

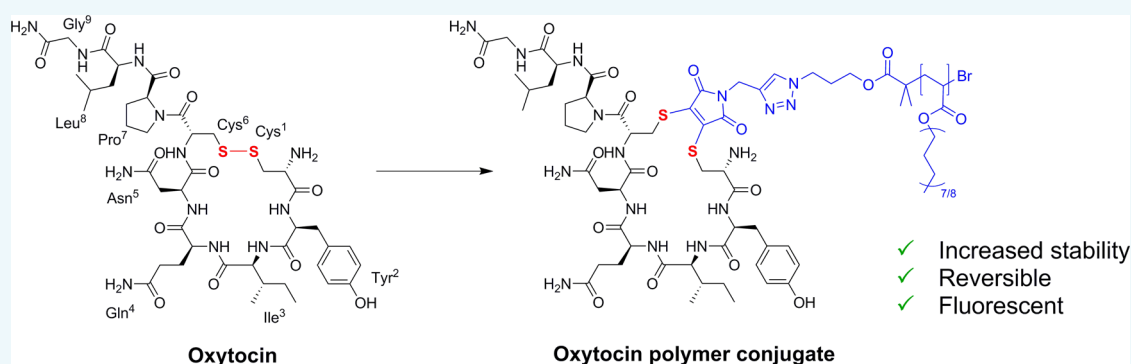
In Situ Conjugation of Dithiophenol Maleimide Polymers and Oxytocin for Stable and Reversible Polymer–Peptide Conjugates

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S Supporting Information



ABSTRACT: The in situ one-pot synthesis of peptide–polymer bioconjugates is reported. Conjugation occurs efficiently without the need for purification of dithiophenol maleimide functionalized polymer as a disulfide bridging agent for the therapeutic oxytocin. Conjugation of polymers was demonstrated to be reversible and to significantly improve the solution stability of oxytocin.

Postpartum hemorrhaging (PPH) (bleeding in excess of 500 mL after birth) is one of the major causes of maternal morbidity. The WHO-recommended drug for its prevention is oxytocin (Figure 1),^{1–3} a neurohypophyseal uterotonic nonapeptide (cyclic [Cys–Tyr–Ile–Gln–Asn–Cys]–Pro–Leu–Gly–NH₂), also released from the pituitary gland in the hypothalamus.^{4–6} Oxytocin helps to prevent PPH by causing uterine contractions which prevent large blood losses postbirth.¹ This occurs by oxytocin binding to and causing activation of the oxytocin binding receptor, a G-protein coupled receptor, which ultimately stimulates uterine contractions through production of intracellular Ca²⁺ and prostaglandins.^{7,8} Structurally, Asn⁵ and Tyr² are key residues for activity at the uterus, while Ile³, Gln⁴, Pro⁷, and Leu⁸ are important for receptor binding.⁹

Oxytocin has very limited stability in aqueous solutions, particularly at elevated temperatures with degradation causing a loss of activity of the drug.^{10–12} This is particularly problematic in warm climates often found in many developing countries where approximately 99% of the world's maternal deaths occur. Maternal mortality rates in these countries can be as high as 1000 women per 100 000 live births.^{13,14} Oxytocin degradation occurs via a variety of processes, but particularly at the Cys¹–Cys⁶ disulfide bond, which is prone to dimerization, oxidation, tri/tetrasulfide formation, β -elimination, and the formation of

larger aggregates.¹¹ As a result of this, injectable aqueous oxytocin formulations require refrigeration (2–8 °C). As a consequence, researchers have been looking for methods to improve the stability of the drug, which allows for better handling in clinical applications, in particular, in the developing world where establishment of refrigerated supply chains is often not possible.^{15–19} Various research has been carried out to modify the structure of oxytocin to change the sequence or nature of amino acids and analysis of remaining biological activity.²⁰ Due to the cysteine residues of the disulfide bond not being implicated in receptor binding, or activity, a variety of modifications at this position have previously been evaluated. In particular, various disulfide bond alternatives have been considered including removal or substitution of a sulfur atom from the disulfide bridge. On decreasing the structural ring size through peptide engineering, the result was a significant loss of activity.²¹ Substitution of one sulfur atom on the disulfide bond (such as with selenium), however, is more highly tolerated, and of particular note are the thioether bridged oxytocin analogues which efficiently conserve binding affinity and functional activity.^{22,23}

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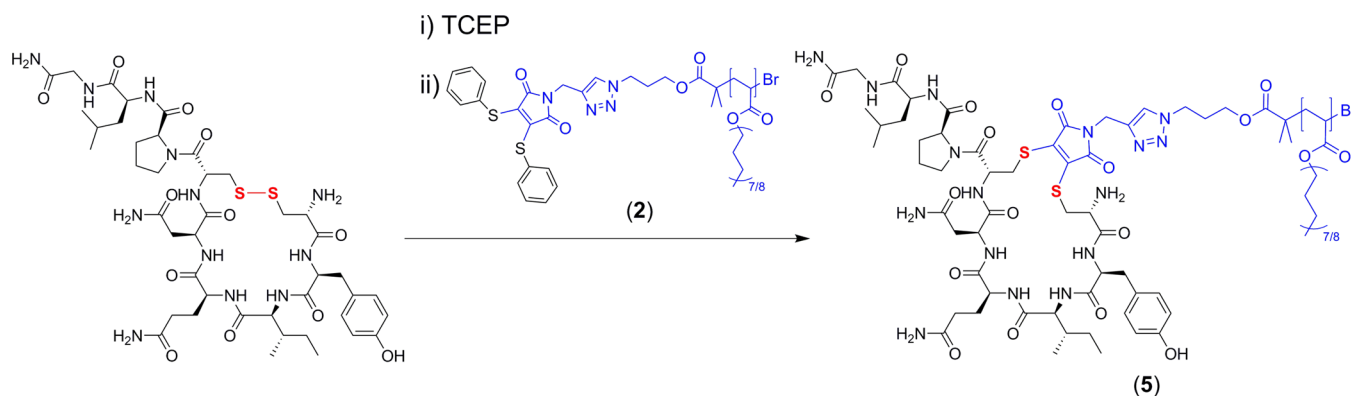


Figure 1. Structure of therapeutic peptide oxytocin and conjugation reaction with maleimide functional polymer.

Conjugation of synthetic polymers to peptides and proteins can lead to a variety of enhanced properties depending on the polymer used, including improved drug solubility and stability.^{24–26} In particular, amine groups, such as those at the N-terminus and constituent lysine residues, are useful covalent handles for the synthesis of well-defined conjugates. However, most peptides or proteins possess multiple amino groups, which complicates the monofunctionalization, due to competing reactions, and makes site-specific approaches challenging. This has led to modification at cysteine residues and disulfide bonds receiving increased attention as an alternative modification site due to lower relative abundance within the protein. Reactions at cysteine residues, e.g., via thio-Michael addition, can lead to a more site specific conjugation approach.²⁷ While free thiols of reduced cysteine residues can be targeted by the addition of polymers using thio-Michael reactions, this is not always desirable if the peptide contains disulfide bonds, which are often important for the retention of tertiary structure and thus protein activity.^{28–30}

Various protein conjugation techniques have been developed which show the protein structure and function being retained by the formation of a disulfide linking carbon bridge where the polymer is inserted. In 2006, Brocchini et al. suggested a method for targeting disulfide bridges using an α,β -unsaturated β' -monosulfone functional polymer reagent.^{31–33} More recently, Baker et al. described several different maleimide reagents for protein conjugation, using both the reactions of single cysteines as well as rebridging of disulfides via a two-carbon bridge.^{34–36} Both dibromo and dithio polymeric maleimides were demonstrated to be efficient at reversibly rebridging reduced disulfide bonds in a variety of peptides, including somatostatin and salmon calcitonin.^{37–39}

The use of “poly(PEG)” functional polymers for conjugation to biomolecules can be advantageous when compared to linear equivalents. For the synthesis of higher molecular weight polymers, designed to slow down/prevent renal clearance from the body, end functional poly(PEG) can be more easily prepared in comparison to linear high-molecular-weight PEG. A major disadvantage of linear PEG is the accumulation of PEG as vacuoles in certain organs.^{26,40,41} In addition, the viscosity of poly(PEG)s with short pendant PEG chains are lower, as they exist as rigid rods in solution where viscosity is not dependent on chain length, whereas linear PEG equivalents dissolve as random coils with an increase in viscosity on increasing molecule weight. This manifests itself where administration is required over long time periods in chronic illnesses.

Progress in synthetic polymerization techniques in combination with highly efficient modification reactions has provided a versatile platform for the synthesis of highly functional polymers and materials tailor-made for the conjugation to proteins and peptides.^{42–45} Synthesis of polymers using Cu(I) mediated polymerization atom-transfer radical polymerization (ATRP) from a radical compatible dithiophenol maleimide initiator resulted in appropriate α -functional polymers.⁴⁶ Following purification of the functional polymer, conjugation onto the peptide salmon calcitonin in an efficient and site-selective manner was achieved. It was later discovered that dithioalkyl maleimide molecules show fluorescence, with the effect being significantly quenched for dithiophenol maleimides, which enables monitoring of the conjugation.⁴⁷

Herein, we describe the synthesis of α -functional maleimide polymers by single electron transfer living radical polymerization (SET-LRP) and their in situ, reversible conjugation to oxytocin, which is shown to enhance the stability and thus the potential storage capacity/shelf life of the peptide prior to administration.

To obtain the dithiophenol functional polymers (Figure 2A), a dithiophenol maleimide (DTM) initiator was prepared and the polymerizations were conducted at ambient temperature in 80 vol % DMSO to guarantee the dissolution of the functional initiator. Importantly, Cu(I)Br was allowed to disproportionate fully in water upon addition of Me₆Tren prior to monomer and initiator addition as a single solution in DMSO.⁴⁸ Three poly(methoxy poly(ethylene glycol) acrylate)s (PmPEGA) were synthesized using [M]:[I] = 20, 50, and 100 (2–4). In all cases high conversions were achieved and quantitative functionalization of the polymers with the maleimide end group was observed by ¹H NMR (Figure 2B). SEC analysis of the polymers (measured in DMF relative to narrow molecular weight PMMA calibrants) indicated the synthesis of polymers with relatively narrow dispersities (Figure 2C).

An interesting phenomenon was observed during the polymerization of the higher molar mass polymers (3) and (4) whereby the polymer “precipitated/separated” out of the polymerization solution (Figure S3). Very little polymer was detected in the upper layer, which consisted of largely the solvents and catalyst, and this is particularly convenient for the purification of these polymers.

For the in situ conjugation (Figure 1), the disulfide bond of oxytocin was initially cleaved using tris(2-carboxyethyl)-phosphine (TCEP) to generate two free cysteine residues. Following polymerization, an aliquot of the polymerization mixture was taken directly from the polymerization solution

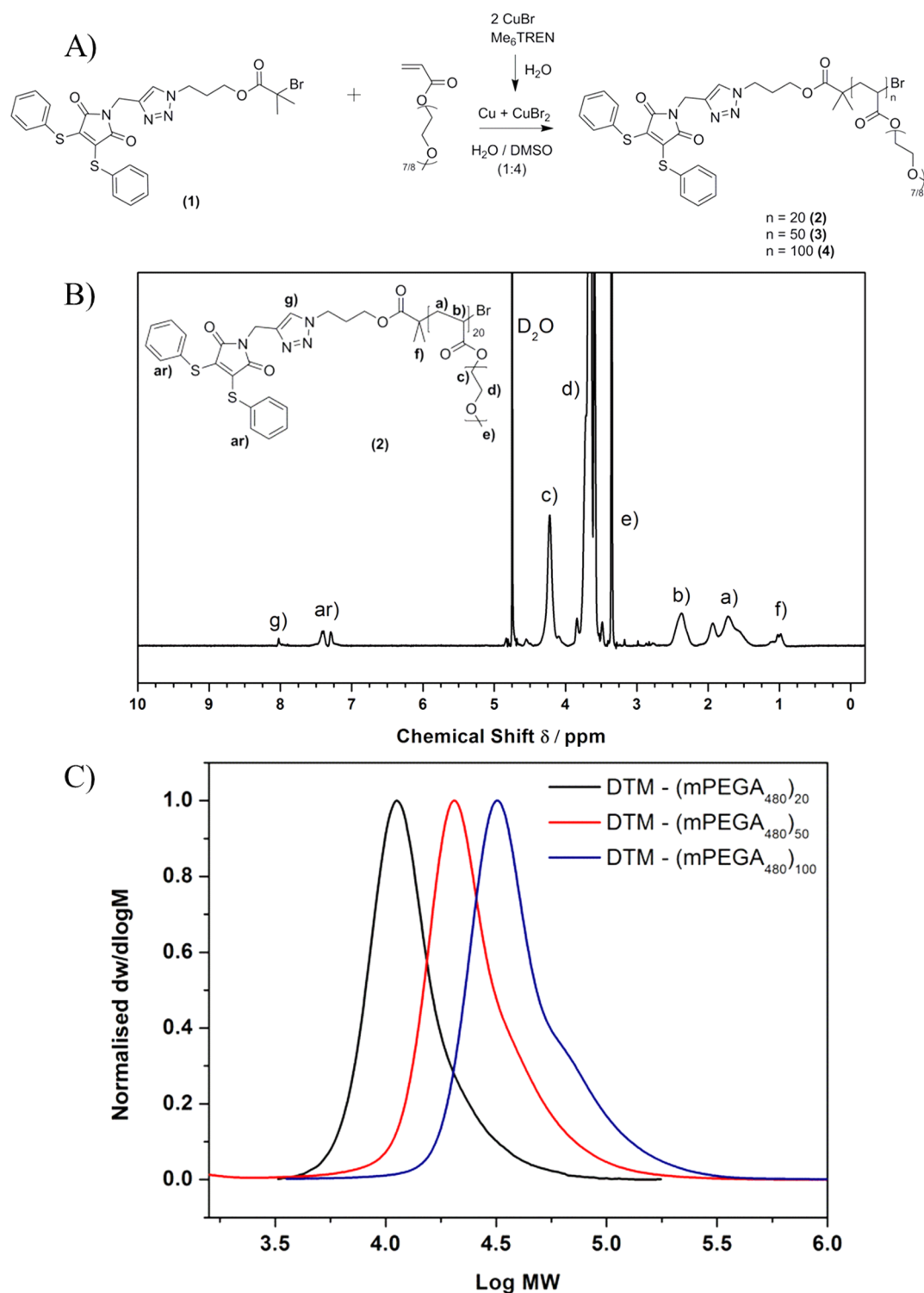


Figure 2. (A) Polymerization of methoxy poly(ethylene glycol) acrylate monomer with initiator (1). (B) ^1H NMR spectra (D_2O) of dithiophenol functional polymer. (C) SEC analysis (DMF) of polymerization reaction with $[\text{M}]: [\text{I}] = 20, 50, \text{ and } 100$.

and added to a solution of the reduced peptide. After leaving the reaction at 10°C overnight a sample of the reaction was analyzed by RP-HPLC both with UV ($\lambda = 280\text{ nm}$) and fluorescence ($\lambda_{\text{ex}} = 341\text{ nm}$; $\lambda_{\text{em}} = 502\text{ nm}$) detection (Figure 3A). A shift in retention time was observed from the dithiophenol maleimide polymer to a new peak corresponding to the oxytocin polymer conjugate, as well as the appearance of a coincident fluorescence peak. The use of a slight excess of

polymer coupled with the performance of the reaction not proceeding with 100% efficiency potentially results in a small amount of unreacted polymer remaining in the solution.

Following purification, RP-HPLC analysis shows that all thiophenol and remaining oxytocin were removed and there is a clear shift in retention time between the polymer and the oxytocin polymer conjugate, as well as the new fluorescence (Figure 3B). SEC analysis showed the expected increase in

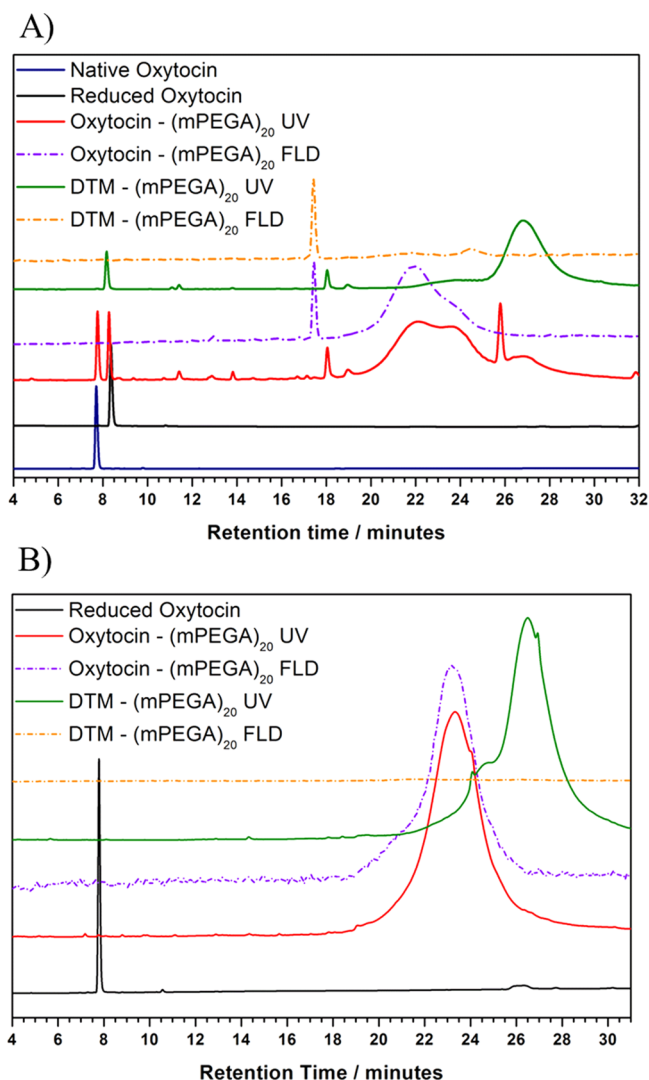


Figure 3. (A) Reaction monitoring of in situ disulfide bridging conjugation reaction by RP-HPLC. (B) RP-HPLC of dithiophenol maleimide polymer and oxytocin conjugate following purification of the in situ product.

molar mass (Figure S12). Fluorescence photometer studies showed that the amount of fluorescence observed increased from the native polymer to the peptide conjugate by more than a factor of 50 (Figure S18). This is in agreement with previous work that suggests that the exchange of the thiophenol groups on the maleimide for insertion to the peptide increases observed fluorescence.⁴⁷ Post-polymerization reaction with purified polymers and reduced oxytocin yielded similar successful conjugation as the in situ approach, as observed by RP HPLC (Figure S18).

It was theorized that the stability of the oxytocin polymer conjugates in comparison to native oxytocin should have increased following conjugation with PEG polymers. This was investigated via an accelerated stability test whereby the oxytocin polymer conjugates, along with native oxytocin, were individually dissolved in water to a concentration of 1 mg mL⁻¹ and immersed in a water bath set at 50 °C. Aliquots of the samples were periodically removed and analyzed by RP-HPLC across 28 days for an investigation of degradation at this elevated temperature. The amount of oxytocin and oxytocin polymer conjugate remaining, respectively, was calculated as a

percentage of the relevant peak before immersion in water bath to the other degradation products at the different time intervals (Figure 4A). The amount of degradation that is observed was

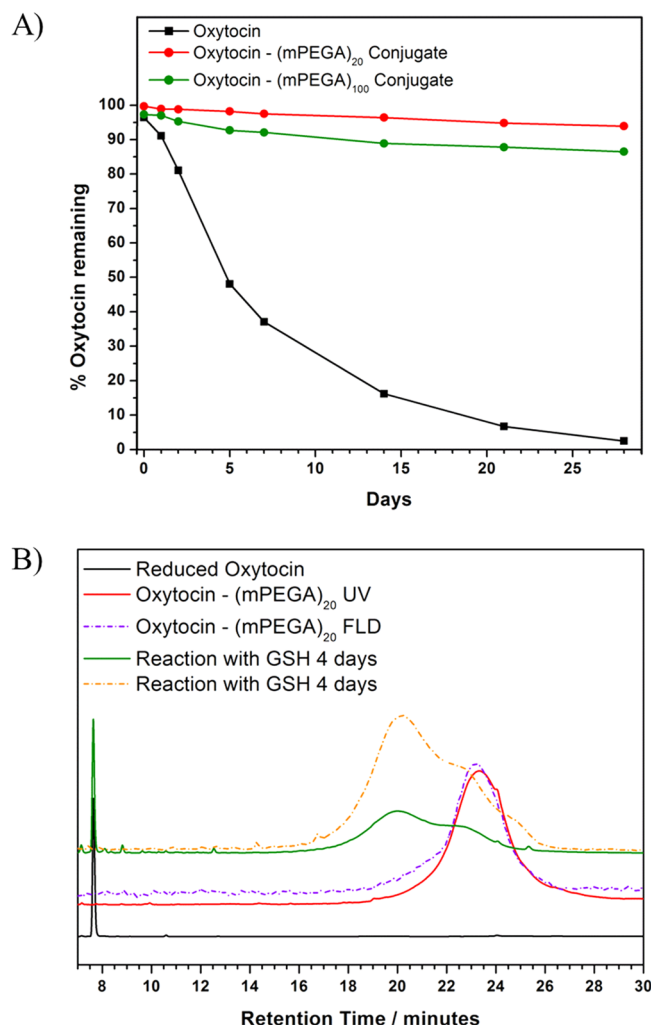


Figure 4. (A) Degradation profiles for aqueous solutions of native oxytocin and oxytocin polymer conjugates at 50 °C. (B) RP-HPLC monitoring of reversibility of oxytocin polymer conjugate with glutathione, releasing the native peptide.

severely suppressed by the conjugation of the dithiophenol maleimide polymers bridging the disulfide bond, whereby after 28 days only 2.5% of native oxytocin remained, whereas for the polymer conjugates oxytocin-(mPEGA₄₈₀)₂₀ and oxytocin-(mPEGA₄₈₀)₁₀₀, 93.9% and 86.5% remained, respectively. The major peaks that can be observed for the conjugates after 28 days coincide with the conjugate peaks present prior to the stability test. The minor peaks are attributed to oxytocin or degradation products of oxytocin where identical peaks were observed in the equivalent stability test on unconjugated oxytocin. This could be attributed to previous research that suggests a large amount of the degradation products observed for oxytocin are from routes occurring from reactions centered around the disulfide bond.^{10,11} This indicates that insertion of the maleimide functional polymers in this manner have improved the stability of oxytocin by preventing or hindering degradation around the disulfide bond.

Reversibility of conjugation is another important criterion for bioconjugation techniques with different reversible conjugation

strategies, arising from site selective conjugations between proteins/peptides and polymers that are well-known in the literature.^{49–53} Release of polymers from peptides can restore the native (unmodified) peptide and therefore confer resumption of biological activity. In order to investigate the reversibility of the polymer conjugation under biological conditions, studies were carried out in an excess of glutathione (GSH). GSH is a tripeptide containing glutamate, cysteine, and glycine, and exists in the cytoplasm of healthy cells of the body in concentrations 0.2–10 mM.⁵⁴ Maleimides conjugated onto disulfide bonds have previously been found to be reversible in the presence of an excess of GSH.^{34,39} An excess of GSH equivalent to 6 mM, being in the biorelevant range, was added to a 5 mg mL⁻¹ solution of oxytocin polymer conjugate (5) which had been purified by dialysis. Sampling of the reaction after 4 days and monitoring by RP-HPLC showed the successful release and reformation of native oxytocin, as well as a shift in the retention time for the GSH substituted polymer from the polymer conjugate. Fluorescence is maintained on the polymer, where two molecules of GSH add onto the maleimide in the previous place of the peptide (Scheme S4). The released peptide was confirmed as native oxytocin using MALDI-TOF MS (Figure S23).

In summary, we have demonstrated the in situ synthesis of disulfide bridging polymer peptide conjugates of oxytocin without the need for prior purification of the polymers. Many different approaches to site-selective PEGylation can be considered for enhancing properties of peptides, possessing advantages and disadvantages depending on a multitude of factors. Disulfide bridge insertion using dithiophenol maleimide chemistry proved particularly useful for this peptide, due to the manner of oxytocin degradation. In this case the subsequent conjugates showed significantly higher stability than the native peptide at elevated temperatures. The reversible nature of this conjugation approach further highlights the possibility of reformation of the native peptide, potentially preventing a loss of biological activity. Future studies will focus on biological testing to establish the uterotonic activity of the polymer conjugates and GSH released oxytocin in comparison to native oxytocin.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental procedures and additional data including NMR, SEC, RP-HPLC, MALDI-TOF, and fluorescence spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Maughan, K. L., Heim, S. W., and Galazka, S. I. M. S. (2006) *Am. Fam. Physician* 73, 1025.
- (2) Van Dongen, P. W., Van Roosmalen, J., De Boer, C. N., and Van Rooij, J. (1991) *Pharm. Weekbl., Sci. Ed.* 13, 238.
- (3) Moir, C. (1939) *Proc. R. Soc. Med.* 32, 928.
- (4) Du Vigneaud, V., Ressler, C., and Trippett, S. (1953) *J. Biol. Chem.* 205, 949.
- (5) Du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., and Katsoyannis, P. G. (1954) *J. Am. Chem. Soc.* 76, 3115.
- (6) Ohno, A., Kawasaki, N., Fukuhara, K., Okuda, H., and Yamaguchi, T. (2010) *Magn. Reson. Chem.* 48, 168.
- (7) Vrachnis, N., Malamas, F. M., Sifakis, S., Deligeoroglou, E., and Iliodromiti, Z. (2011) *Int. J. Endocrinol.* 2011, 350546.
- (8) Manning, M., Misicka, a, Olma, a, Bankowski, K., Stoev, S., Chini, B., Durroux, T., Mouillac, B., Corbani, M., and Guillon, G. (2012) *J. Neuroendocrinol.* 24, 609.
- (9) Walter, R., Schwartz, I. L., Darnell, J. H., and Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 1355.
- (10) Hawe, A., Poole, R., Romeijn, S., Kasper, P., van der Heijden, R., and Jiskoot, W. (2009) *Pharm. Res.* 26, 1679.
- (11) Wiśniewski, K., Finnman, J., Flipo, M., Galyean, R., and Schteingart, C. D. (2013) *Biopolymers* 100, 408.
- (12) Gard, J. W., Alexander, J. M., Bawdon, R. E., and Albrecht, J. T. (2002) *Am. J. Obstet. Gynecol.* 186, 496.
- (13) Hogan, M. C., Foreman, K. J., Naghavi, M., Ahn, S. Y., Wang, M., Makela, S. M., Lopez, A. D., Lozano, R., and Murray, C. J. L. (2010) *Lancet* 375, 1609.
- (14) Khan, K. S., Wojdyla, D., Say, L., Gülmezoglu, A. M., and Van Look, P. F. A. (2006) *Lancet* 367, 1066.
- (15) Prankerd, R. J., Nguyen, T.-H., Ibrahim, J. P., Bischof, R. J., Nassta, G. C., Olerile, L. D., Russell, A. S., Meiser, F., Parkinson, H. C., Coleman, H. A., Morton, D. A. V., and McIntosh, M. P. (2013) *PLoS One* 8, e82965.
- (16) Avanti, C., Permentier, H. P., Dam, A., van Poole, R., Jiskoot, W., Frijlink, H. W., and Hinrichs, W. L. J. (2012) *Mol. Pharmaceutics* 9, 554.
- (17) Avanti, C., Hinrichs, W. L. J., Casini, A., Eissens, A. C., Dam, A. V. A. N., Kedrov, A., Driessen, A. J. M., Frijlink, H. W., and Permentier, H. P. (2013) *J. Pharm. Sci.* 102, 1734.
- (18) Avanti, C., Amorij, J.-P., Setyaningsih, D., Hawe, A., Jiskoot, W., Visser, J., Kedrov, A., Driessen, A. J. M., Hinrichs, W. L. J., and Frijlink, H. W. (2011) *AAPS J.* 13, 284.
- (19) Avanti, C., Oktaviani, N. A., Hinrichs, W. L. J., Frijlink, H. W., and Mulder, F. (2013) *Int. J. Pharm.* 444, 139.
- (20) Berde, B., Doepfner, W., and Konzett, H. (1957) *Br. J. Pharmacol. Chemother.* 12, 209.
- (21) Muttenthaler, M., Andersson, A., de Araujo, A. D., Dekan, Z., Lewis, R. J., and Alewood, P. F. (2010) *J. Med. Chem.* 53, 8585.
- (22) Wiśniewski, K., Alagarsamy, S., Galyean, R., Tariga, H., Thompson, D., Ly, B., Wiśniewski, H., Qi, S., Croston, G., Laporte, R., Rivie, P. J., and Schteingart, C. D. (2014) *J. Med. Chem.* 57, 5306.
- (23) De Araujo, A. D., Mobli, M., Castro, J., Harrington, A. M., Vetter, I., Dekan, Z., Muttenthaler, M., Wan, J., Lewis, R. J., King, G. F., Brierley, S. M., and Alewood, P. F. (2014) *Nat. Commun.* 5, 3165.
- (24) Veronese, F. M., and Pasut, G. (2005) *Drug Discovery Today* 10, 1451.
- (25) Harris, J. M., and Chess, R. B. (2003) *Nat. Rev. Drug Discovery* 2, 214.
- (26) Knop, K., Hoogenboom, R., Fischer, D., and Schubert, U. S. (2010) *Angew. Chem., Int. Ed.* 49, 6288.
- (27) Stenzel, M. H. (2013) *ACS Macro Lett.* 2, 14.

- (28) Jones, M. W., Gibson, M. I., Mantovani, G., and Haddleton, D. M. (2011) *Polym. Chem.* 2, 572.
- (29) Slavin, S., Khoshdel, E., and Haddleton, D. M. (2012) *Polym. Chem.* 3, 1461.
- (30) Jones, M. W., Mantovani, G., Ryan, S. M., Wang, X., Brayden, D. J., and Haddleton, D. M. (2009) *Chem. Commun.*, 5272.
- (31) Brocchini, S., Godwin, A., Balan, S., Choi, J., Zloh, M., and Shaunak, S. (2008) *Adv. Drug Delivery Rev.* 60, 3.
- (32) Balan, S., Choi, J.-W., Godwin, A., Teo, I., Laborde, C. M., Heidelberger, S., Zloh, M., Shaunak, S., and Brocchini, S. (2007) *Bioconjugate Chem.* 18, 61.
- (33) Shaunak, S., Godwin, A., Choi, J.-W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M., and Brocchini, S. (2006) *Nat. Chem. Biol.* 2, 312.
- (34) Smith, M. E. B., Schumacher, F. F., Ryan, C. P., Tedaldi, L. M., Papaioannou, D., Waksman, G., Caddick, S., and Baker, J. R. (2010) *J. Am. Chem. Soc.* 132, 1960.
- (35) Tedaldi, L. M., Smith, M. E. B., Nathani, R. I., and Baker, J. R. (2009) *Chem. Commun.*, 6583.
- (36) Castañeda, L., Wright, Z. V. F., Marculescu, C., Tran, T. M., Chudasama, V., Maruani, A., Hull, E. A., Nunes, J. P. M., Fitzmaurice, R. J., Smith, M. E. B., Jones, L. H., Caddick, S., and Baker, J. R. (2013) *Tetrahedron Lett.* 54, 3493.
- (37) Schumacher, F. F., Nobles, M., Ryan, C. P., Smith, M. E. B., Tinker, A., Caddick, S., and Baker, J. R. (2011) *Bioconjugate Chem.* 22, 132.
- (38) Jones, M. W., Strickland, R. A., Schumacher, F. F., Caddick, S., Baker, J. R., Gibson, M. I., and Haddleton, D. M. (2012) *J. Am. Chem. Soc.* 134, 1847.
- (39) Moody, P., Smith, M. E. B., Ryan, C. P., Chudasama, V., Baker, J. R., Molloy, J., and Caddick, S. (2012) *ChemBioChem* 13, 39.
- (40) Pasut, G., and Veronese, F. M. (2007) *Prog. Polym. Sci.* 32, 933.
- (41) Webster, R., Elliott, V., Park, B. K., Walker, D., and Taupin, P. (2009) In *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pp 127–146, Birkhäuser, Basel.
- (42) Broyer, R. M., Grover, G. N., and Maynard, H. D. (2011) *Chem. Commun.* 47, 2212.
- (43) Le Droumaguet, B., and Nicolas, J. (2010) *Polym. Chem.* 1, 563.
- (44) Grover, G. N., and Maynard, H. D. (2010) *Curr. Opin. Chem. Biol.* 14, 818.
- (45) Tolstyka, Z. P., and Maynard, H. D. (2012) In *Polymer Science: A Comprehensive Reference*, pp 317–337, Vol 9, Elsevier B.V..
- (46) Jones, M. W., Strickland, R. A., Schumacher, F. F., Caddick, S., Baker, J. R., Gibson, M. I., and Haddleton, D. M. (2012) *Chem. Commun.* 48, 4064.
- (47) Robin, M. P., Wilson, P., Mabire, A. B., Kiviaho, J. K., Raymond, J. E., Haddleton, D. M., and O'Reilly, R. K. (2013) *J. Am. Chem. Soc.* 135, 2875.
- (48) Zhang, Q., Wilson, P., Li, Z., Mchale, R., Godfrey, J., Anastasaki, A., Waldron, C., and Haddleton, D. M. (2013) *J. Am. Chem. Soc.* 135, 7355.
- (49) Gong, Y., Leroux, J., and Gauthier, M. A. (2015) *Bioconjugate Chem.*, DOI: 10.1021/bc500611k.
- (50) Pasut, G. (2014) *Polymers (Basel)* 6, 160.
- (51) Pasut, G., and Veronese, F. M. (2012) *J. Controlled Release* 161, 461.
- (52) Greenwald, R. B., Choe, Y. H., Mcguire, J., and Conover, C. D. (2003) *Adv. Drug Delivery Rev.* 55, 217.
- (53) Filpula, D., and Zhao, H. (2008) *Adv. Drug Delivery Rev.* 60, 29.
- (54) Anderson, M. E. (1998) *Chem. Biol. Interact.* 111–112, 1.