending on the conjugate), and the results were found to be reproducible.

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 the solution from colorless to orange, indicative of the formation of diazo adducts. In addition, the color 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 gradually lowering the pH to 5.0, this peak was observed to disappear, with the formation of a band at λ = 372 nm (Figure 4). 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 showed a marked change with the pH, with a band at approximately 500 nm with intensity that increased with the pH, in line with the behavior 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

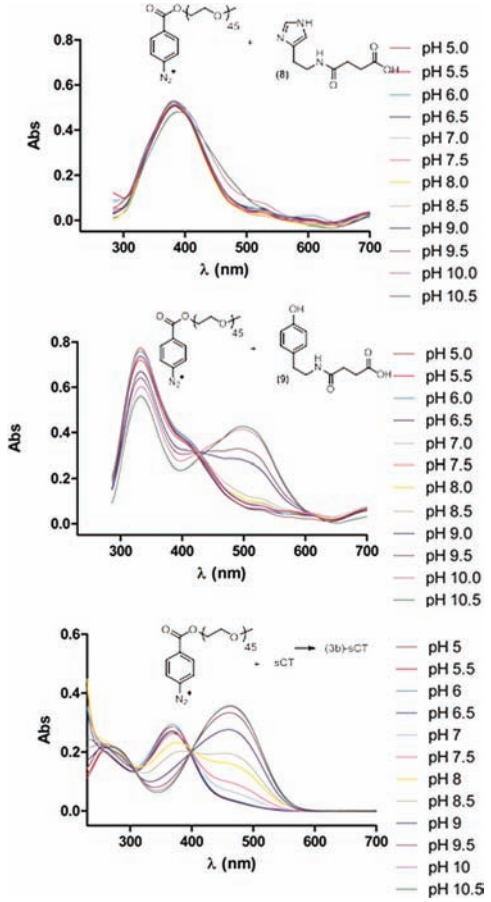

Figure 4. UV−vis spectra of **3b**-sCT and purified conjugates of **3b** with histidine (**8**) and tyrosine (**9**) mimics at various pH.

a chromophore formed by deprotonation of a diaza-phenolic moiety.[39] Further titration studies allowed us to estimate $pK_a \approx$ 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.

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 peptide's amino acid attachment site by NMR, and also provided a means to discriminate the nitrogen atom introduced in the peptides from the nitrogens already present, facilitating the characterization of the peptide conjugates by mass spectrometry.

Conjugation reactions of **3a** to [D-ala^2]-leucine enkephalin, and aniline-functional linear **3b** and comb **6** polymers to sCT, were 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-trisubstituted 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 for 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

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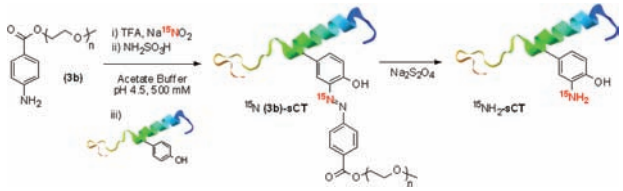
cleaved by colon azoreductases, releasing the required active drugs.[50,51] Several drug delivery systems based on polymer-containing diazo linkers have also been extensively investigated.[51−53] Reversible PEGylation of peptides and colon release are beyond the scope of the present work.

The polymers were cleaved from the conjugated peptides using sodium dithionite. The reaction was optimized and investigated using a model tyrosine conjugate 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 equiv of sodium dithionite reducing agent, an immediate loss of the characteristic orange color of the model conjugate was observed, indicating rapid and quantitative cleavage of the azobenzene linker, yielding the parent aniline functional polymers and 2-amino-4-methylphenol, as confirmed by ^{1}H NMR, UV−vis, and MALDI-TOF-MS analysis (see Supporting Information).

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). Again, upon

Scheme 5. ^{15}N Isotopic Labeling 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 $Na_2S_2O_4$



addition of sodium dithionite to a solution of all sCT conjugates at pH 7.2, an immediate loss of the characteristic orange color of the linker was observed, and the reduction was 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

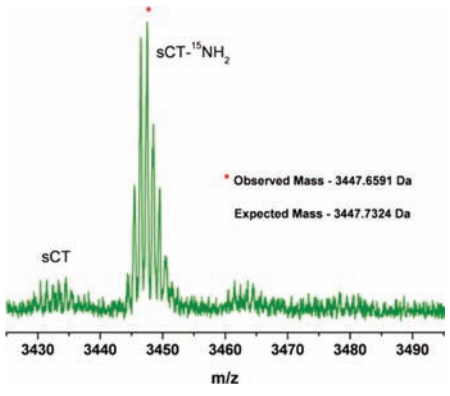


Figure 5. MALDI-TOF spectrum of sCT-$^{15}NH_2$ adduct obtained by cleavage of the **6**-sCT azo-linker using sodium dithionite.

signals corresponding to unmodified sCT and traces of 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 toward 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 was subjected to trypsin digestion, which cleaved it at the *C*-terminus of the 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).

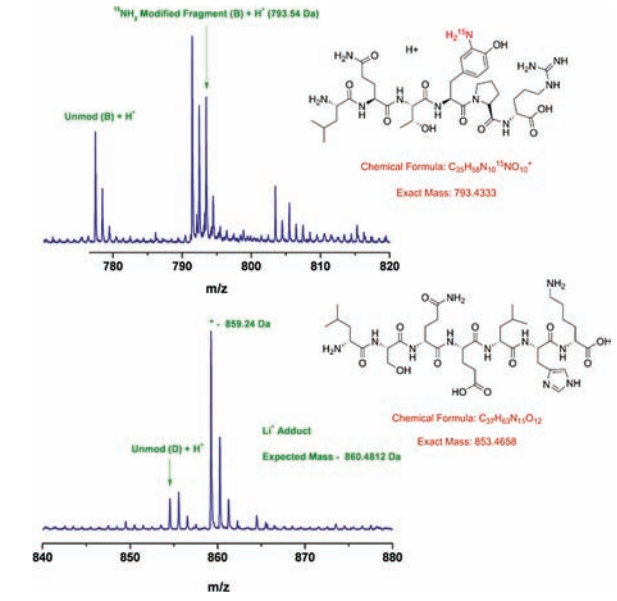


Figure 6. Trypsin digestion of **6**-sCT conjugate: MALDI-TOF-MS analysis of Leu12-Lys18 and Leu19-Arg24.

All of the other trypsin digest fragments were identical to those of native sCT, which is consistent with little, if any, conjugation 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, *in 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.

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In vitro results revealed that **3b**-sCT was able to significantly elevate intracellular cAMP levels (Figure 7) with no loss in

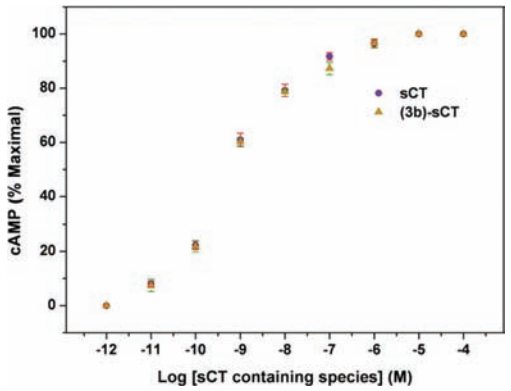

Figure 7. ELISA measurement of the increase of intracellular cAMP induced in T47D human breast cancer cells bearing sCT receptors by **3b**-sCT and native sCT.

bioactivity compared to native sCT. In these experiments two negative controls were employed, PBS alone and polymer **3b** in PBS. 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 min after injections into the tail vein, and the plasma total calcium was analyzed. The **3b**-sCT conjugate was shown to have ability for lowering plasma calcium comparable to that of unmodified sCT (Figure 8). Negative control data using i.v.

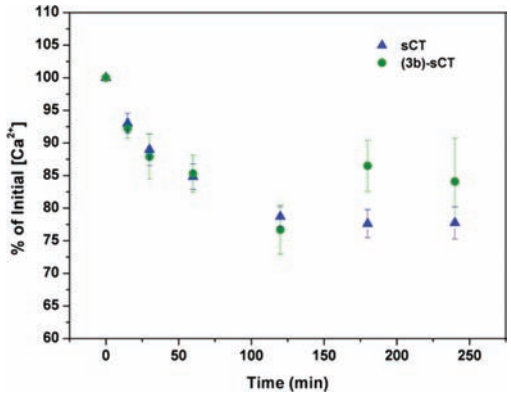

Figure 8. Plasma total calcium profiles of male Wistar rats following intravenous injections of sCT (blue triangles) and **3b**-sCT conjugate (green circles).

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.

In summary, this work describes a novel general approach to direct polymer conjugation to peptides, based on diazonium-terminated materials with various macromolecular architectures. A short oligoethylene glycol, a well-defined linear PEG, and poly($mPEG_{(475)}$ methacrylate) prepared by atom transfer radical polymerization were synthesized and employed to target 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 aimed 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.[55−57] 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 preferential (or sometimes exclusive) targeting of 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 pK_a 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}, no loss of bioactivity occurred *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 an ability to lower plasma total $[Ca^{2+}]$ 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, to the best of our knowledge, the first example of site-specific direct polymer conjugation at Tyr residues.

■ ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare the following competing financial interest(s): D. M. Haddleton is a Director of Warwick Effect Polymers Ltd.

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