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A new approach to bioconjugates for proteins and peptides ("pegylation") utilising living radical polymerisation†

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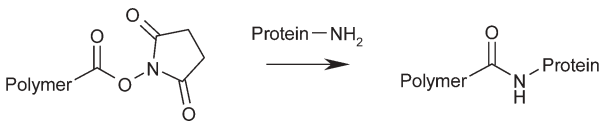
The synthesis of protein–polymer bioconjugates is reported using *N*-succinimidyl ester functionalised polymers from transition metal mediated living radical polymerisation.

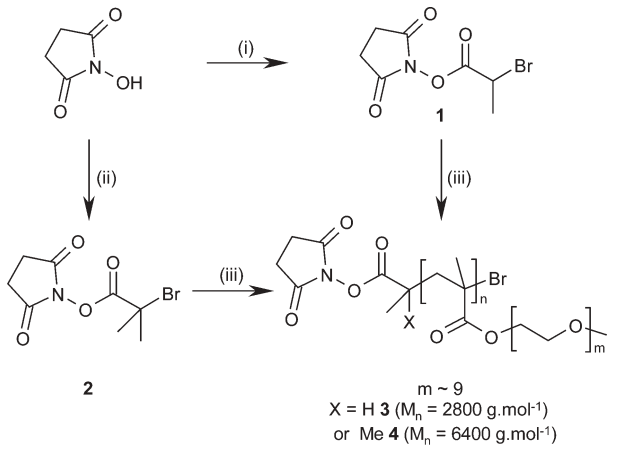
Protein bioconjugation has been an area of increasing interest since the pioneering work by Abuchowski *et al.*[1] Bioconjugation is the covalent binding of a protein to a synthetic or natural polymer chain to form a new macromolecule. Bioconjugation offers the opportunity to substantially alter the pharmacokinetics and pharmacodynamic properties of protein therapeutics. Poly(ethylene glycol methyl ether) (mPEG), a non-toxic polymer, has been extensively used for conjugation, "pegylation".[2] In most cases, suitable functionalisation of the polymer is the first step in the conjugation. This process is the "activation" step and the conjugation group is chosen to match the available groups on proteins.[3] A common route for protein conjugation is the reaction of an appropriate terminally-functionalised polymer with the ε-amino group of lysine residues and the *N*-terminal amino group present in the polypeptides. A well developed method of activation is the transformation of the α-terminus of polymers with succinimidyl esters, which leads to the formation of stable amido linkages with proteins (Scheme 1).[4] It is noted that while the chemistry for the conjugation of polymers to proteins has been widely developed, hitherto few polymer types have been used with mPEG remaining popular due to its non-hazardous and non-immunogenic properties, as well as its ability to solubilise biological molecules. Nevertheless a few examples of conjugation have been reported using polystyrene as the synthetic polymer to form amphiphilic block copolymers, or super amphiphiles.[5] In this report, we described the synthesis of *N*-succinimidyl ester functionalised initiators and their subsequent use in the transition metal mediated living radical polymerization (TMM-LRP) of poly(ethylene glycol) methyl ether methacrylate (mPEGMA). Living radical polymerisation provides an excellent method for the polymerisation of a diverse range of vinyl monomers (acrylates, methacrylates and styrenes) in a controlled manner,[6] with excellent tolerance towards a variety of functional groups. Moreover this is a facile way to incorporate an initiator site into a molecule leading to a wide range of functionalised polymers.[7]

The *N*-succinimidyl functional initiators were prepared as shown in Scheme 2. *N*-Succinimidyl 2-bromo-2-methylpropionate **2** was obtained in very high yield by acylation of *N*-hydroxysuccimide with 2-bromopropionyl bromide, while an analogous synthetic protocol applied to the synthesis of **1** yielded a quite complicated mixture of products. Initiator **1** was subsequently prepared in very good yield by coupling of 2-bromo propionate and *N*-hydroxysuccimide in the presence of DCC. Living radical polymerisation of mPEGMA was carried out at 30 °C using Cu(I)Br/*N*-(ethyl)-2-pyridylmethanimine as catalyst[8] to give mono-functionalised polymers with structure **3** and **4**. Catalyst residues were removed by ultrafiltration to yield white products.† The presence of the *N*-succinimidyl ester moiety at the chain ends was confirmed by ^{1}H NMR analysis as a singlet at 2.8 ppm. The synthesis of pure mono-functionalised polymers with zero contamination from α, ω disubstituted polymer is a significant asset of this synthetic route. A disadvantage of conventional linear PEGylating reagents is the presence of di-functionalised byproducts inherent to the ring-opening polymerisation. These can result in unwanted crosslinking between proteins during the conjugation. Gel permeation chromatography (GPC) showed that the molecular weight distribution was very narrow ($M_w/M_n < 1.15$) for all of the materials obtained. This is essential as a polydisperse polymer reflects on the polydispersity of the protein–polymer conjugates.[9]

The reactivity of *N*-succinimidyl ester function in polymers **3** and **4** was evaluated by monitoring the rate of hydrolysis by online ^{1}H NMR experiments, whereby the reaction was carried out in an NMR tube within the cavity of the spectrometer (Fig. 1) in various deuterium oxide buffers. It was found that the hydrolytic stability of the activated polymers was strongly dependent on the pH of the medium. At mild acidic pH (pH = 6), the cleavage of the succinimde group was almost negligible, whilst, as expected, under basic conditions the hydrolysis of the functionalised polymers was very fast (at pH = 9, $t_{1/2}$(**4**) = 0.2 h).[10] The hydrolytic stability experiments also demonstrated that polymers initiated by **1** were much more reactive towards water, and hence towards nucleophiles such as amines, than polymers initiated by **2**. This agrees with the observations of Harris and coworkers for α-branched *N*-succinimidyl esters-mPEG.[3,4] Although this difference in reactivity is often ascribed to a different steric hindrance on the



Scheme 1 Coupling of proteins to *N*-succinimidyl ester functionalised polymers.



Scheme 2 Reagents and conditions: (i) 2-Bromopropionic acid, dicyclohexylcarbodiimide, CH_2Cl_2; (ii) 2-bromo-2-methylpropionyl bromide, triethylamine, CH_2Cl_2; (iii) Cu(I)Br, *N*-(ethyl)-2-pyridylmethanimine, poly(ethylene glycol) methyl ether methacrylate, toluene 50% v/v; [PEGMA]:[initiator]:[Cu(I)Br]:[ligand] = 5:1:1:2.

† Electronic supplementary information (ESI) available: Experimental procedures on prepared compounds and characterisation. See http://www.rsc.org/suppdata/cc/b4/b407712a/

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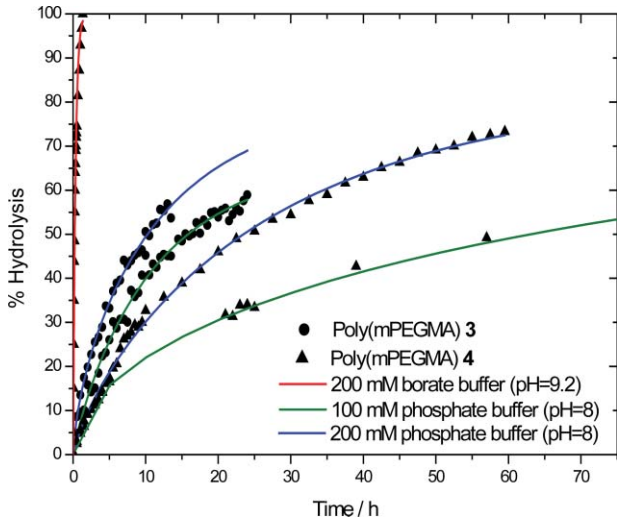

Fig. 1 Kinetic plots for the hydrolysis of various Poly(mPEGMA)-NHS at different pH and buffer ionic strength.

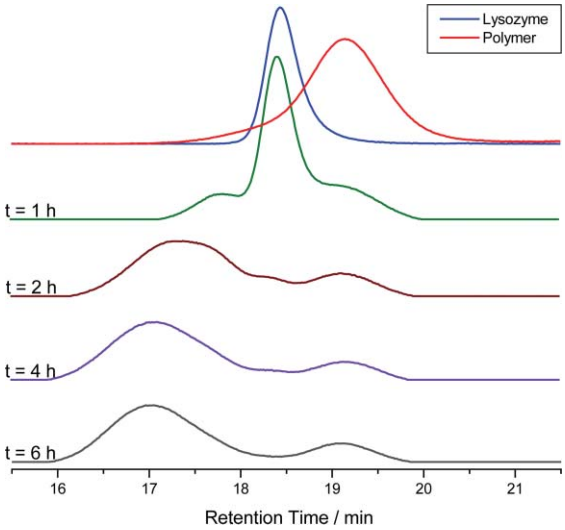

Fig. 2 SEC HPLC traces following the reaction of *N*-succinimidyl esters functionalised Poly(mPEGMA) (M_n = 6400 g mol^{-1}, M_w/M_n = 1.11) with lysozyme.

electrophilic centre, electronic contributions provided by the α-substituents cannot be ruled out.

Lysozyme was used as a model protein for conjugation experiments to both polymers[11] in anhydrous DMSO in the presence of triethylamine. Size-exclusion HPLC was used to monitor the evolution of the conjugate (Fig. 2). In the case of polymer **4**, no reaction was observed after 24 h. Further analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) confirmed the absence of any conjugates. For polymer **3**, the reaction was fast and the HPLC trace showed the complete disappearance of the lysozyme after 6 h. SDS-PAGE analysis confirmed the absence of the lysozyme starting material and revealed that an approximate number of 6 to 7 polymeric chains were conjugated (Fig. 3).[12] It is noted that lysozyme has 6 lysine groups in addition to the terminal amine.

In summary, transition metal mediated living radical polymerisation has been successfully employed for the synthesis of two different types of *N*-succinimidyl ester functionalised poly(mPEGMA) with different reactivities depending on the type of initiator used. This versatile synthetic approach provides a novel route to protein–synthetic polymer bioconjugates using living radical polymerization. This strategy has been applied using lysozyme as a model protein and poly(mPEGMA) as the synthetic polymer, it looks to be very general and will be extended to other

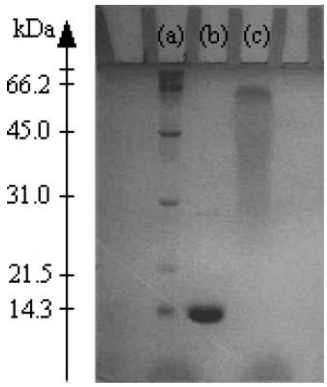

Fig. 3 SDS-PAGE for the conjugation of lysozyme with **3**. (a) Protein standards. (b) Lysozyme. (c) Protein–polymer bioconjugate.

polypeptides and other hydrophilic monomers polymerisable under TMM-LRP conditions (acrylates, methacrylates and styrenes).

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