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Stability Enhancing *N*-Terminal PEGylation of Oxytocin Exploiting Different Polymer Architectures and Conjugation Approaches

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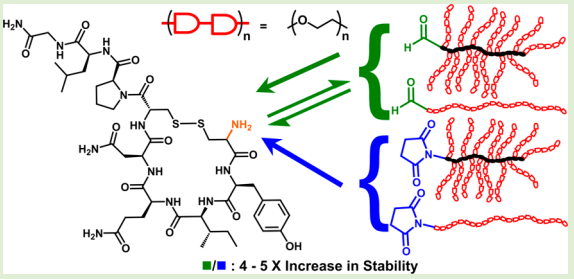
Supporting Information

ABSTRACT: Oxytocin, a cyclic nine amino acid neurohypophyseal hormone therapeutic, is effectively used in the control of postpartum hemorrhaging (PPH) and is on the WHO List of Essential Medicines. However, oxytocin has limited shelf life stability in aqueous solutions, particularly at temperatures in excess of 25 °C and injectable aqueous oxytocin formulations require refrigeration (<8 °C). This is particularly problematic in the hot climates often found in many developing countries where daytime temperatures can exceed 40 °C and where reliable cold-chain storage is not always achievable. The purpose of this study was to develop *N*-terminal amine targeted PEGylation strategies utilizing both linear PEG and polyPEG "comb" polymers as an effective method for stabilizing solution formulations of this peptide for prolonged storage in the absence of efficient cold-chain storage. The conjugation chemistries investigated herein include irreversible amine targeted conjugation methods utilizing NHS ester and aldehyde reductive amination chemistry. Additionally, one reversible conjugation method using a Schiff base approach was explored to allow for the release of the native peptide, thus, ensuring that biological activity remains unaffected. The reversibility of this approach was investigated for the different polymer architectures, alongside a nonpolymer oxytocin analogue to monitor how pH can tune native peptide release. Elevated temperature degradation studies of the polymer conjugates were evaluated to assess the stability of the PEGylated analogues in comparison to the native peptide in aqueous formulations to mimic storage conditions in developing nations and regions where storage under appropriate conditions is challenging.



1. INTRODUCTION

There is an unacceptably large difference in the death of mothers during child birth between the developed and developing world. Maternal mortality in developing regions is still 14× higher than in the developed regions. The UN lists reducing the maternal mortality rate to <70 women in every 100000 live births by 2030 as its #1 "Goal 3 Target".[1] Since 1990, worldwide maternal mortality rates have improved; however, rates are still unacceptably high in developing nations that will account for 99% of the maternal deaths that occur in 2016. Postpartum hemorrhaging (PPH) is one such complication, manifesting as excessive and uncontrolled bleeding due to insufficient contraction of the uterus after delivery. It is understood that in developing countries, where home births are common and access to well-resourced health care facilities and professionals are limited, that obstetric hemorrhage continues to be a leading cause of maternal deaths and, sadly, a conservative estimate is that over 140000 women per year die needlessly of complications arising from PPH.

Oxytocin is a World Health Organization (WHO) recommended drug currently used across the world as a uterotonic for the induction of labor and prevention of PPH and is on the WHO List of Essential Medicines.[2−6] PPH is a worldwide problem, being one of the leading causes of maternal morbidity, and oxytocin (cyclic [Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly-NH_2; marketed under trade names Syntocinon and Pitocin) prevents large amounts of post birth bleeding by causing the uterus to contract. A significant problem with the use of oxytocin is that it rapidly undergoes degradation in aqueous solutions, with degradation occurring by a variety of processes including deamidation, β-elimination, tri/tetrasulfide formation, dimerization, and the formation of larger aggregates.[7,8] Oxytocin formulations therefore do not have long shelf lives and this leads to the requirement of cold-chain supply and storage, which is not always practical, particularly within the developing world due to complex economic and social reasons.

Various approaches have been established in attempts to somewhat resolve this problem with an aim to providing longer shelf life stability to oxytocin.[9−11] There has been a significant amount of research developed since the discovery of oxytocin into various oxytocin analogues with a focus on analysis of structural changes and the effect this can have on peptide activity and stability.[12−16] Structurally the amino acids required

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for uterine activity are Tyr[2] and Asn[5] with Ile[3], Gln[4], Pro[7], and Leu[8] also being important for binding to the receptor.[17] Recently, we reported the synthesis of dithiophenol maleimide functional polyPEGs and conjugated these site-specifically onto oxytocin by bridging of the Cys[1]−Cys[6] disulfide bond.[9] This led to an increase in the stability of aqueous solutions of the peptide highlighting the potential of applying PEGylation as a method for reducing degradation of this therapeutic. Moreover, previous modifications at the *N*-terminal amine where the free amino group is lacking from the structure of the peptide (desamino-oxytocin) have shown high levels of uterine contractility proving potentially more potent than the native peptide as an oxytocic drug.[18−22] A further oxytocin analogue that has proved fairly successful is carbetocin (1-deamino-1-monocarba-[2-*O*-methyltyrosine]-oxytocin), which also has changes to the amino groups, along with other structural modifications. This octapeptide oxytocin alternative has been approved for human and veterinary use in several countries and provides effective uterotonic activity while maintaining a higher stability than the native peptide.[23,24] Amino targeting chemistry is site selective for oxytocin as the only amine group present on the peptide structure is that at the *N*-terminus, leading to the possibility of only one site of polymer attachment. The *N*-terminal amine also spatially is very close to the Cys[1]−Cys[6] disulfide bond, one of the sites where a large proportion of the degradation products arise from, and amine targeted conjugation strategies, would potentially provide some protection across this highly susceptible degradation position. Targeting of the *N*-terminus in oxytocin could prove advantageous as an attachment position, as previous studies for different oxytocin analogues where the *N*-terminus has been functionalized have still maintained a high amount of oxytocic activity.[18,19]

The attachment of polymer chains such as poly(ethylene glycol) (PEG) to peptides and proteins (PEGylation) can offer a high number of advantages including improved water solubility, longer blood circulation times, as well as reducing degradation thus improving shelf life and stability under stressed conditions.[25−30] As a result, there are an increasing number of FDA approved PEGylated products in the clinic and many more in clinical studies.[31−35] To date, various coupling chemistries have been evaluated for PEGylation that can react in a site-specific manner at different amino acids found on peptide and proteins. Targeted strategies among others have included the site-selective conjugation at lysine,[36−38] cysteine,[39−43] arginine,[44] and tyrosine[45] residues.

Unfortunately, many polymer-modification procedures result in a peptide/protein loss of activity following polymer conjugation.[46−48] In order to overcome this problem, a significant amount of research has been conducted into different reversible conjugation approaches, which are able to, under certain conditions, restore the peptide/protein so that full function and activity can be maintained.[49−58]

As an alternative to the conjugation of linear PEG onto bioactive molecules there have been efforts to incorporate different architectures such as branched "comb" PEGs onto proteins and peptides.[59−65] This can provide a higher density of PEG and leads to differences in physical properties which can offer an alternative to linear equivalents.[66] Potential advantages of comb polymers include possessing a decreased viscosity with respect to solutions of linear polymers due to being rigid rods as opposed to random coils in solution and reduced polymer crystallization and subsequent organ vacuolization.[67] The synthesis of tailor-made polymers bearing low molar mass PEG side chains by implementing (meth)acrylate PEG monomers have been more accessible since the development of living radical polymerization techniques. These are prepared in a controlled manner by a variety of polymerization techniques such as atom transfer radical polymerization (ATRP)[68,69] and Cu(0)-mediated living radical polymerization.[70−72] A variety of α-end group functionalities can be incorporated, and the controlled nature of the polymerization leads to targeted molecular weight (degree of polymerization) polymers with low dispersities capable of undergoing post polymerization conjugations onto biological molecules. There have been several different targeting strategies previously described for the "grafting-to" of polyPEGs onto proteins or peptides via the incorporation of polymer end group functionalities. Strategies include the conjugation at thiol (cysteine) groups using maleimide[9,41,73−75] and arsenic[76] functional polymers and at amino (lysine or *N*-terminal) groups using aldehyde (Schiff base formation/reductive amination)[66,77,78] or *N*-hydroxysuccinimide (NHS) ester[79,80] chemistry.

Herein we describe both linear and polyPEG architectures for site specific conjugation onto oxytocin utilizing NHS ester chemistry for the synthesis of thermally stable conjugates and aldehyde-amine reactions for the formation of both irreversible and reversible oxytocin conjugates.

2. MATERIALS AND METHODS

2.1. Materials. Oxytocin (*c*-[Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly-NH_2) was gifted from PolyPeptide Laboratories (Hillerød, Denmark) and used as received. Functional poly(ethylene glycol)s: succinimidyl ester (NHS) functional poly(ethylene glycol) (molecular weight = 2000 and 5000 Da) and formyl (aldehyde) functional poly(ethylene glycol) (molecular weight = 2000 and 5000 Da) were purchased from Rapp Polymere (Germany) and stored in a freezer. All other chemicals were purchased from Sigma-Aldrich and used without further purification. Copper wire was pretreated by washing in hydrochloric acid (35%) for 20 min, then rinsed with water, and dried under nitrogen immediately prior to use. *N*,*N*,*N*′,*N*′,*N*″,*N*″-Hexamethyl-[tris(aminoethyl)amine] (Me_6-TREN) was synthesized according to a reported procedure and stored at 4 °C.[81]

2.2. Instruments and Analysis. Nuclear magnetic resonance (NMR) spectra were acquired on Bruker DPX-300 MHz, HD-300, and HD-400 spectrometers with samples prepared in deuterated solvents ($CDCl_3$ or DMSO-d_6) and chemical shifts were reported in parts per million (ppm) with reference to solvent residual peaks. 1D ^{1}H and 2D ^{1}H NMR spectra for unreacted oxytocin and the reaction products with butyraldehyde were acquired at 298 K on a Bruker Avance III 600 NMR spectrometer operating at 600.13 MHz for ^{1}H nuclei using the reaction mixture (2 mM) with 10% D_2O for field-frequency lock (further details in SI). Size exclusion chromatography (SEC) was performed on an Agilent Polymer Laboratories GPC50 fitted with differential refractive index (RI). Separations were performed on a pair of Agilent Polargel Medium Columns eluting with *N*,*N*-dimethylformamide containing 0.1 M LiBr as an additive at 50 °C with a flow rate of 1 mL/min. Molecular weights were calculated relative to narrow poly(methyl methacrylate) (PMMA) standards. Analytical reverse phase high performance liquid chromatography (RP-HPLC) was performed on Agilent 1260 infinity series stack equipped with an Agilent 1260 binary pump and degasser. Samples were injected using Agilent 1260 autosampler. The HPLC was fitted with a Phenomenex Lunar C_{18} column (250 × 4.6 mm) 5 μm packing (100 Å). Detection was achieved using an Agilent 1260 variable wavelength detector monitoring at 280 nm. Mobile phases consisted of acetonitrile and H_2O with TFA as an additive (0.04%). Semi-preparative HPLC was performed on the same HPLC system, using the same mobile phases as above fitted with a Phenomenex Jupiter C_{18} column (250 × 21.2 mm) with 5 μm packing (300 Å). Detection was

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achieved using an Agilent 1260 multiple wavelength detector monitoring at λ = 280 and 225 nm. HPLC gradients are described in the Supporting Information. MALDI-ToF mass spectrometry was conducted using a Bruker Daltonics Autoflex MALDI-ToF mass spectrometer. MALDI-ToF samples were made by mixing a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in methanol as a matrix (10.8 μL), sodium iodide in tetrahydrofuran (THF; 1.0 mg/mL) as catonization agent (4.2 μL), and sample in THF with 1 drop of water (1.0 mg/mL; 10.8 μL) and 0.7 μL of this mixture was applied to target plate. Spectra were recorded in reflector mode calibrating with methoxy PEG (2000 Da). Electrospray ionization mass spectra (ESI) were recorded on an Agilent 6130B Single-Quad.

2.3. Methods. *N-Hydroxysuccinimide-2-bromo-2-methylpropionate.* This procedure was adapted from a previously reported procedure.[79] *N*-Hydroxy succinimide (4.45 g, 38.7 mmol) was dissolved in anhydrous DCM (100 mL) and triethylamine (7.16 mL, 51.5 mmol) was added. The flask was cooled to 0 °C before the addition of α-bromoisobutyryl bromide (5.25 mL, 42.6 mmol) in DCM (20 mL) dropwise under the flow of nitrogen. The mixture was stirred at 0 °C for 45 min, before warming to room temperature and stirring for a further 3 h. The reaction mixture was then poured into ice−water (200 mL), and the organic layer was separated and washed with $NaHCO_3$ (2 × 50 mL) followed by water (2 × 50 mL) and again with $NaHCO_3$ (2 × 50 mL). The organic layer was dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by recrystallization with Et_2O to afford *N*-hydroxysuccinimide-2-bromo-2-methylpropionate as an off-white powder (8.46 g, 32.0 mmol, 83%). ^{1}H NMR ($CDCl_3$, 400.05 MHz) δ (ppm): 2.06 (6H, s, $C(CH_3)_2Br$), 2.85 (4H, s, $(CH_2CO)_2N$). ^{13}C NMR ($CDCl_3$, 100.59 MHz) δ (ppm): 25.54 ($(CH_2CO)_2N$), 30.59 ($C(CH_3)_2Br$), 51.17 ($C(CH_3)_2Br$), 167.40 (NOCO), 168.59 ($(CH_2CO)_2N$).

2-(2,2-Dimethoxyethoxy)ethyl-2-bromo-2-methylpropionate. The two-step protected aldehyde initiator synthesis was adapted from reported procedure.[82] Potassium hydroxide (40 g, 0.71 mol) and ethylene glycol (100 mL, 1.79 mol) were added together and gradually heated to 115 °C with stirring. Chloroacetaldehyde dimethyl acetal was slowly added dropwise, and the solution was stirred for 48 h at 115 °C. The solution was cooled to ambient temperature before the addition of water (200 mL). The solution was then extracted with DCM (2 × 150 mL) and washed with brine (2 × 100 mL) before the organic fractions were dried with $MgSO_4$, and the solvent was removed under reduced pressure, yielding 2-(2,2-dimethoxy-ethoxy)-ethanol as a yellow oil (18.1 g, 0.12 mol, 16.9%), which was used for the subsequent step without further purification. ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ (ppm): 3.37 (6H, s, $CH(OCH_3)_2$), 3.52 (2H, t, J = 5.09 Hz, $CHCH_2O$), 3.56−3.73 (4H, m, OCH_2CH_2OH), 4.50 (1H, q, J = 5.09 Hz, $CH(OCH_3)_2$). ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ (ppm): 52.83 ($CH(CH_3O)_2$), 61.54 (CH_2CH_2OH), 70.51 (CH_2CH_2OH), 72.82 ($CHCH_2O$), 102.47 ($CH(CH_3O)_2$).

A solution of 2-(2,2-dimethoxyethoxy)-ethanol (11 g, 73.2 mmol) and triethylamine (12 mL, 86.2 mmol) were dissolved in DCM (150 mL) and cooled to 0 °C under nitrogen. α-Bromoisobutyryl bromide (8.5 mL, 69.7 mmol) in DCM (50 mL) was added dropwise. The solution was stirred for 1 h at 0 °C and overnight at ambient temperature, and the resulting suspension was filtered. The solution was then washed with saturated $NaHCO_3$ solution (3 × 100 mL) and dried with $MgSO_4$, and the solvent was removed under reduced pressure, yielding a crude yellow oil (18 g). A total of 6.5 g of this oil was purified by silica column chromatography (petroleum ether: diethyl ether; 19:1 to 3:1), yielding 2-(2,2-dimethoxyethoxy)ethyl-2-bromo-2-methylpropionate as a colorless oil (4.06 g, 12.9 mmol). ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ (ppm): 1.89 (6H, s, $C(CH_3)_2Br$), 3.34 (6H, s, $(OCH_3)_2CH$), 3.51 (2H, d, J = 5.09 Hz, $CHCH_2O$), 3.71 (2H, t, J = 4.71 Hz, OCH_2CH_2OCO), 4.27 (2H, t, J = 4.71 Hz, OCH_2CH_2OCO), 4.45 (1H, t, J = 5.09 Hz, $(OCH_3)_2CH$). ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ (ppm): 30.09 ($C(CH_3)_2Br$), 53.31 ($(CH_3O)_2CH$), 55.00 ($C(CH_3)_2Br$), 64.44 (CH_2O), 68.41 (CH_2O), 70.32 (CH_2O), 101.98 ($(CH_3O)_2CH$), 170.85 ($COC(CH_3)_2Br$).

General Procedure for Cu(0)-Mediated Living Radical Polymerization of mPEGA$_{480}$. Copper wire (1.25 mm diameter) was activated by washing in hydrochloric acid for 20 min, before being sufficiently rinsed with water and dried under a nitrogen blanket. Cu(II) bromide (3.7 mg, 0.0167 mmol) was dissolved in DMSO (1.5 mL) in a Schlenk tube and Me_6TREN ligand (16.1 μL, 0.0602 mmol) was added. Functional initiator (0.334 mmol) in DMSO (1 mL) and mPEGA (2.94 mL, 6.68 mmol) in DMSO (1 mL) were added. The solution was degassed with nitrogen bubbling for 20 min prior to the addition of pre activated copper wire under a positive nitrogen pressure and the Schlenk tube sealed with a rubber septum. The polymerization was sampled under a positive pressure of nitrogen for NMR conversion analysis (DMSO-d_6) and SEC molecular weight analysis (DMF).

Polymer Purification. The succinimidyl terminated polymers were purified by repeated precipitation into diethyl ether/hexane (1:1), and the protected aldehyde polymers were purified by dialysis against water (regenerated cellulose, 1 kDa MWCO, 3 days).

Deprotection of Acetal Group. The acetal groups were removed by dissolving polymer (1 g) in a (1:1) solution of TFA/H_2O (30 mL) and stirring for 48 h. The aldehyde functional deprotected polymer was purified by dialysis against water (regenerated cellulose, 1 kDa MWCO, 3 days).

PEGylation of Oxytocin with Linear Succinimidyl Polymer. Oxytocin (20 mg, 19.9 μmol) and NHS-PEG (Rapp-Polymere, 2000 Da; 60 mg, 30 μmol) were separately dissolved in 1 mL of DMF, and the solutions were added together at 10 °C, along with 20 μL of triethylamine. After stirring overnight at T = 10 °C, 20 μL of the solution was removed and dissolved in 1 mL of H_2O for RP-HPLC.

PEGylation of Oxytocin with PolyPEG Succinimidyl Polymer. Oxytocin (1 mg, 0.99 μmol) was dissolved in 1 mL of phosphate buffer (pH 6.5, 0.1 M) and added to succinimide functional polyPEG (65 mg, 10 μmol) dissolved in 1 mL of the same buffer. The reaction was stirred overnight at ambient temperature before a sample was removed for RP-HPLC analysis.

Example PEGylation of Oxytocin with Linear or "Comb" Aldehyde PEG. Aldehyde functional PEG (40 mg, 20 μmol) was dissolved in sodium phosphate buffer (5 mL, pH 6.2, 100 mM) and left at 10 °C for 1 h. Oxytocin (15 mg, 14.9 μmol) was stirred in the same buffer (5 mL) at 10 °C for 15 min. Both solutions were added together and a freshly prepared solution of $NaCNBH_3$ (25 mmol) was added. After stirring overnight at T = 10 °C a 100 μL aliquot was taken and dissolved in 1 mL of H_2O for RP-HPLC analysis.

Example Reversible Conjugation of Oxytocin with Butyraldehyde or Linear or "Comb" Aldehyde PEG. Aldehyde functional linear PEG (200 mg, 40 μmol) dissolved in phosphate buffer (3 mL, pH 8, 0.1 M) was added to oxytocin (20 mg, 20 μmol) dissolved in the same buffer (2 mL). The reaction was allowed to proceed at room temperature overnight before a sample was removed for RP-HPLC analysis..

Irreversible Conjugation of Butyraldehyde onto Oxytocin. To oxytocin (10 mg, 9.9 μmol) in H_2O (4 mL), butyraldehyde (4.4 μL, 5.0 μmol) was added after which $NaCNBH_3$ (25 mM, 1 mL) was added. The reaction was stirred at ambient temperature overnight before a sample was removed for RP-HPLC.

Reversibility Studies of Butyraldehyde Oxytocin Conjugates. Butyraldehyde Schiff base conjugated oxytocin solution directly succeeding conjugation (0.5 mL, 4 mM) was diluted with 1.5 mL of different pH solutions (1 mM). After stirring for 24 h, the samples were analyzed by RP-HPLC for any increase in concentration of oxytocin.

Reversibility Studies of Oxytocin−Polymer Conjugates. Immediately postconjugation to 1 mL of the conjugation solution (4 mM) was added H_2O (3 mL) and the solution dialyzed (3.5 kDa MWCO, 3 day) against water which had been pH adjusted with 0.1 M NaOH to pH 8. After removal from dialysis a sample was submitted for RP-HPLC to ascertain minimum oxytocin content. A total of 0.5 mL of the dialysis solution was diluted with 1.5 mL of solutions at different pHs and stirred for 18 days with periodic sampling by RP-HPLC.

Thermal Stability Assays. Oxytocin and oxytocin−polymer conjugates (1 μmol unless otherwise stated) were dissolved in HPLC grade water before being sealed and placed in an oven set at 80

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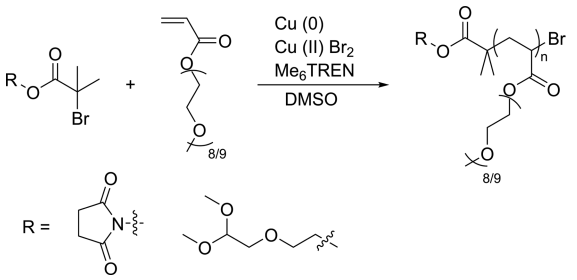
°C. After 24 h, the samples were removed and allowed to cool to room temperature before analysis by RP-HPLC (UV: λ = 280 nm). All solutions were prepared in triplicate and analysis of the remaining oxytocin (or conjugate) peak was with respect to a 5 point concentration calibration plot.

3. RESULTS AND DISCUSSION

In this work the syntheses of oxytocin−PEG conjugates are described using amine reactive succinimidyl ester and Schiff base/reductive amination aldehyde chemistry comparing linear and branched architectures in terms of providing potential stabilization effects. In order to do this, polyPEGs were first synthesized using Cu(0)-mediated living radical polymerization with targeted molar masses using two different initiators for α-end functionality (Scheme 1), resulting in polymers that have a "comb" type architecture capable of conjugating onto the *N*-terminal amine of oxytocin.

Scheme 1. Cu(0)-Mediated Living Radical Polymerization of mPEGA$_{480}$ Using Different Initiators for α-End Functionality Capable of Undergoing Post-Polymerization Peptide Conjugation



3.1. PolyPEG Synthesis for Post-Polymerization Conjugations onto Peptides.

N-Succinimidyl 2-bromo-2-methylpropionate was synthesized in one step by reacting *N*-hydroxysuccinimide with 2-bromoisobutyryl bromide in a high yield with little purification required.[79] An alternative initiator, which contains a protected aldehyde (acetal) group and has previously been reported for the controlled synthesis of poly(mPEGMA) polymers by copper-mediated living radical polymerization was synthesized in a previously reported two-step procedure.[66,82]

The succinimidyl ester initiator was used in the Cu(0)-mediated living radical polymerization of poly(ethylene glycol) methyl ether acrylate (avg M_n = 480 g mol^{-1}, mPEGA$_{480}$), which was carried out using anhydrous DMSO as solvent with Cu(II)Br$_2$, Cu(0) wire, and tris[2-(dimethylamino)ethyl]amine (Me$_6$TREN) as ligand ([I]/[M]/[Me$_6$TREN]/[CuBr$_2$] = 1:20:0.18:0.05 and 5 cm Cu(0) wire). Samples were removed periodically and diluted in DMSO-d_6 for ^{1}H NMR analysis and DMF for SEC molecular weight data in order to follow the kinetics of the reaction. Conversions were measured by comparing the disappearance of vinyl groups (δ = 5.8−6.4 ppm) against a CH$_2$ signal in the emerging polymer from the PEG repeat unit (δ = 4.1 ppm). There was an initial inhibition time to the polymerization, where there was no presence of polymer by ^{1}H NMR or SEC after 60 min with conversion rising to 23% after 2 h followed by almost quantitative conversion (98%) achieved after 24 h (Figure 1A). The dispersities of the polymers remained low throughout the reaction ($Đ$ < 1.15), with SEC chromatograms showing monomodal peaks, and molecular weights increasing throughout the polymerization (Figure 1B). The linear increase of molecular weight with conversion (which also correlated well with theoretical molecular weights) shows evidence of the controlled nature of this polymerization (Figure 1C,D).

A succinimidyl functional polymer capable of undergoing conjugation to oxytocin was synthesized targeting a molecular weight of 6.5 kDa using the same conditions as for the kinetic experiments but with [M]/[I] ratio =13:1. After 24 h high conversion was achieved (97%) and the resulting polymer was purified by repeated precipitation into Et$_2$O/hexane (1:1) to yield a succinimidyl ester end functionalized polymer. Characterization of the purified polymer by ^{1}H NMR confirmed that the succinimide group was still present with

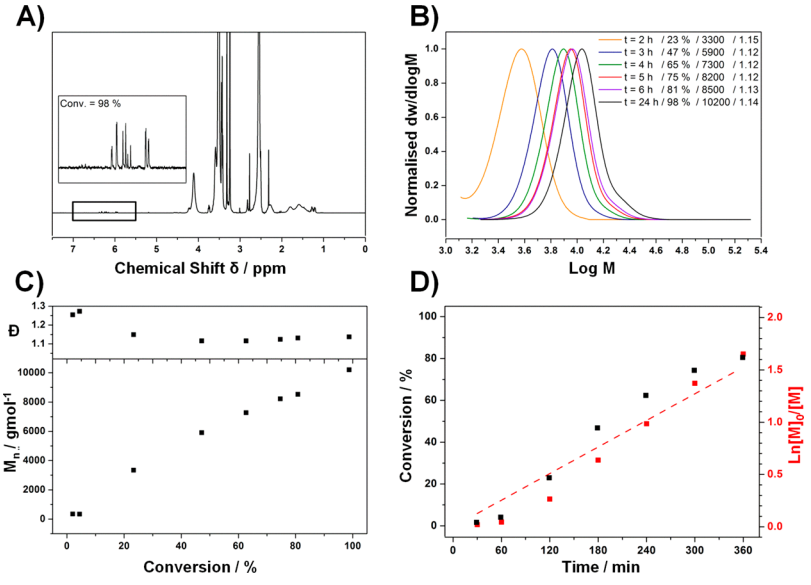


Figure 1. Cu(0)-mediated polymerization of mPEGA$_{480}$ with succinimide ester initiator in DMSO with Me$_6$TREN as ligand showing (A) final conversion by ^{1}H NMR (DMSO-d_6, 300.13 MHz), (B) SEC (DMF + 0.1 M LiBr) analysis of polymerization, (C) kinetic plot of molecular weight vs conversion, and (D) kinetic plot of ln([M]$_0$/[M]$_n$) and conversion vs time.

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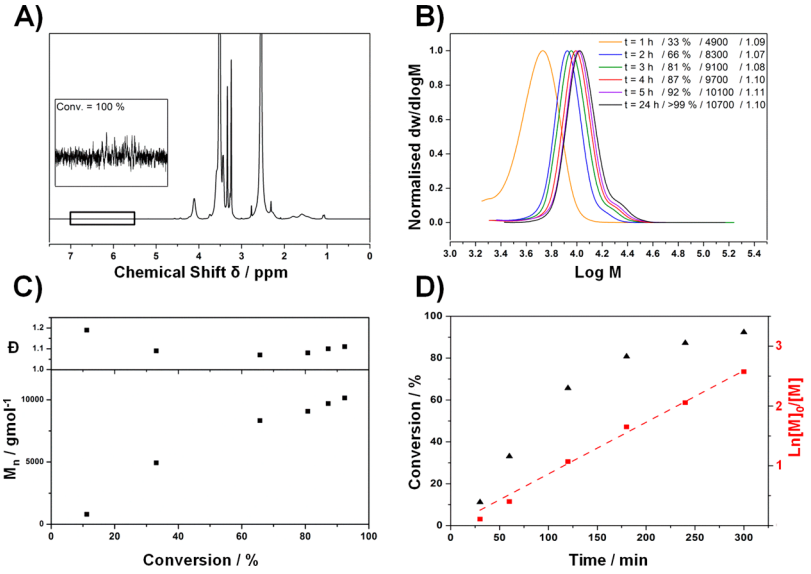

Figure 2. Cu(0)-mediated polymerization of $mPEGA_{480}$ with acetal-protected initiator in DMSO with Me_6TREN as ligand showing (A) final conversion by ^1H NMR (DMSO-d_6, 300.13 MHz), (B) SEC (DMF + 0.1 M LiBr) analysis of polymerization, (C) kinetic plot of molecular weight vs conversion, and (D) kinetic plot of $\ln([M]_0/[M]_n)$ and conversion vs time.

the peak at δ = 2.81 ppm representing the four protons in the succinimide ring. By a comparison between these protons and the protons from the first CH_2 of the PEG repeat unit (δ = 4.1 ppm), the average DP was calculated to be 13, yielding an experimental molecular weight of 6500 g mol^{-1} with low dispersity confirmed by SEC ($Đ$ = 1.08; SI, Figure S3).

The Cu(0)-mediated living radical polymerization of $mPEGA_{480}$ using the protected aldehyde initiator was carried out under the same conditions as for the succinimide initiator ([I]/[M]/[Me_6TREN]/[$CuBr_2$] = 1:20:0.18:0.05 and 5 cm Cu(0) wire) for a targeted molar mass of 10 kDa. The polymerization proceeded at a faster rate compared to that with the succinimide initiator, with 66% conversion after 2 h and complete conversion being achieved after 24 h (Figure 2A). As with the succinimide initiated polymers the molecular weight distributions remained monomodal and narrow throughout the polymerization (<1.12), alongside good correlation between the theoretical and experimental molecular weights (Figure 2B). The kinetics of the reaction revealed controlled characteristics with a linear evolution of molecular weight (M_n) with conversion as well as $\ln([M]_0/[M])$ increasing linearly with time (Figure 2C,D).

Two aldehyde functional poly($mPEGA_{480}$) polymers were therefore synthesized for conjugation onto oxytocin using the acetal-protected aldehyde initiator targeting molecular weights of 10 and 25 kDa, respectively (equivalent to [M]/[I] of 20 and 50) with [I]/[Me_6TREN]/[$CuBr_2$] of 1:0.18:0.05 and 5 cm Cu(0) wire; overnight).

The α-end group functionality of the purified (dialysis, 1 kDa MWCO, 3 days) polymers was confirmed by ^1H NMR where peaks representing the acetal protecting group appear at δ = 4.4 and 3.3 ppm. The experimental molecular weight was calculated by a comparison between the 6 protons of the isobutyryl group in the α-end group of the polymer (δ = 1.07 ppm) and the first CH_2 group of the PEG repeat unit (δ = 4.1 ppm) giving final DPs of 21 and 53, representing molecular weights of 10 kDa and 26 kDa (SI, Figure S4). Furthermore, SEC analysis (DMF) showed that narrow molecular weight distributions were maintained, with final dispersity values of $Đ$ = 1.12 and 1.16, respectively.

The polymers were deprotected by addition of aqueous solutions of trifluoroacetic acid (50% v/v), resulting in removal of the acetal protecting group, thus, yielding α-aldehyde functionality on the polymer chains. The aldehyde functionality was confirmed by ^1H NMR (DMSO-d_6) where there is the appearance of a characteristic aldehyde peak at 9.5 ppm and a disappearance of the acetal peaks at 4.4 and 3.3 ppm (SI, Figure S5). After deprotection of the acetal group, the molecular weight distribution remained narrow ($Đ$ = 1.15/1.18) and monomodal, and only a small decrease in average molecular weight was observed (Table 1 and SI, Figure S6).

Table 1. Summary of the ^1H NMR (DMSO-d_6) Conversion and SEC (DMF Eluent) Molecular Weight Data for Synthesis of NHS Ester and Acetal-Protected/Deprotected Aldehyde Polymers

polyPEG	conversion[a] (%)	M_n[b] (g mol^{-1})	$Đ$[b]	M_n[b] (g mol^{-1}; deprotected)	$Đ$[b] (deprotected)
6.5 kDa NHS	97	6500	1.08		
10 kDa NHS	98	11700	1.15		
10 kDa acetal	100	11000	1.12	10500	1.15
25 kDa acetal	99	21300	1.16	20500	1.18

[a]Determined by ^1H NMR (DMSO-d_6). [b]Determined by SEC (DMF + LiBr) using narrow PMMA standards.

3.2. Activated Ester Polymer Conjugation onto Oxytocin. Succinimide ester (NHS ester) linear PEGs of two different molecular weights (2 and 5 kDa) were conjugated onto oxytocin in DMF containing 1% TEA to yield amide-linked linear PEG conjugates (**1**; Figure 3A). After 18 h, a sample was taken and submitted for RP-HPLC analysis (UV λ: 280 nm), which showed the appearance of a broad peak at a higher retention time (t = 14.7 min) with respect to the native

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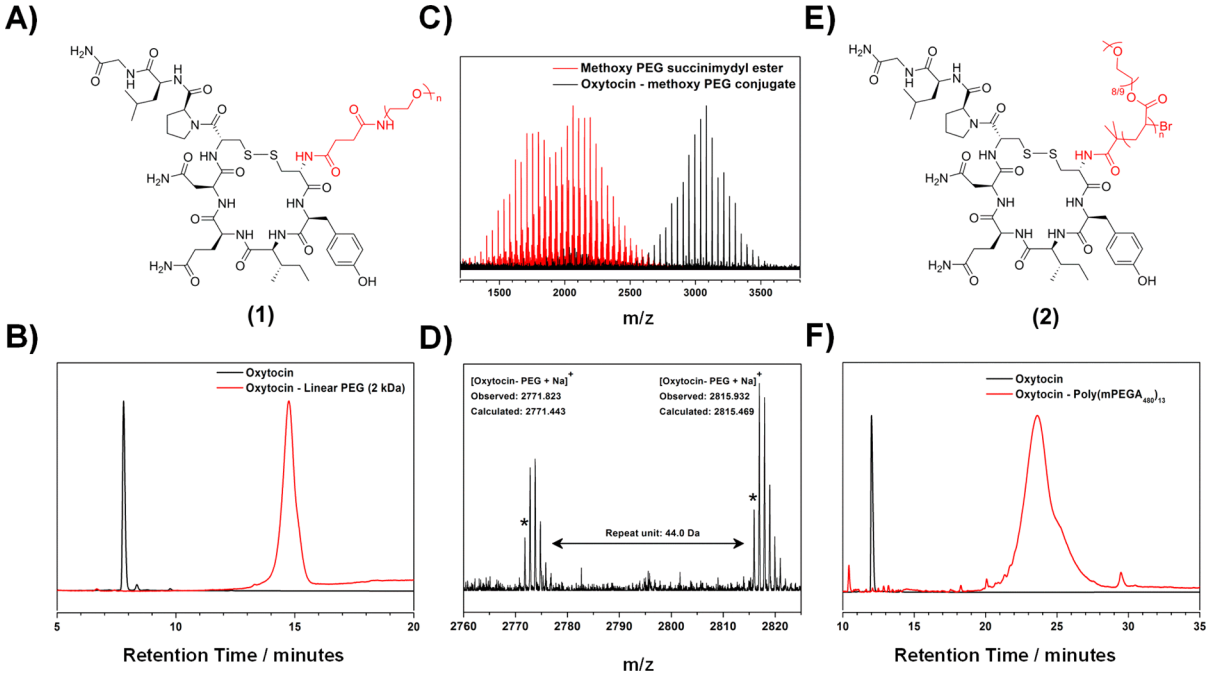

Figure 3. (A) Structure of linear succinimydyl PEG−oxytocin conjugate (**1**), (B) analysis by RP-HPLC, (C) MALDI-TOF MS of shift in molecular weight from polymer to polymer conjugate and (D) MALDI-TOF MS of polymer conjugate (**1**), (E) Structure of "comb" succinimidyl polyPEG−oxytocin conjugate (**2**), (F) analysis by RP-HPLC.

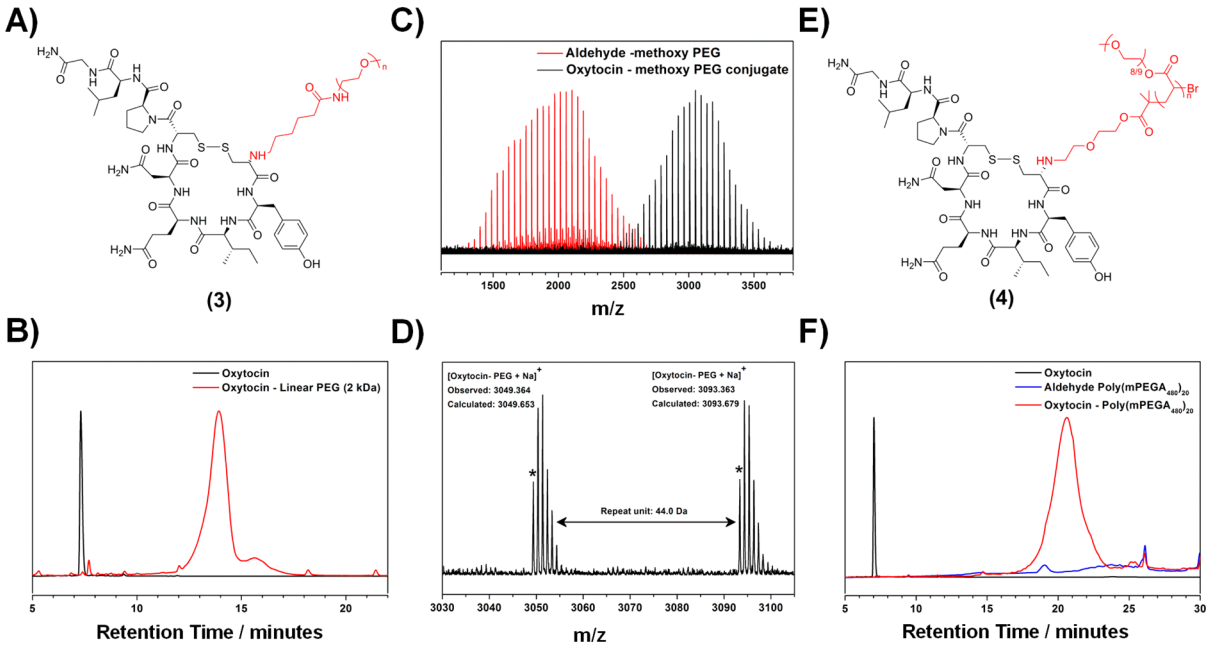

Figure 4. (A) Structure of linear aldehyde PEG−oxytocin conjugate (**3**), (B) analysis by RP-HPLC, (C) MALDI-TOF MS of shift in molecular weight from polymer to polymer conjugate and (D) MALDI-TOF MS of polymer conjugate (**3**), (E) Structure of "comb" aldehyde functional polyPEG−oxytocin conjugate (**4**), (F) analysis by RP-HPLC.

peptide (t = 7.8 min; Figure 3B). The presence of native oxytocin was observed in the reaction mixture for both conjugations, indicating that the reaction was not quantitative. Conjugation was confirmed by MALDI-TOF MS, whereby a clear shift in molecular weight was observed in the MALDI-TOF spectrum from the NHS ester polymer to the polymer−peptide conjugate (Figure 3C). After conjugation (with purification by semipreparative RP-HPLC) there is only one major peak distribution present with good agreement between the exact masses achieved from the spectra and the theoretical (calculated) values and distances between peaks representative of the ethylene glycol repeat unit (44.03 Da), thus, validating the expected conjugate structure (Figure 3D).

Conjugation of oxytocin with the 6.5 kDa α-succinimide polyPEG "comb" was then attempted, which would give a conjugate with equivalent amide linkage between the peptide and the polymer, but with a different PEG architecture (Figure 3E). However, under the same conditions as the linear

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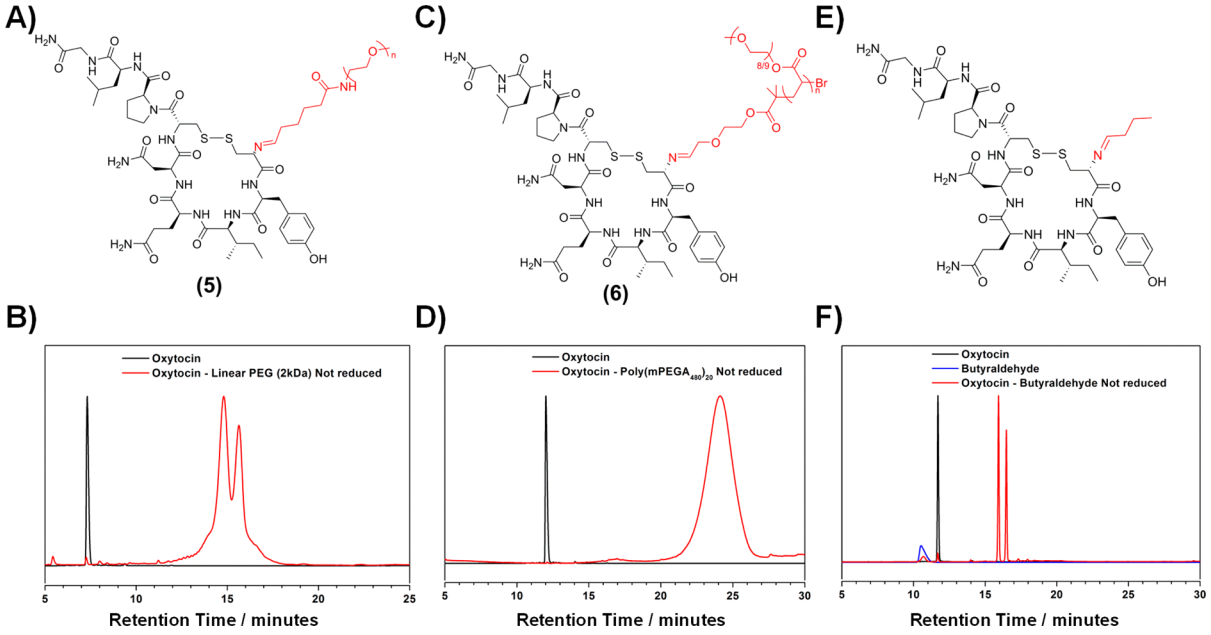

Figure 5. Formation of potentially reversible peptide−polymer conjugates: (A) structure and (B) RP-HPLC trace for formation of linear oxytocin conjugate (**5**); (C) structure and (D) RP-HPLC trace for formation of "comb" polymer oxytocin conjugate (**6**); and (E) structure and (F) RP-HPLC trace for formation of butyraldehyde oxytocin conjugate.

analogues ([oxytocin]/[polymer] = 1:1.2), no conjugation was observed by RP-HPLC within 24 h. The reaction was then performed using a higher excess of the polymer (10 equiv). After 24 h, analysis by RP-HPLC (UV λ= 280 nm) showed the appearance of a broad peak at a higher retention time (t = 23.7 min) compared to the native peptide (t = 12.0 min) attributed to the polyPEG conjugate (**2**; Figure 3F). The requirement for a high excess of polymer is likely due to a combination of the rapid hydrolysis of the succinimide end group causing the polymer to no longer have an activated ester end functionality and issues arising from the steric nature of the polyPEG chains.[83] From the RP-HPLC as observed for the linear PEGylation with succinimidyl functional polymer there is still some native oxytocin present after conjugation (t = 12.2 min), which was easily removed during purification by dialysis.

3.3. Aldehyde Functional Polymer Conjugation onto Oxytocin. Typically when aldehyde chemistry is employed to modify the amines in proteins and peptides a two-step process is employed, the first involving the reversible formation of a Schiff base intermediate. Then the irreversible product (a secondary amine linked peptide−polymer conjugate) is generated via reductive amination by the subsequent addition of a reducing agent such as sodium cyanoborohydride ($NaCNBH_3$).

The irreversibly linked linear aldehyde-PEG conjugation to oxytocin was carried out in phosphate buffer (0.1 M, pH 6.2) with the addition of $NaCNBH_3$ (25 mM) at two different molecular weights (2 kDa and 5 kDa; **3**; Figure 4A). After 24 h, a sample was submitted for RP-HPLC analysis which confirmed the formation of a new product, with a new broad peak being observed with a longer retention time (t = 14.0 min) than the native peptide (t = 7.4 min), confirming the presence of the linear PEG−oxytocin conjugate (Figure 4B). Any remaining native peptide was removed during purification of the polymer conjugate by dialysis (1 kDa MWCO, 3 days), after which the PEGylated oxytocin product was confirmed by MALDI-TOF MS. There is a clear shift on the MALDI spectra between the aldehyde polymer and the polymer conjugate relating to the MW of oxytocin (Figure 4C). The MALDI spectrum for the polymer conjugate showed only one major distribution with experimental masses close to theoretical (calculated) values and differences between peaks corresponding to the ethylene glycol repeat unit (44.03 Da; Figure 4D).

The conjugation of an aldehyde functional polyPEG onto oxytocin was carried out in the same manner as the conjugation of linear aldehyde PEG, resulting in an amine-linked "comb" polymer conjugate (**4**; Figure 4E). RP-HPLC analysis revealed the appearance of a substantial broad peak, at a higher retention time (t = 20.6 min) than for oxytocin (t = 7.1 min), signifying the synthesis of a newly formed peptide−polymer conjugate (Figure 4F). There was a variety of smaller baseline peaks that were also observed on the HPLC trace, although a sample of the purified polymer reagent revealed peaks at identical retention times, suggesting minor impurities in the polymer solution. Although it is likely that the purified conjugates still contained some residual unconjugated polymers (in the case of both linear and polyPEG), when these reagents were separately analyzed by RP-HPLC (UV, λ = 280 nm) no obvious peaks were observed in similar regions to those realized after conjugation. This suggests that the broad conjugate peaks in all cases were attributable to peptide−polymer conjugates.

3.4. Use of Aldehyde Functional Polymers for Reversible PEGylation of Oxytocin. As the first step of the aldehyde conjugation is a reversible reaction, it was proposed that this linkage could be used to form reversible oxytocin conjugates, whereby the polymer could be removed by an external stimulus (such as a change in pH), allowing the therapeutic to maintain complete activity. The reversible conjugation of linear PEG onto oxytocin was performed in phosphate buffer (0.1 M, pH 6.2) with the exclusion of the reducing agent forming the Schiff base product (**5**; Figure 5A). After 24 h, a sample was submitted for RP-HPLC analysis, and for both molecular weights of PEG (2 and 5 kDa), the RP-HPLC chromatograms showed the appearance of a bimodal

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peak at a higher retention time (t = 14.8 and 15.6 min) than for oxytocin (t = 7.3 min; Figure 5B). The reaction was subsequently performed at a higher pH (pH 8.0) to investigate the formation of these two products under mildly basic conditions, potentially minimizing acid hydrolysis. However, even at higher pH, the presence of a bimodal product peak was further observed, suggesting that two distinct conjugate products were being formed at both pHs. The ratio between the peaks was approximately equivalent for both the mildly acidic and mildly basic pHs, although it was noted that following longer reaction monitoring, the second peak started to become more prominent, regardless of the pH used in the conjugation process. An investigation of the speed of the reaction showed that there was a higher formation of both conjugate products with respect to oxytocin at pH 8, with a smaller proportion of the native peptide remaining (SI, Figure S7). It was proposed that these two peaks could represent the different imine stereo isomers (E/Z) that can be formed during Schiff base formation.

In a similar manner the aldehyde functional polyPEG (10 kDa) was conjugated onto oxytocin without the aid of a reducing agent, leading to the formation of a Schiff base linked "comb" polymer conjugate (**6**; Figure 5C). This conjugation was carried out in phosphate buffer (0.1 M. pH 8), and sampling by RP-HPLC after 24 h revealed the presence of a newly formed single broad peak at a higher retention time (t = 24.1 min) than for the native peptide (t = 12 min; Figure 5D).

In order to more thoroughly analyze the formation of the two different products formed in the reversible reaction between oxytocin and aldehyde functional linear PEG, a model reaction was examined using butyraldehyde as a comparable water-soluble small aldehyde (Figure 5E). The reaction was performed in phosphate buffer (0.1 M, pH 8) with a sample removed for RP-HPLC analysis of the reaction mixture after 24 h. This showed the formation of two distinct peaks; butyraldehyde conjugate peak 1 (**BCP1**) at t = 15.9 min and butyraldehyde conjugate peak 2 (**BCP2**) at 16.5 min, both at higher retention times than for the native peptide (t = 11.7 min; Figure 5F). These distinct peaks were more resolved than those products observed for the linear PEGylated products, with peak shape and peak intensity being much more comparable to the native peptide. It was also noted that when the conjugation of butyraldehyde and the polymers onto oxytocin were compared, only residual amounts of native peptide remained after 24 h in the butyraldehyde conjugation, suggesting that this reaction was more efficient. The two butyraldehyde conjugate products, **BCP1** and **BCP2** were then separated and individually collected using RP-HPLC and analyzed by ESI-MS for differences in molecular weight. The major product observed in both cases was the oxytocin−butyraldehyde product $[M + H^+]$ = 1061.4 Da, although some native peptide was also observed as a minor component $[M + H^+]$ = 1007.3 Da. Some Schiff base hydrolysis occurred due to the low pH (~2.1) of the resting solutions from RP-HPLC as a result of the TFA additive, particularly after a prolonged exposure. This is particularly notable for **BCP1**, where after long storage times, only residual conjugate is remaining; although **BCP2** also shows a high decrease in concentration of the conjugate, the only major product observed in both cases was confirmed as regenerated oxytocin by RP-HPLC and ESI-MS (SI, Figure S8).

In order to confirm that this appearance of more than one product/isomer is a particular characteristic of the reversible conjugation, the conjugation of butyraldehyde was also carried out with the addition of $NaCNBH_3$ leading to the irreversibly linked secondary amine conjugate. As with the two aldehyde PEGylations with addition of reducing agent $NaCNBH_3$, RP-HPLC analysis showed the formation of a single peak at a higher retention time (t = 13.6 min) than for oxytocin (t = 12.1 min) accompanied by an almost complete disappearance of the peak corresponding to the native peptide (SI, Figure S9). The irreversibly formed butyraldehyde conjugate was confirmed by ESI-MS, where the only major peak observed was relatable to the predicted product $[M + Na^+]$ = 1085.3 Da.

In a separate study, one experiment was performed with "in situ" reduction (as with standard procedure), while the other was performed with sequential Schiff base formation and reduction. In the sequential experiment, the distinct isomers converged to a signal peak upon reduction, identical to that obtained from the "in situ" reduction experiment (SI, Figure S10).

NMR analysis was conducted on the butyraldehyde conjugate reaction mixture (without addition of $NaCNBH_3$) and compared to that of the native peptide (SI, Figure S11). This showed that for the Schiff base conjugate there was the presence of two new doublet peaks within the imine region of the spectra could be observed (7.84/7.56 ppm), likely due to Schiff base formation. The two imine proton signals showed TOCSY connectives of two different signals, both assignable to the α proton of the Cys^1 residue. Additionally, the peaks representing the aromatic groups on the tyrosine residues show a chemical shift postconjugation (native peptide: δ_H = 7.16, ε_H = 6.83 ppm; butyraldehyde: δ_H = 7.09, ε_H = 6.85 ppm), where Tyr^2 is the next amino acid from the N-terminal amine within the peptide structure. The reaction mixtures also included excess aldehyde, for which peaks are still visible for the unreacted aldehyde as well as the hydrate formed during the hydrolysis of the aldehyde after the addition of water where the peaks at δ = 5.00 ppm are particularly prominent. These results confirm that the conjugation has occurred in the predicted manner, and the appearance of two different imine peaks assists in clarifying that there are two stereoisomers formed.

3.5. Reversibility Studies of Schiff Base Linked Oxytocin Conjugates. The reversibility of the Schiff base linked (reversible) conjugates (linear PEGylated oxytocin, polyPEGylated oxytocin, and butyraldehyde conjugated oxytocin) were studied using RP-HPLC to highlight changes in concentrations of the peptide or the conjugates. The studies were performed under different pH conditions to investigate the stability of the linkages and the practicability of recovering a product analogous to the native peptide, with an element of control over pH stimulation. Under these pH stimulated conditions, the native peptide itself remains stable for the time scale of the experiment, meaning that the released oxytocin should have retained the properties of the native peptide prior to conjugation (SI, Figure S12).

For the butyraldehyde−oxytocin conjugate, this reversibility experiment was carried out immediately post-conjugation due to the high efficiency of the reaction that resulted in minimal remaining oxytocin. A sample of the conjugation solution was diluted with two buffers, one at pH 5.0 (citrate buffer, 0.1 M) and the other pH 7.4 (phosphate buffer, 0.1 M), to give overall concentrations of 0.2 mg/mL of peptide. After 4 days, the pH 5.0 solution showed a high (10-fold) increase in the amount of oxytocin present in the solution (from 68.2 to 682 mAUs), which also corresponded to a decrease in the peak area for both

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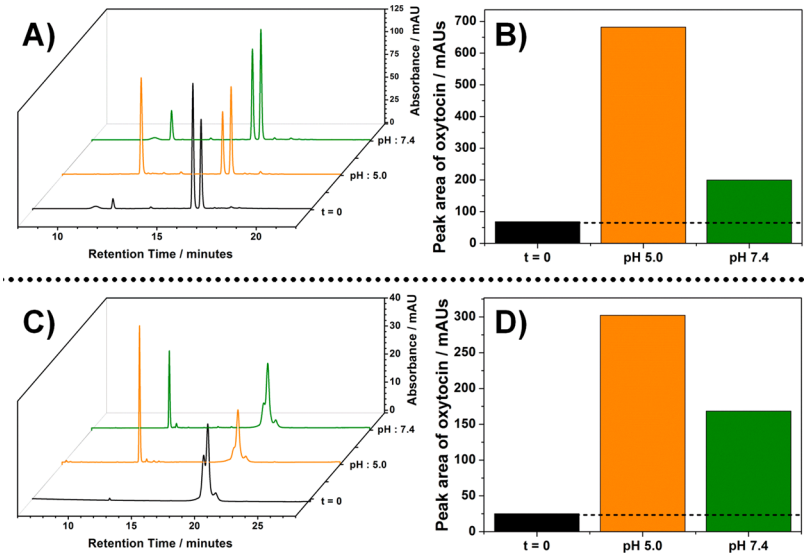

Figure 6. (A) RP-HPLC traces and (B) increase in oxytocin observed for reversal of oxytocin−butyraldehyde conjugates at pH 5.0 and pH 7.4, (C) RP-HPLC traces, and (D) increase in oxytocin observed for reversal of oxytocin−linear PEG conjugates at pH 5.0 and pH 7.4.

of the conjugate peaks (**BCP1** and **BCP2**; Figure 6A,B). Analysis of the two conjugate peaks revealed that **BCP2** showed a much smaller decrease in area, signifying that there was not a large change in concentration for this isomer (**BCP1**, 49% decrease; **BCP2**, 3% decrease; overall, 30% decrease; SI, Figure S13). For the pH 7.4 buffer, a moderate (3-fold) increase in the amount of oxytocin present in solution could still be observed (68.2−199 mAUs), but there was not a high accompanying decrease in the area of the combined conjugate peaks (**BCP1** + **BCP2**: 8% decrease). That said, the relative ratios of **BCP1** and **BCP2**, with a decrease in **BCP1** and an increase in **BCP2** respectively, suggest that **BCP2** is the more stable conjugate product (0.58:0.42 to 0.45:0.55).

This difference between the two pHs observed in these data clearly shows that the reversal of the conjugation can be somewhat controlled by using pH as a means to induce release of native peptide. For further investigation, the reversible nature of the Schiff base linked PEGylated oxytocin (both linear and polyPEG) was analyzed for oxytocin release and conjugate disappearance at different pHs. For both the polyPEG and linear PEG conjugates, two pH conditions were again examined: pH 5.0 and 7.4. The appearance of oxytocin was monitored to investigate whether it was possible to stimulate a higher release of the native peptide under a particular pH, as observed with the nonpolymer analogue.

For the linear PEGylated conjugate, the oxytocin release was monitored by RP-HPLC after storage in the different pH solutions for a 3 week period. The RP-HPLC traces show the appearance of oxytocin at both pHs when compared to t = 0 samples (Figure 6C). After 2 weeks the amount of oxytocin released at pH 5.0 showed a 12-fold increase, approximately double that released at pH 7.4 (t = 0:25 mAUs, pH 5:302 mAUs; pH 7.4:168 mAUs; Figure 6D). The disappearance of conjugates was also monitored, with similar behavior to the butyraldehyde conjugate being observed whereby the first conjugate peak is much more prone to hydrolysis releasing the native peptide. This was evaluated at both pH values but is particularly prominent under more acidic conditions (60% decrease at pH 5.0; 38% decrease at pH 7.4; SI, Figure S13).

The release was further monitored at several different time points, and it revealed that the initial level of oxytocin released was approximately the same at both pH 5 and 7.4, but at pH 7.4 this starts to plateau in the observed amount of oxytocin released after 4−6 days (SI, Figure S14). The oxytocin concentration, however, continued to gradually increase for the pH 5 solution across the study, showing potential for a sustained release of oxytocin from the conjugates over time.

For the polyPEG conjugates, there was no appreciable reversal of the peptide conjugation or release of the native peptide observed by the end of the study. From the RP-HPLC there was very little change to the peptide region of the chromatogram for the reappearance of native oxytocin and minimal concentration changes to the single broad conjugate peak, regardless of pH. This suggests that the reverse reaction was not occurring to release the native peptide over the same time scale. It can therefore be hypothesized that the structural design of the conjugates plays an important role in the ability to reverse the conjugation under stimulated conditions, with larger and more branched polymers proving less prone to reversion.

3.6. Effect of PEG Architecture on Peptide Stabilization. In order to determine the extent of improved shelf life stability for the irreversibly linked oxytocin conjugates at high temperature (80 °C), a heat stressed stability assay was devised. The high temperature was needed in order to give an appreciable degradation of oxytocin, so that reliable stability comparisons could be achieved over a reasonable time frame. The thermal stability was tested for the native peptide, each type of oxytocin conjugate linkage (linear and polyPEG both by succinimide and Schiff base/reductive amination conjugation) as well as solutions containing oxytocin with similar (non-conjugated) PEGs added as excipients into the solution (SI, Table S1). All samples were tested in triplicate, with the concentrations of remaining substances being calculated from peak areas using UV (λ = 280 nm) absorbance of the peaks on RP-HPLC examined with respect to calibrations performed on nondegraded samples (Figure 7).

Under the conditions of this heat stability assay oxytocin (1 mM) showed a high level of degradation after 24 h, with a very low oxytocin recovery (19.2 ± 1.10%), which is consistent with

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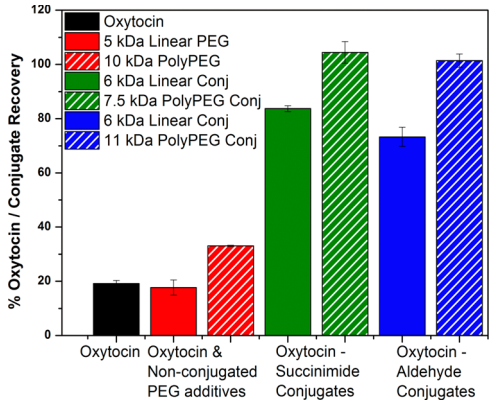

Figure 7. % Recovery of native oxytocin, oxytocin solutions containing polymer additives, and oxytocin polymer conjugates after heating at 80 °C for 24 h analyzed by RP-HPLC.

similar high temperature heat stressed assays which have been carried out previously for oxytocin.[8,84] A comparison of the HPLC traces before and after the stability assay shows the appearance of numerous degradation products, which supports previous research on the different manners that the peptide undergoes degradation.[7,8,85] Next the effect of nonconjugated PEGs of both different architectures (linear and polyPEG) were evaluated whereby polymers with similar molar mass but no conjugating groups were used to evaluate the influence of noncovalent polymer−peptide interactions. Upon addition of linear methoxy PEG (5 kDa) as a 1:1 mixture with the peptide, the degradation of oxytocin was still very high, with the polymer proving inefficient at providing any additional stabilization (oxytocin recovery: 17.7 ± 2.78%). Poly(mPEGA$_{480}$) was synthesized by Cu(0)-mediated living radical polymerization, using ethyl α-bromoisobutyrate (EBIB) as the initiator targeting a DP of 20 (94% conversion, M_n = 12300 g mol^{-1}, $Đ$ = 1.06), which could be used in the same manner as a polyPEG excipient in the oxytocin solution. A 1:1 mixture of the poly(mPEGA$_{480}$) and oxytocin in water showed marginal improvement in the recovery of oxytocin (33.1 ± 0.21%), highlighting that the polyPEGs possibly have potentially better protection properties on the peptide. As the polyPEG sample was synthesized as 20 repeat units of a smaller linear mPEGA monomer, the stability study was repeated using a ratio of 20:1 of a small linear methoxy PEG (350 Da) to oxytocin. For this higher ratio of small PEG chains, no further stabilizing effect was observed for the peptide with oxytocin recovery remaining low (19.0 ± 0.56%).

The conjugation of polyPEG onto oxytocin at the disulfide bond has previously been shown to dramatically enhance the stability of the peptide, and therefore it was anticipated that the *N*-terminally conjugated polymers would also exhibit improved stability.[9] When the irreversibly conjugated oxytocin conjugates underwent the high temperature thermal stability assay the results suggested that this was the case with high increases in conjugate recovery compared to the native peptide. The degradation was measured by comparing the broad conjugate peak observed on RP-HPLC at t = 0 and 24 h, to calculate any change in concentrations, and all reductions attributed to degradation of the conjugates. For all four different conjugates (linear PEG and polyPEG linked by aldehyde/reductive amination as well as succinimide chemistry) stabilities were seen to increase leading to oxytocin-conjugate recovery of over 70% for all samples. The comb polymer architecture is particularly noteworthy, as degradation remained very low with oxytocin-conjugate recoveries remaining around 100%. These results prove very promising in showing that the conjugation of PEG onto oxytocin via the *N*-terminal amine can prove beneficial for reducing degradation and extending shelf life stability at high temperatures.

4. CONCLUSION

Different methods of conjugating linear PEG and polyPEG site specifically to the *N*-terminal amine of the peptide therapeutic oxytocin have been explored, with an aim of improving the shelf life stability of the aqueous formulations of the drug. This has the potential for extending the application of this WHO essential medicine to help address maternal mortality in the developing world. Well-defined "comb" polymer synthesis was achieved utilizing copper-mediated living radical polymerization, and alongside linear polymer equivalents, conjugation strategies have incorporated well-developed succinimide and Schiff base/reductive amination couplings. The reversible conjugation of linear PEG aldehydes to the *N*-terminal amine suggests potential methods for use of these types of conjugations as a release mechanism, recovering the active form of the peptide. Upon conjugation two different isomers (E/Z) can be formed, each exhibiting different reversibility characteristics and the reversibility of the conjugation was found to be tunable by externally changing the pH, leading to high releases of native peptide, particularly at lower pH. In contrast, the polyPEG Schiff base conjugates did not release oxytocin significantly over the time frame of our experiments. In general, the conjugation of the polymers led to dramatic increases in stability of the peptide under high temperature conditions, resulting in a reduction of inactive degradation products of the peptide. Following on from this work, it is important to note that the uterotonic activity of the irreversible polymer conjugates will be investigated. These will be compared to the reversible conjugates at different stages of oxytocin return, as well as the native peptide, for an evaluation of any potential losses of activity post-conjugation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.6b00919.

HPLC gradient conditions, ^{1}H NMR, ^{13}C NMR, and RP-HPLC traces, and data not included in the body of this manuscript and tabulated results from the thermal stability assay (PDF).

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Notes

The authors declare no competing financial interest.

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