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Communication

Comb poly(oligo(2-ethyl-2-oxazoline)methacrylate)-peptide conjugates prepared by aqueous Cu(0)-mediated polymerization and reductive amination

Jennifer Collins, Sacha J. Wallis, Alexandre Simula,[#] Michael R. Whittaker, Michelle P. McIntosh, Paul Wilson, Thomas P. Davis, David M. Haddleton,* Kristian Kempe*

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J. Collins, S. J. Wallis, Dr. A. Simula, Dr. P. Wilson, Prof. T. P. Davis Prof. D. M. Haddleton, Dr. K. Kempe

Chemistry Department, University of Warwick, Coventry, CV4 7AL, UK
E-mail: D.M.Haddleton@warwick.ac.uk

Dr. M. R. Whittaker, Dr. P. Wilson, Prof. T. P. Davis, Prof. D. M. Haddleton, Dr. K. Kempe
ARC Centre of Excellence in Convergent Bio-Nano Science & Technology, Monash Institute of
Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia

Michelle P. McIntosh

Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia

E-mail: kristian.kempe@monash.edu

#Present address: POLYMAT and Kimika Aplikatua Salia, University of the Basque Country UPV/EHU,
Joxe Mari Korta Center, Avda. Tolosa 72, 20018 Donostia-San Sebastián, Spain

The controlled synthesis of poly(oligo(2-ethyl-2-oxazoline)methacrylate) (P(OEtOxMA)) polymers by Cu(0)-mediated polymerization in water/methanol mixtures is reported. Utilizing an acetal protected aldehyde initiator for the polymerization, well-defined polymers were synthesized (> 99 % conversion, $\bar{D} < 1.25$) with subsequent post-polymerization deprotection resulting in α -aldehyde end group containing comb polymers. These P(OEtOxMA) were subsequently site-specifically conjugated, via reductive amination, to a dipeptide (NH₂-Gly-Tyr-COOH) as a model peptide, prior to conjugation to the functional peptide oxytocin. The resulting oxytocin conjugates were evaluated in comparison

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to poly(oligo(ethylene glycol) methyl ether methacrylate) P(OEGMA) combs synthesized in the same manner for potential effects on thermal stability in comparison to the native peptide.

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Introduction

The conjugation of polymers to peptides or proteins can have a beneficial effect on their pharmacokinetics and can increase the efficacy and safety of peptide based medicines. The larger hydrodynamic volume of the conjugates in combination with altered rigidity and charge can offer beneficial factors, which can decrease renal clearance and depending on the type of polymer used also increase the blood circulation time of the conjugate. In this context, the covalent attachment of poly(ethylene glycol) (PEG) to drugs, also known as PEGylation, has led to the development of an impressive range of FDA approved clinical products.^{1,2} However, an issue with the conjugation of linear polymers to drugs is their increased viscosity due to the relatively high hydrodynamic volumes of the conjugate.³ This gives rise to an increased solution viscosity at a given concentration which in turn results in the requirement of either higher pressure for administration or wider bore needles. These are negative effects for the injection of the conjugates, which is the general approach for systemic administration. Comb-shaped poly(oligo(ethylene glycol)) (PolyOEG) systems have been found to offer a potential solution for this problem while maintaining increased blood half-life times as the comb polymers are rigid rods in solution and align with flow causing a large decrease in solution viscosity.⁴ These beneficial properties have attracted significant interest towards comb/brush-shaped OEG systems in recent years and has resulted in the development of a range of polymerization strategies which enable the preparation of well-defined comb-shaped PolyOEG with defined end groups which can be exploited post-polymerisation, e.g. for peptide conjugations.⁵⁻¹²

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A polymer class exhibiting similar properties to PEG are poly(2-oxazoline)s (POx).¹³ This highly functional polymer class combines modularity^{14,15} with biocompatibility¹⁶ and thus has emerged as a valuable PEG alternative. POx are synthesised by cationic ring-opening polymerization (CROP) of 2-substituted-2-oxazolines and depending on the 2-substituent, polymers with different properties can be obtained.^{17,18} Short aliphatic 2-substituents such as methyl or ethyl have been shown to be of particular interest as they lead to the formation of hydrophilic, non-toxic polymers, which possess low-fouling properties and a 'stealth' behaviour similar to PEG.^{13,19} The living character of the CROP enables the synthesis of well-defined amphiphilic block copolymers with modular end groups by the selection of appropriate monomers and functional initiators and/or terminating agents.¹⁵ When terminating the polymerization with (meth)acrylic acid POx macromonomers are obtained, which can be further polymerized giving the opportunity for a further level of functionality.²⁰ To date, the latter have been polymerised by free radical polymerization (FRP),²¹ group transfer polymerization (GTP),²² reversible addition-fragmentation chain transfer (RAFT) polymerization,²³ and atom transfer radical polymerization (ATRP)²⁴ to give comb-shaped POx (co)polymers with tuneable properties. In contrast to comb-shaped PolyOEG the potential of comb-shaped POx has not been fully exploited yet. Most studies on comb-shaped POx have been concerned with their polymerization behaviour and the alteration of their aqueous solution properties.^{23,24} However, the previously employed controlled polymerization strategies often achieved only moderate conversions, requiring complicated purification, high catalyst loadings²⁴ or high temperatures,²³ which is not always

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compatible, particularly for polymerisations of monomers exhibiting lower critical solution temperature (LCST) behaviours. Recently, the toolbox of comb-shaped POx has been expanded by copolymerizations with other functional monomers, which has allowed the further modification of comb-shaped POx and their use in a layer-by-layer approach for the fabrication of targeted,²⁵ degradable²⁶ and low-fouling²⁷ POx capsules. Up to date, with regard to bioconjugation, only comb-shaped POx with glycosylated²⁸ and antibody-functionalised²⁹ side chains have been prepared using α -glycosylated and α -azide POx macromonomers, respectively. However, to the best of our knowledge, the preparation of well-defined main chain linked comb-shaped POx-peptide/protein conjugates has not been described.

Herein, we report for the first time, the aqueous Cu(0)-mediated polymerization of POx macromonomers. Cu(0)-mediated polymerizations have proven useful for the synthesis of a variety of different monomers with high end group fidelity.³⁰ Utilising the rapid pre-disproportionation of Cu(I) the polymerisation of monomers that have previously proven problematic such as acrylamides are highly accessible, and recently the controlled aqueous synthesis of poly(methacrylates) has been reported.^{31,32} This versatile and highly tuneable controlled polymerisation technique has been reported in water,^{33,34} complex aqueous and alcoholic mixtures,^{35,36} and under biologically relevant conditions³⁷ including for the synthesis of peptide-polymer conjugates.³⁸

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In this contribution, it is demonstrated that Cu(0) mediated reversible deactivation radical polymerisation (RDRP) enables the rapid synthesis of well-defined comb-shaped POx with quantitative monomer conversions (> 99 %) under benign conditions. Application of a protected aldehyde initiator in combination with high end-group fidelity provided straightforward access to POx combs adept for post-polymerization peptide modification after deprotection. Peptide-polymer conjugations were performed by reductive amination of the α -aldehyde end group of the comb-shaped POx with two model peptides to demonstrate the feasibility of the approach.

Results and Discussion

Conditions have previously been developed for the controlled synthesis of methacrylic polymers utilizing the disproportionation of $[\text{Cu}^{\text{I}}(\text{PMDETA})\text{Cl}]$ in water to yield Cu(0) and $[\text{Cu}^{\text{II}}(\text{PMDETA})\text{Cl}]\text{Cl}$ in solutions containing NaCl as an external additive.³¹ The addition of halide salts (to a concentration of at least 1M) is vital in retaining control during the polymerization by limiting halide dissociation from the copper complex.³⁹ These conditions were employed and optimized for the Cu(0)-mediated polymerization of oligo(2-ethyl-2-oxazoline)methacrylate (OEtOxMA), a promising macromonomer with regard to its 'low fouling' properties, whose polymerization has proven to be challenging in the

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past. Two initiators were used in the present study, a dihydroxy functional initiator well-established in aqueous Cu(0)-mediated polymerizations (**1**),³³ and an acetal protected aldehyde initiator, previously introduced for post-polymerization protein/peptide modifications (**2**).⁷

Initially, oligo(ethylene glycol) methyl ether methacrylate (OEGMA) ($M_n = 475 \text{ g mol}^{-1}$) (a commercially available macromonomer, previously employed under similar conditions) was polymerized with initiators (**1**) and (**2**). The reactions were performed at ambient temperature with $[I]:[M]:[PMDTA]:[CuCl] = 1:50:0.8:0.8$, in water-methanol solutions containing 1.6 M of NaCl, with periodic monitoring of the conversion (^1H NMR) and molecular weights (SEC). High conversions were rapidly achieved for these polymerizations, particularly prominent for the P(OEGMA) synthesis using initiator (**2**), where a conversion of 85 % was obtained after 2.5 minutes ($M_n = 16900 \text{ g mol}^{-1}$, $\bar{D} = 1.21$) rising to 97 % after 30 minutes ($M_n = 20000 \text{ g mol}^{-1}$, $\bar{D} = 1.19$). The high polymerization rates complicated the sampling of the polymerization (initiator (**1**): $t = 2.5$ minutes, 65 % conversion, $M_n = 14300 \text{ g mol}^{-1}$, $\bar{D} = 1.34$; initiator (**2**): $t = 2.5$ minutes, 85 % conversion, $M_n = 16900 \text{ g mol}^{-1}$, $\bar{D} = 1.21$). Nonetheless, even for high conversions relatively narrow ($\bar{D} < 1.35$) and mono-modal molecular weight distributions were observed throughout (Figures S1 and S2, Supporting Information).

The OEtOxMA macromonomer ($DP\ 5$; $M_{n,NMR} = 600 \text{ g mol}^{-1}$), which was obtained by CROP with subsequent end capping with MAA (Figure S3, Supporting Information),²⁰ was polymerized under the same conditions (ambient temperature, in water-methanol containing 1.6 M NaCl,

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[I]:[M]:[CuCl]:[PMDTA]= 1:50:0.8:0.8). The kinetic experiments were conducted for the synthesis of P(OEtOxMA) with initiator (**1**) utilizing these polymerization conditions, which achieved an almost quantitative conversion after 60 minutes (98 %). The monomer conversion was calculated by a comparison between the macromonomer vinyl peaks (δ = 5.6 – 6.00 ppm) and the 10 CH₂ protons of the EtOx repeating units (δ = 2.29 ppm). A linear first-order kinetic plot ($\ln([M]_0/[M]_t)$ against time) was observed accompanied by a linear trend of M_n against conversion and narrow and mono-modal molecular weight distributions ($\mathcal{D}$ < 1.3) (Figure S4, Supporting Information).

For the polymerization of OEtOxMA with initiator (**2**) a linear evolution of molecular weight with respect to conversion was observed with narrow molecular weight distributions attained throughout ($\mathcal{D}$ < 1.25), which shows evidence of the controlled nature of the polymerization (Figure 1, right; Figure S5, Supporting Information). A plot of $\ln([M]_0/[M]_t)$ against time presented a linear increase throughout the polymerization with the polymerization rapidly achieving high conversion (68% conversion attained after 10 minutes, rising to quantitative conversion at t = 60 minutes). The results of these two polymerization kinetics demonstrate the potential of Cu(0)-mediated polymerization to prepare well-defined comb-shaped P(OEtOxMA) with quantitative conversions under benign reaction conditions. This represents a clear advantage over previous controlled polymerization techniques described, which have struggled to achieve full monomer conversions,²³ or required excessive catalyst ratios ([I]:[Cu(I)]:[Cu(II)]:[bpy]= 1:1:9:22).²⁴

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Subsequently, the polymerizations were performed again using $[M]:[I]$ ratios for initiators (1) and (2) of 10, 20 and 50 to give a range of polymers of different molecular weights using identical $[CuCl]:[PMDETA]:[NaCl]$ ratios (Table 1). In all cases quantitative conversions were attained (> 99 %), with molecular weight distributions remaining narrow ($\mathcal{D} < 1.25$) and mono-modal. The M_n values obtained from SEC analysis differed significantly from theoretical values, which is due to differences in hydrodynamic volumes of the polymer from the PMMA calibrant. This results in much smaller values being attained, particularly at increasing molecular weights (DP = 50, M_n (theoretical) = 30 kDa, M_n (SEC) = 13.6 kDa). However, on further analysis of the NMRs of the final purified polymers synthesized with (2) the exact M_n can be evaluated by a comparison of the $(CH_3O)_2CH$ peak ($\delta = 4.42$ ppm) on the initiating group, and the pendant CH_3 group ($\delta = 2.9$ ppm) of the macromonomer side chain. Resulting experimental results for M_n showed good agreement to targeted molecular weights with final DPs of 13, 21 and 45, confirming the controlled synthesis of comb-shaped P(OEtOxMA) in a molecular weight range of 8 – 30 kDa. These results again highlight the efficiency of these polymerization conditions for full and rapid conversion of different methacrylic macromonomers, resulting in polymers with well-controlled molecular weights, and specific α -end functionalities.

Polymers obtained from initiator (2) contain an α -acetal end group, which upon deprotection reveal an α -aldehyde end group that is available for post-polymerization modifications with amines via reductive amination. The acetal protecting group was removed by simple treatment with aqueous

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solutions of trifluoroacetic acid (50 % v/v). The end group was confirmed by ^1H NMR where there was a complete disappearance of the peaks representing the acetal protecting group ($\delta = 4.42$ ppm), and the appearance of characteristic aldehyde peaks ($\delta = 9.57$ ppm) (Figure 2, Figure S6-S9, Supporting Information). Following deprotection, analysis by SEC confirmed that there was no decrease in molecular weight of the polymers, verifying that there was no cleavage of the OEtOxMA side chains from the polymer backbone.

A commonly applied method for the conjugation of polymers to peptides and proteins is reductive amination between a carbonyl group (such as an α -aldehyde functional polymer) and an amine (found in peptides and proteins on lysine residues, or at the *N*-terminus). Initially in a model reaction a dipeptide, namely $\text{NH}_2\text{-Gly-Tyr-COOH}$ [Gly-Tyr] was used for the reductive amination reaction between the α -aldehyde functional P(OEtOxMA)_{50} and the *N*-terminus of the dipeptide, upon the addition of NaCNBH_3 . The conjugation was monitored by RP-HPLC (UV, $\lambda = 280$ nm) which revealed the disappearance of the Gly-Tyr signal alongside the appearance of a broad conjugate peak at a higher retention time to the native peptide (Figure S10, Supporting Information). ^1H NMR of the polymer conjugate revealed the appearance of aromatic peaks ($\delta = 6.5 - 7.0$ ppm) consistent with the tyrosine signals observed within the dipeptide, confirming successful conjugation (Figure S11, Supporting Information).

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Previously, our group have utilized reductive amination chemistry for the conjugation of linear and comb PEG polymers containing aldehyde functionality onto the therapeutic peptide oxytocin (cyclic [Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly).⁴⁰ Oxytocin is an important therapeutic recommended globally as an uterotonic for the prevention of postpartum hemorrhaging and is classified by the World Health Organization as an essential medicine. However, it is restricted in use where there is no guaranteed refrigeration supply chain as problems arise with the aqueous stability of the peptide, particularly at elevated temperatures.^{41,42} As with the dipeptide [Gly-Tyr], the only amine available for polymer conjugation on the peptide structure is the *N*-terminal amine, and therefore polymer attachment occurs site-specifically. The reductive amination of the three P(OEtOxMA) and P(OEGMA) polymers onto oxytocin, were performed at pH 6.2, with the addition of NaCNBH₃ as a reducing agent, leading to stable secondary amine linkages between the peptide and the polymer. RP-HPLC monitoring revealed a disappearance of oxytocin and the presence of a new broad conjugate peak at a higher retention time to the native peptide, and at a similar retention time to the [Gly-Tyr]-polymer conjugate (Figure 3a, Figure S12-S15, Supporting Information). All three P(OEtOxMA) polymers reacted with similar efficiencies with an almost complete disappearance of oxytocin observed on RP-HPLC, 5 days after addition of the polymers.

PEGylated conjugates (including comb-shaped P(OEGA) architectures) have previously been shown to possess better thermal stability than native equivalents under high thermal stressing.^{40,43} In order

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to evaluate the potential benefits that conjugation of comb-shaped P(OEtOxMA) might have on the stability of oxytocin, accelerated high temperature thermal studies were conducted. Aqueous solutions of the P(OEtOxMA) and P(OEGMA) conjugates alongside the native peptide were placed in an oven at 80 °C and left for 24 hours. The thermal stability was then determined by RP-HPLC by monitoring concentration changes post-storage in comparison to samples before thermal degradation (Figure 3b). The results showed that the native peptide is highly unstable under these conditions with only 15 % ($\pm$ 3.4) recovery being obtained, alongside the observation of a wide variety of degradation products (Figure S16, Supporting information). The comb-shaped polymer conjugates for both P(OEtOxMA) and P(OEGMA) (both DP 50) showed remarkably improved stability with over 95% recovery of the peptide-polymer conjugates being attained. When comparing different chain lengths of the P(OEtOxMA) combs (DP 13, 21 and 45) the stability was not affected, with all conjugate recoveries remaining close to 100% (Table S1, Supporting Information). Values in excess of 100 % are due to evaporation of water under the conditions used within the study and relate to approximately full conjugate recoveries.

These results highlight the potential for P(OEtOxMA) polymers as potential PEG alternatives for the stabilization of aqueous solutions of unstable peptides, such as oxytocin under highly temperature stressed conditions. The P(OEtOxMA) combs could be synthesized and conjugated onto a model

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peptide as efficiently as P(OEGMA), providing similar thermal stabilities, and thereby showing promise as alternatives to PEGylation.

Conclusions

In summary, using Cu(0)-mediated polymerization comb-shaped P(OEtOxMA) were successfully synthesized in aqueous solution using functional initiators, including a deprotected aldehyde initiator. Near quantitative conversions could be achieved in 60 minutes at ambient temperature whilst maintaining linear polymerization kinetics and narrow molecular weight distributions (< 1.3). Post-polymerization the polymer α -end group was successfully modified into a reactive carbonyl group and used for the site specific *N*-terminal conjugation onto two model peptides, namely [Gly-Tyr] and oxytocin via reductive amination. The aqueous heat stability of P(OEtOxMA)-peptide conjugates was improved post-conjugation analogous to similar P(OEGMA) combs. This is the first reported example of the synthesis of these polymers utilizing Cu(0)-mediated polymerizations, which are post-polymerization available for subsequent peptide conjugation procedures leading to novel peptide-polymer bioconjugates. The synthesis of other reversible conjugates and their testing as alternative uterotonic therapeutics is the focus of our ongoing work.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 1. Left: Overview of the Cu(0)-mediated polymerization of OEtOxMA and OEGMA with initiators (1) and (2) in water:MeOH (1.5:1) ([I]:[M]:[PMDETA]:[CuCl] = 1:DP:0.8:0.8, 1.6 M of NaCl); Right: Polymerization kinetics for DP = 50 P(OEtOxMA) using initiator (2).

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Figure 2. Deprotection of acetal protected P(OEtOxMA) yielding α-aldehyde end groups: Top: Reaction scheme; Bottom: ¹H NMR (DP 10, DMSO-*d*₆) and SEC (DP 10, 20 and 50, CHCl₃ eluent).

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Figure 3. Conjugation by reductive amination reaction scheme, HPLC chromatograms (a) of native oxytocin and P(OEtOxMA)₂₀-oxytocin conjugate and before and after thermal degradation and thermal stability results for all polymer conjugates (b, 24 h, 80 °C).

Table 1. Summary of characterization data for Cu(0)-mediated synthesis of different functional P(OEtOxMA).

Initiator	[M]:[I] ^{a)}	Conv. ^{b)} [%]	M _{n,NMR} ^{c)} [g mol ⁻¹]	M _{n,SEC} ^{d)} [g mol ⁻¹]	Đ ^{d)}	M _{n,SEC} ^{d)} Deprotected	Đ ^{d)} Deprotected
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[g mol ⁻¹]							
1	10	> 99	-	7500	1.08	-	-
1	20	>99	-	10900	1.12	-	-
1	50	> 99	-	13600	1.21	-	-
2	10	> 99	7800	6000	1.23	6500	1.14
2	20	> 99	12400	8000	1.18	7900	1.15
2	50	> 99	27200	12800	1.23	12500	1.24

^{a)}Polymerization conditions = [I]:[M]:[PMDTA]:[CuCl] = 1:DP:0.8:0.8, 1.6 M of NaCl; ^{b)}Obtained at t = 24 h from ¹H NMR analysis (CDCl₃, 400.13 MHz); ^{c)}Calculated from NMR analysis (DMSO-*d*₆, 400.13 MHz); ^{d)}Extracted from SEC analysis (CHCl₃ eluent)

Table of Contents

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Comb poly(oligo(2-ethyl-2-oxazoline)methacrylate)-peptide conjugates prepared by aqueous Cu(0)-mediated polymerization and reductive amination

Jennifer Collins, Sacha J. Wallis, Alexandre Simula, Michael R. Whittaker, Michelle P. McIntosh, Paul Wilson, Thomas P. Davis, David M. Haddleton*, Kristian Kempe*

The controlled Cu(0)-mediated polymerization of OEtOxMA is reported in water/alcohol mixtures. The facile incorporation of α -end functionality capable of undergoing post-polymerization peptide conjugation led to well-defined peptide-polymer conjugates.

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Author/s:

Collins, J;Wallis, SJ;Simula, A;Whittaker, MR;McIntosh, MP;Wilson, P;Davis, TP;Haddleton, DM;Kempe, K

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