

## $\alpha$ -Aldehyde Terminally Functional Methacrylic Polymers from Living Radical Polymerization: Application in Protein Conjugation "Pegylation"

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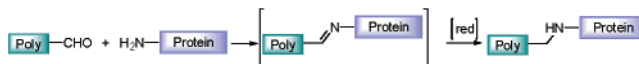
Application of proteins and peptides as human therapeutics is expanding rapidly as drug discovery molecules emerge from -omics research programs. Accompanying the discovery of novel peptides and proteins a better understanding of the mechanism of action in vivo is emerging.<sup>1,2</sup>

Unfortunately, many peptide-based molecules have properties that are not always conducive to oral delivery and therefore must be injected at great cost and inconvenience to patients. The oral route of administration of these substances remains unavailable because they are generally destroyed by the digestive system, while injected proteins can be removed from the body by proteolytic digestion or rapid renal excretion.<sup>2</sup> Many biological properties of polypeptide-based drugs, such as plasma half-lives, stability, and therapeutic potency can be substantially improved by reaction of the latter with appropriate chain-end functionalized poly(ethylene glycol) (PEG), "pegylation".<sup>1-5</sup> In some cases, the PEG chains can also be predictably detached in vivo from the conjugate, introducing the benefits of controlled release of native protein therapeutics.<sup>6</sup>  $\alpha$ -Aldehyde terminally functional poly(ethylene glycol)s have been termed "second generation" pegylation agents. This class of derivatives has been developed in order to circumvent problems often related to pegylation chemistry, such as low purity of the reagents, side reactions, and poor selectivity in substitution, which can lead to single site attachment at the terminal amine under appropriate reaction conditions.<sup>1</sup>

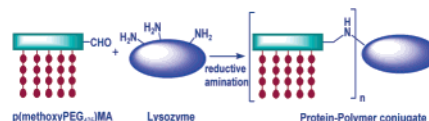
Conjugation reactions occur via a reductive amination pathway, in which an imino linkage is formed prior to reduction in situ to a stable secondary amine with basicity comparable to that of the parent primary amine (Scheme 1).<sup>7</sup> This is a very important parameter, especially when the amino positive charge is critical for the retention of biological activity.<sup>4,8</sup>

Although the chemistry of peptide-polymer conjugation has been widely developed, few examples of the use of polymers other than PEG have been reported thus far.<sup>9,10</sup> In this present work we have explored the use of new  $\alpha$ -aldehyde-functional poly(methoxyPEG)methacrylates obtained via Cu(I) mediated living radical polymerization and application of these materials in conjugation reactions with a model protein, lysozyme (Scheme 2).<sup>11</sup> It is noted that graft polymers of poly(methoxyPEG)methacrylates have been previously investigated as components of oral drug delivery copolymers.<sup>12</sup> Living radical polymerization (LRP) is a route to polymer architectures unavailable by other synthetic techniques, allowing many functional monomers to be incorporated into macromolecules with designed architecture and function.<sup>13</sup> The possibility to obtain polymers with a narrow molecular weight distribution is also a crucial parameter as polydisperse polymers reflect in polydispersity of the target polypeptide-polymer conjugate.<sup>4</sup> An acetal initiator has been previously used for living anionic ring-opening polymerization of ethylene oxide to give aldehyde functional polymers;<sup>14</sup> it is noted that living radical polymerization is extremely diverse in the number of potential

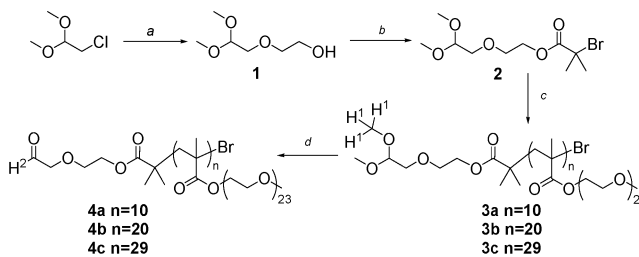
**Scheme 1.** Conjugation of  $\alpha$ -Aldehyde-Functional Polymer with Proteins



**Scheme 2.** Multisite Conjugation of Polymer and Lysozyme



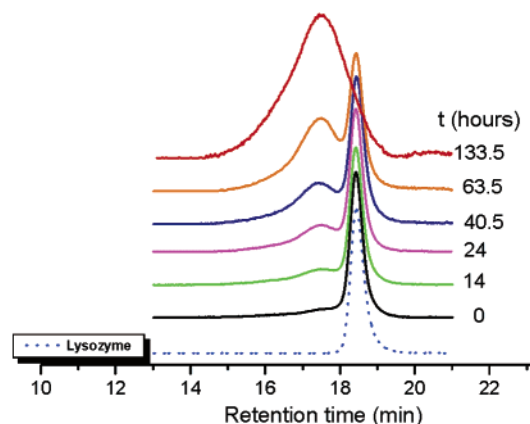
**Scheme 3**<sup>a</sup>



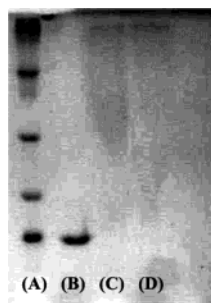
<sup>a</sup> Reagents and conditions: (a) ethylene glycol, KOH, 115 °C; (b) 2-bromoisobutyryl bromide, Et<sub>3</sub>N, 0 °C; (c) Cu<sup>I</sup>X (X = Cl, Br)/N-(ethyl)-2-pyridylmethanimine, methoxyPEG(1100)methacrylate, toluene; T = 80 °C (X = Cl) or T = 50 °C (X = Br); (d) CF<sub>3</sub>COOH/H<sub>2</sub>O 1:1, room temperature.

monomers in addition to being robust toward protic groups in the system.<sup>13</sup> Our synthetic strategy is summarized in Scheme 3. In the first step an  $\alpha$ -functional polymer was obtained with LRP using methoxyPEG(1100)methacrylate monomer and initiator **2**. Subsequently the resulting polymer was conjugated with lysozyme.

Initiator **2** was obtained by treatment of the commercially available 2-chloro-1,1-dimethoxyethane with ethylene glycol under basic conditions, followed by acylation with 2-bromoisobutyryl bromide. Polymerization of (PEG<sub>1100</sub>-methyl ether)methacrylate in the presence of Cu<sup>I</sup>Cl and an iminopyridine ligand<sup>15</sup> furnished the intermediate **3a**. The product was isolated from the relatively high mass monomer (1100 g mol<sup>-1</sup>) by ultrafiltration and subsequent freeze-drying. The polymer was subsequently deprotected with TFA/water to give the  $\alpha$ -aldehyde-terminally functional polymer **4a**. The reaction was monitored by <sup>1</sup>H NMR with H<sup>1</sup> and H<sup>2</sup> visible at 3.38 and 9.72 ppm respectively (Scheme 3). The molecular weight distribution of the polymer before ( $M_n = 11\,000$ , PDI = 1.14) and following end group hydrolysis ( $M_n = 11\,000$ , PDI = 1.16) confirmed that no backbone hydrolysis occurred during this deprotection. Two further  $\alpha$ -aldehyde-terminally functional polymers were synthesized with  $M_n = 22\,000$  (PDI = 1.09), **3b/4b** and 32 000 (PDI = 1.11) g mol<sup>-1</sup> **3c/4c** respectively. In these reactions Cu<sup>I</sup>Br was used in place of Cu<sup>I</sup>Cl resulting in a faster polymerization



**Figure 1.** SEC-HPLC analysis of the reaction mixture obtained in the conjugation reaction of polymer **4a** with lysozyme.



**Figure 2.** SDS-PAGE for the conjugation of lysozyme with **4a**: (A) protein standards; (B) lysozyme; (C) protein-polymer bioconjugate (reaction at pH 5); (D) protein-polymer bioconjugate (reaction at pH 6).

with the reaction temperature set higher accordingly (see Supporting Information). In all of the polymerization reactions, the kinetics showed a linear dependence with respect to the monomer concentration and the polymers all had a narrow molecular weight distribution (PDI ranging from 1.09 to 1.15), consistent with living polymerization. Thus, this approach enables the synthesis of  $\alpha$ -aldehyde terminally functional polymers with predictable  $M_n$  and narrow PDI.

The polymers were screened for their reactivity toward protein conjugation with lysozyme as a model substrate. Conjugation reactions were carried out at pH 5 and 6 using an excess of polymer with  $\text{NaCNBH}_3$  as an in situ reducing agent. The reactions were followed by size-exclusion (SEC)-HPLC and showed a similar trend for all of the three polymers, with the signal from free lysozyme decreasing with time and a new peak corresponding to the conjugates increasing (Figure 1). It is noted that the free polymer gives only a weak signal at  $\lambda = 225$  nm.

Conjugation occurred faster at pH 5 than at pH 6 ascribed to the fact that at lower pH the formation of the imino linkage between the polymer and the protein may be favored. SDS-PAGE analysis of the conjugates confirmed the disappearance of the lysozyme starting material and the appearance of high molecular weight conjugates<sup>16</sup> (Figure 2). Lysozyme has seven free amino groups from lysine residues and the terminal group with the electrophoresis indicating multi site attachment.

This makes it an ideal candidate as a model protein, however, it is noted that pegylation with even a single 12 kDa linear polymer has been previously shown to eliminate lysozyme activity.<sup>6b</sup>

In summary, we have described the synthesis of new  $\alpha$ -aldehyde-terminated poly(methoxyPEG)methacrylates obtained via Cu(I) mediated living radical polymerization and their application for the conjugation of proteins using lysozyme as a model. Living radical polymerization with a functional initiator leads to pure monofunctionalized products with absence of difunctional impurities which can lead to unwanted proteins cross-linking. This method appears to be very general and may be extended to many proteins and most of the monomers amenable to metal-catalyzed living radical polymerization. Future work will focus on investigation of proteins where biological activity is expected to be retained following conjugation.

**Acknowledgment.** We would like to thank the POLYCAT EC RTN network (HPRN-CT-2000-00010) and the University of Warwick (L.T. and F.L.) for funding this work and Drs. A. Jarvis, A. J. Carmichael, and A. G. Steward of Warwick Effect Polymers for helpful discussions.

**Supporting Information Available:** Text and schemes giving synthesis and characterization of initiators, polymerization procedure, and conjugation conditions, tables summarizing the synthesis data, and figures showing kinetic plots, dependence of  $M_n$  on conversion, <sup>1</sup>H NMR spectra, comparison of GPC traces, and SEC-HPLC chromatography. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA0456454