Polymer–protein conjugates from ω-aldehyde endfunctional poly(*N*-vinylpyrrolidone) synthesised *via* xanthate-mediated living radical polymerisation†

Gwenaelle Pound,*[a] Jean M. McKenzie,*[b] Ronald F. M. Lange*[c] and Bert Klumperman*[a]

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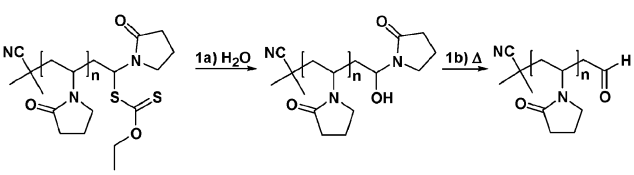
Aldehyde ω-endfunctional poly(*N*-vinylpyrrolidone) was synthesised *via* quantitative conversion of a xanthate endfunctional precursor obtained *via* RAFT-mediated polymerisation.

Poly(*N*-vinylpyrrolidone) (PVP) has a long history as a synthetic polymer for biomedical applications. After its use as a plasma expander during the Second World War,[1] PVP was the first polymer reported for the preparation of injectable polymer–drug conjugates.[2] PVP has also been identified as an alternative to poly(ethylene glycol) (PEG) for peptide/protein modification. Particularly promising is bioconjugation with PVP for cancer therapy due to the enhanced plasma half-life and reduced tissue distribution of PVP compared to other synthetic polymers.[3] Until recently, limitations in the use of PVP for bioconjugation resided in its polymerisation characteristics. Polymerisation of *N*-vinylpyrrolidone (NVP) has only been reported *via* a free-radical process. Conventional free-radical polymerisation produces polymers with broad molecular weight distribution and typically provides poor control over the end-groups, whereas bioconjugation requires that the polymer be endfunctional. Additionally, control over the molecular weight is required to enable tuning of the half-life of the polymer-drug conjugates in the body. Free-radical polymerisation of NVP in the presence of hydroxyl-, carboxylic acid- or carboxylic ester-functional irreversible chain-transfer agents was the method of choice up to this point for the preparation of chain-end functional NVP oligomers.[4] Nonetheless, the range of end-groups accessible *via* this method is limited and the polymerisation does not yield low polydispersity index PVP. The recent development of living radical polymerisation techniques has enabled the preparation of PVP with controlled molecular architectures including control over the end-groups and the molecular weight distribution.[5] It has recently been reported that reversible addition-fragmentation chain transfer (RAFT)-mediated polymerisation of NVP can be applied to prepare PVP with thiol end-groups, which yield reducible protein conjugates *via* disulfide bond formation with cysteine residues.[6] We show that well-defined aldehyde end-groups can be obtained *via* facile post-polymerisation modification of the same xanthate end-functional PVP precursor and used to prepare non-reducible protein-conjugates.

Xanthate endfunctional PVP was prepared *via* (*S*)-(2-cyano-2-propyl) *O*-ethyl xanthate-mediated polymerisation. The ω-xanthate end-group was easily modified using the procedure presented in Scheme 1. ω-Aldehyde endfunctional PVP was obtained *via* hydrolysis. A solution of the polymer in distilled water (100 mg mL^{-1}) was stirred at 40 °C for 16 h. The solution was dialyzed at room temperature and the polymer recovered *via* freeze-drying. The resulting ω-hydroxyl endfunctional PVP was heated at 120 °C at 1 mbar for 20 h to yield ω-aldehyde endfunctional PVP with more than 90% conversion (by ^{1}H NMR spectroscopy). The ^{1}H NMR spectra in Fig. 1 clearly indicate the disappearance of the xanthate end-groups, formation of hydroxyl and finally aldehyde end-groups. The structures of the end-groups were further confirmed *via* ^{13}C and ^{1}H/^{13}C-heteronuclear single quantum coherence NMR spectroscopy (see ESI†) and matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-ToF-MS). The main distribution in the MALDI-ToF-MS spectrum in Fig. 2 matched the calculated isotopic pattern for PVP with α-2-cyano-2-propyl and ω-aldehyde endgroups ($I + K^+$ in Fig. 2). Low intensity peaks were attributed to ω-unsaturated ($II + K^+$) and unmodified ω-xanthate endgroups ($III + K^+$). Unsaturated chain-ends may form *via* thermal elimination of the xanthate[7] or may be the result of fragmentation of the xanthate during MALDI-ToF-MS analysis.

An important parameter is the pH at which hydrolysis is carried out. Quantitative conversion to aldehyde end-groups was obtained when the pH ranged from 4–5 (pH of the solution when water is added to the polymer) to 10 (adjusted



Scheme 1 Modification of PVP xanthate chain-ends into hydroxyl and aldehyde end-groups. *Reagents and conditions*: (1a) distilled water (pH = 4–10), 40 °C, 16 h; (1b) 120 °C, 1 mbar, 20 h. In this study, $15 < n < 150$.

[a] *Department of Chemistry and Polymer Science, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa. E-mail: gwen@sun.ac.za; bklump@sun.ac.za; Fax: +27(0)21 808 4967; Tel: +27(0)21 808 3986*
[b] *Central Analytical Facility, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa. E-mail: jmck@sun.ac.za; Tel: +27(0)21 808 3338*
[c] *BASF AG. Polymer Laboratories, GKT – B1, 67056 Ludwigshafen, Germany. E-mail: ronald.lange@basf.com*

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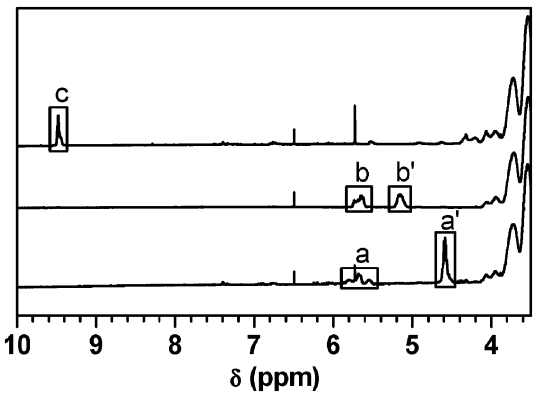


Fig. 1 ^{1}H NMR spectra of PVP. The three spectra correspond to PVP in DMSO-d_6 precipitated three times from diethyl ether (bottom), heated in water at 40 °C for 16 h, dialyzed and freeze-dried (middle) and after an extra 20 h at 120 °C in the dry state at 1 mbar (top). Peak assignment: (a) C*H*(N)SCS, (a′) SC(S)OC*H*$_2$, (b) CH(N)O*H*, (b′) C*H*(N)OH, (c) CH$_2$C*H*O.

with KOH). Above 10, a significant fraction of thiol/disulfide chain-end functionalities were observed (see ESI†). To the best of our knowledge, the thiocarbonyl thio end-group of a polymer chain has never been directly replaced by a hydroxyl functionality. Aldehyde end-groups were reported before as side-products in PVP prepared *via* conventional free-radical polymerisation with hydrogen peroxide.[8] However, the quantitative conversion of the thiocarbonyl thio end-group into an aldehyde is unprecedented.

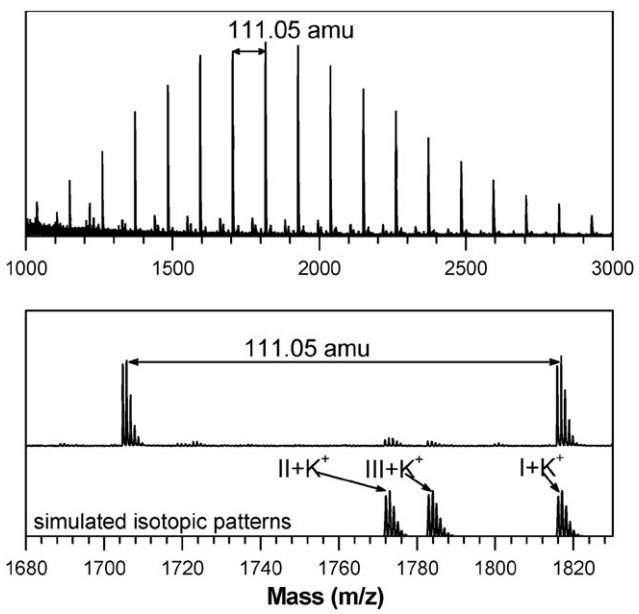


Fig. 2 MALDI-ToF-MS spectra of hydrolyzed PVP. Top: poly(*N*-vinylpyrrolidone) ($M_{n,SEC}$ = 2640 g mol^{-1} (PMMA equivalents in $(CF_3)_2CHOH$) PDI = 1.21) heated at 40 °C in distilled water for 16 h and dialyzed at room temperature, freeze-dried and heated at 120 °C for 20 h; Bottom: enlargement of the experimental spectrum in the region 1680–1830 a.m.u. and calculated isotopic patterns for $C_4H_6N(C_6H_9NO)_{15}CH_2CHO + K^+$ (I + K$^+$), $C_4H_6N(C_6H_9NO)_{14}$-$C_6H_8NO + K^+$ (II + K$^+$) and $C_4H_6N(C_6H_9NO)_{14}C_3H_5OS_2 + K^+$ (III + K$^+$).

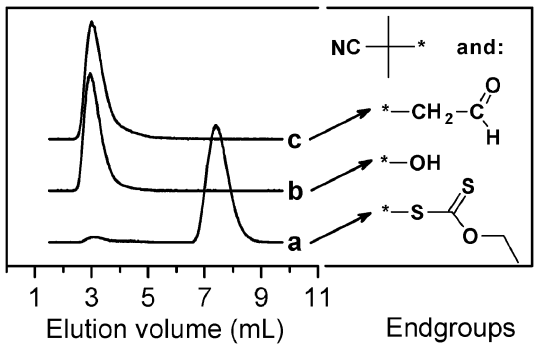
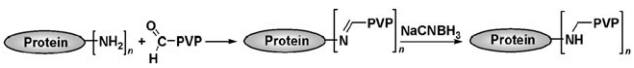


Fig. 3 GPEC chromatograms of PVP with various end-groups. The end-group structures correspond to a minimum of 90% of the chain-ends as determined *via* ^{1}H NMR spectroscopy.

Low molecular weight PVPs (typically M_n = 1000–5000 g mol^{-1}) were prepared in order to enable identification and quantification of the end-groups *via* NMR spectroscopy and MALDI-ToF-MS and separation *via* gradient polymer elution chromatography (GPEC). Separation of the polymer according to the end-groups is presented in Fig. 3. The GPEC chromatograms indicate quantitative conversion of the xanthate end-groups upon treatment of xanthate endfunctional PVP. The molecular weight distribution did not vary significantly upon end-group modification, except where hydrolysis was performed at pH > 11 (see SEC data in ESI†), confirming that the post-polymerisation treatments do not affect the polymer backbone.

Aldehyde endfunctional polymers are of particular interest for the preparation of polymer–protein conjugates due to their ability to react with the primary amine of lysine residues in a protein.[9,10] The reactivity of aldehyde chain-end functional PVP towards bioconjugation was demonstrated by coupling the polymer to lysozyme. The reaction was carried out according to the procedure given in Scheme 2. SDS-PAGE analysis revealed the formation of PVP-lysozyme conjugates with aldehyde chain-end functional PVPs (Fig. 4). As expected, the lower the average degree of polymerisation (DP_n) of the polymer, the further the migration of the conjugate, *i.e.* the smaller the size of the conjugate. A series of blank experiments were carried out where PVPs with xanthate, thiol or hydroxyl end-groups were incubated with lysozyme under the same conditions as the polymer with aldehyde end-groups. Polymers which did not bear aldehyde end-functionality did not produce stable conjugates, as indicated by the presence of free lysozyme.

Bioconjugation of polymers with proteins is a field of research that is receiving considerable attention in recent



Scheme 2 Conjugation of aldehyde endfunctional PVP to a protein. Reaction conditions for coupling to lysozyme: Lysozyme hydrochloride (12.1 mg, 5.7 × 10^{-6} mol of amine) was dissolved in phosphate buffer at pH = 5.4 (5.0 g) and reacted with aldehyde endfunctional PVP (160 mg, 2.1 × 10^{-5} mol of aldehyde, $M_{n,SEC}$ = 7600 g mol^{-1} (PMMA equivalents in $(CF_3)_2CHOH$), PDI = 1.31) at room temperature. After 1 h, $NaCNBH_3$ (0.3 g of 0.25 M solution in distilled water) was added to the solution. After 140 h, the solutions were freeze-dried and the samples analysed with SDS-PAGE.

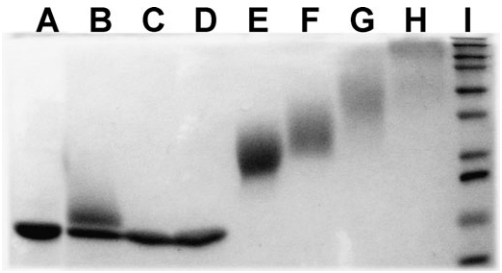


Fig. 4 SDS-PAGE for the conjugation of lysozyme with PVP. Lane A: free lysozyme; B, C and D: lysozyme reacted with PVP with hydroxyl, thiol and xanthate end-groups, respectively; E to H: lysozyme–PVP conjugates with increasing PVP chain-length ($M_{n,SEC}$/g mol^{-1} (PDI) = 2640 (1.21); 3300 (1.21) 7600 (1.31) and 17000 (1.24), respectively).

years. The combination of living radical polymerisation with bioconjugation has been investigated in various ways.[9,11] In the present approach, the R-group of the RAFT agent is not used for conjugation, which means that introduction of additional functionality to the polymer–protein conjugate can still be achieved through the R-group. Conjugation takes place *via* an amine–aldehyde reaction, which is more versatile than the conjugation *via* a cysteine residue as typically performed in the Z-group attachment, and leads to hydrolytically stable bioconjugates.

In conclusion, xanthate-mediated polymerisation is a versatile and facile method for the preparation of PVP with well-defined molecular weight distribution and end-groups, including chain-end functionalities suitable for conjugation to proteins. ω-Aldehyde endfunctional PVP was prepared, analysed and successfully coupled to the model protein lysozyme *via* reductive amination. This methodology opens new perspectives for the use of PVP as a polymeric drug carrier and many more applications.

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