Bioconjugation onto biological surfaces with fluorescently labeled polymers†

Julien Nicolas,‡[a] Ezat Khoshdel[b] and David M. Haddleton*[a]

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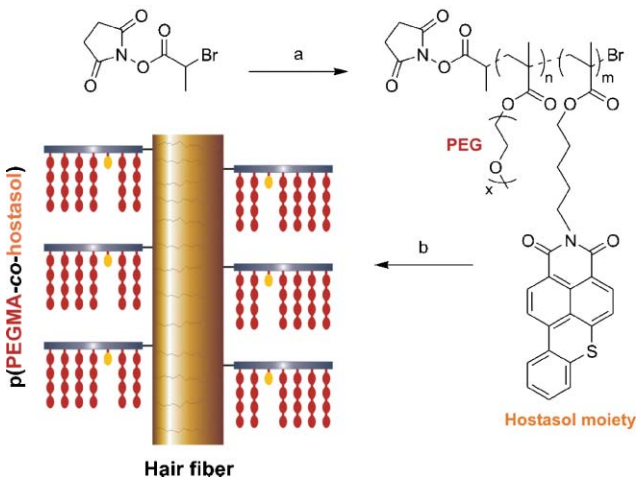
Direct bioconjugation onto hair fibers, monitored by confocal laser scanning microscopy and differential scanning calorimetry, has been performed using NHS α-functional fluorescently tagged polymers synthesised by living radical polymerisation.

Polymer–protein conjugation is an exciting research field that has emerged over the last twenty years in both industry and academia. The attachment of water-soluble synthetic polymers, *e.g.* α-functional poly(ethylene glycol) (PEG), to appropriate proteins and peptides therapeutics,[1] *PEGylation*, has a range of benefits including improved biodistribution and pharmacokinetics, reduced immunogenicity and longer plasma half-lives.[2] Transition-metal-mediated living radical polymerisation (TMM-LRP), often called atom transfer radical polymerisation (ATRP),[3] has been employed to give well-defined α-functionalised poly(methoxy PEG methacrylate) polymers which have been conjugated with amines (both lysine and N-terminal α-amino acid residues) and thiols (cysteine residues) present at the surface of proteins and peptides.[4] We have also recently reported a very simple strategy for the synthesis of fluorescently tagged protein–polymer bioconjugates from protein derived macroinitiators, using either bovine serum albumin (BSA) or lysozyme.[5]

In this present study, we have adapted the "grafting to" strategy (*i.e.* the attachment of a preformed polymer) to a biological protein surface as opposed to a soluble protein in solution. For this purpose, we chose hair as a suitable candidate for several reasons: (i) hair is composed mainly of keratin fibers with free thiol and amine functions at the surface suitable for bioconjugation; (ii) it can be easily handled without any specific requirements and (iii) a broad range of microscopy and surface analysis techniques (optical, scanning electron and confocal microscopy as well as atomic force microscopy) may be used. Our strategy was to synthesize a fluorescently tagged poly(methoxy PEG methacrylate) polymer by ATRP with a terminal α-*N*-hydroxysuccinimide group for subsequent bioconjugation with hair *via* a reaction with the surface amines. Thiol functions were not targeted in this preliminary study (using for example α-maleimide functionalised polymers)[4c] as they are involved in disulfide bridges and thus, in the absence of a suitable reducing agent, poor bioconjugation efficiency would be expected. The hostasol methacrylate (HMA) monomer was selected as the fluorescent tag and was incorporated *in situ* during the synthesis of the polymer by copolymerisation with poly(ethylene glycol) methyl ether methacrylate ($PEGMA_{475}$) initiated by *N*-hydroxysuccinimide-2-bromopropionate, giving a NHS-poly($PEGMA_{475}$-*co*-HMA) copolymer. *N*-Hydroxysuccinimide-2-bromopropionate was selected in favor of *N*-hydroxysuccinimide-2-bromo-2-methylpropionate as even though the initiator efficiency is low during the polymerisation reaction, it has been previously seen that the reactivity towards nucleophiles such as amines, is higher. The overall synthetic approach is represented on Scheme 1.

N-Hydroxysuccinimide-2-bromopropionate was used as initiator for the living copolymerisation of $PEGMA_{475}$ and HMA under mild conditions at 50 °C in toluene under an inert atmosphere (P1). After 48 h high conversion was obtained following linear first-order kinetics (R^2 = 0.9959) indicating a constant concentration of propagating chains throughout the polymerisation (Fig. 1). As expected, the M_n increased linearly with monomer conversion maintaining low polydispersity indexes throughout the reaction. The initiator efficiency was low ($f \sim 35\%$) in agreement with previous work.§ The SEC traces exhibited a shift toward higher molar masses with an increase in conversion.† ^{1}H NMR of the purified copolymer P1 (89 % conv., $M_{n,SEC}$ =



Scheme 1 Synthesis of NHS-poly($PEGMA_{475}$-*co*-HMA) copolymers and subsequent bioconjugation with hair. Reagents and conditions: (a) copper(I) bromide, *N*-(ethyl)-2-pyridylmethanimine, $PEGMA_{475}$, hostasol methacrylate monomer (HMA), toluene, 50 °C, N_2, 48 h (b) Untreated hairs, DMSO–TEA (95 : 5; w/w), ambient temperature, 70 h.

[a]*Department of Chemistry, University of Warwick, Coventry, UK CV4 7AL. E-mail: D.M.Haddleton@warwick.ac.uk; Fax: +44 24 7652 4112; Tel: +44 24 7652 3256*
[b]*Unilever Research, Port Sunlight, Bebington, UK CH63 3JW*
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‡ Permanent address: Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Faculté de Pharmacie, Université Paris-Sud XI, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France.

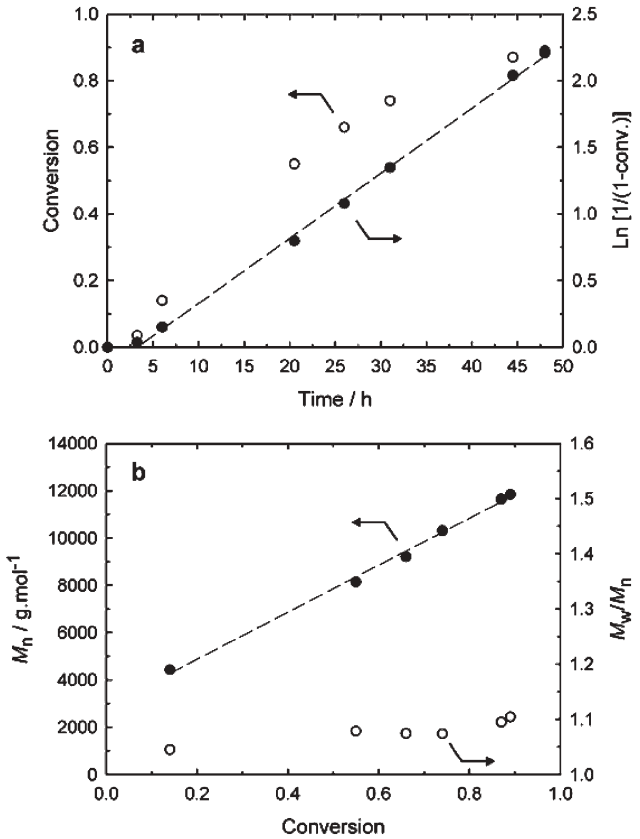

Fig. 1 Living radical copolymerisation of $PEGMA_{475}$ and HMA initiated by *N*-hydroxysuccinimide-2-bromopropionate (P1). (a) Global monomer conversion (○) and ln[1/(1 − conv.)] (●) *vs.* time; (b) M_n, (●) and M_w/M_n, (○) *vs.* global monomer conversion.

11 780 g mol^{-1}, M_w/M_n = 1.12) showed two important features: (i) the presence of the *N*-succinimidyl ester moiety at the chain terminus as confirmed by the broad singlet at 2.8 ppm; and (ii) the incorporation of the fluorescent probe with a good matching with the initial feed ratio.† As expected, the isolated fluorescent copolymer exhibited an intense fluorescence in agreement with the emission/excitation spectra of the HMA monomer[6] corroborating the incorporation of the fluorescent probe during the polymerisation process (Fig. 2).

A range of fluorescently tagged well-defined NHS-poly($PEGMA_{475}$-*co*-HMA) copolymers were targeted with similar reactions conducted with different NHS-initiator : monomer ratios in order to give different final molar masses. As expected, the final molar masses were higher when decreasing the concentration of NHS-initiator relative to monomer and the recovered and purified copolymers were well-defined: P2 (84 % conv., $M_{n,SEC}$ = 14 200 g mol^{-1}, M_w/M_n = 1.08) and P3 (68 % conv., $M_{n,SEC}$ = 45 590 g mol^{-1}, M_w/M_n = 1.48). In order to assess the role of the NHS α-functionality during the bioconjugation reaction, a control fluorescent copolymer was synthesised. An amidation reaction was carried out on the NHS-poly($PEGMA_{475}$-*co*-HMA) copolymer P2 by reaction with benzylamine in dichloromethane over 24 h at ambient temperature to give a Bn-poly($PEGMA_{475}$-*co*-HMA) copolymer P4. The reaction was followed by ^{1}H NMR and the broad characteristic peak of the four protons from the succinimidyl ring attached to the copolymer totally disappeared

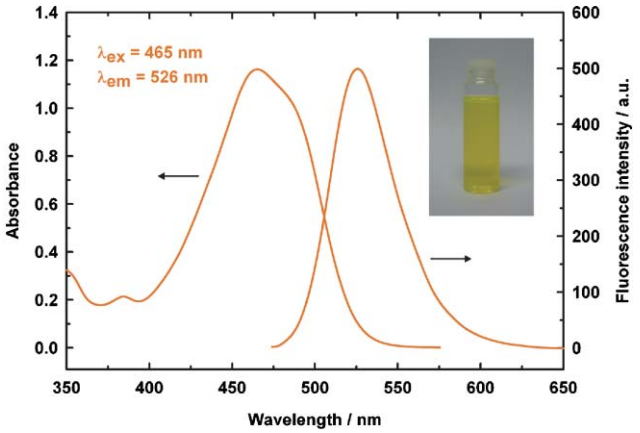

Fig. 2 Absorption and emission spectra of NHS-poly($PEGMA_{475}$-*co*-HMA) fluorescent copolymer P1. Insert: picture of NHS-poly($PEGMA_{475}$-*co*-HMA) fluorescent copolymer P1 at 2 mg mL^{-1} in deionized water.

accompanied by the appearance of those from free *N*-hydroxysuccinimide.† The non-reactive copolymer P4 had $M_{n,SEC}$ = 13 590 g mol^{-1} and M_w/M_n = 1.08.

Bioconjugation reactions were performed with untreated hairs in anhydrous DMSO with 5 wt% TEA for 70 h at ambient temperature with NHS-poly($PEGMA_{475}$-*co*-hostasol) copolymers (Exp. 1–3, with copolymers P1–P3 respectively). The hairs were then intensely rinsed in order to remove unreacted copolymers by gentle stirring in deionized water for several days at ambient temperature. Dried hairs were analyzed by Confocal Laser Scanning Microscopy (CLSM). These conditions were chosen in order to minimize the hydrolysis of the NHS groups.

All hair used for bioconjugation with NHS-poly($PEGMA_{475}$-*co*-HMA) copolymers exhibited strong fluorescence signal under confocal fluorescence microscopy characteristic of the hostasol group (Exp 1–3, Fig. 3). Non-reactive Bn-poly($PEGMA_{475}$-*co*-HMA) fluorescent copolymer (P4) did not lead to a strong fluorescence signal (Exp. 4, Fig. 3), being however, slightly more intense than untreated hairs (Exp. 5, Fig. 3).¶ Thus, the control experiment demonstrated that: (i) both the experimental conditions and the organic compounds involved in the reaction (*i.e.* DMSO and TEA) do not lead to a dramatic increase of hair autofluorescence and (ii) the reactive NHS α-functionality plays

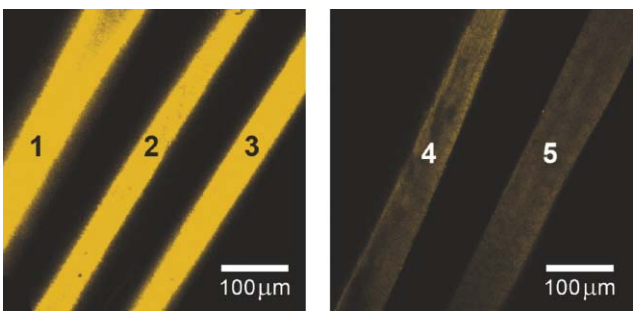

Fig. 3 Confocal fluorescence images of hair reacted with different poly($PEGMA_{475}$-*co*-HMA) copolymers. Left: Exp. 1–3 (NHS-poly($PEGMA_{475}$-*co*-HMA) copolymers P1–P3). Right: Exp. 4 (Bn-poly($PEGMA_{475}$-*co*-HMA) copolymer P4) and Exp. 5 (untreated hairs).

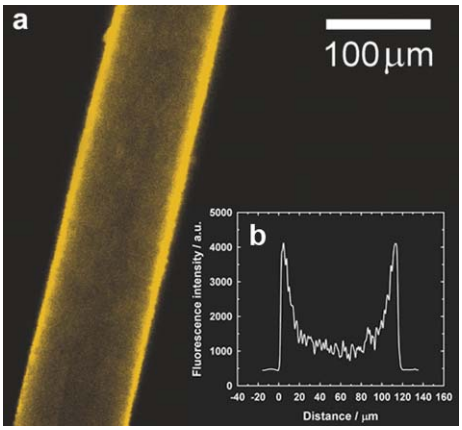

Fig. 4 (a) Confocal fluorescence image of treated hair (Exp. 1) when focusing on the hair side and (b) associated fluorescence intensity cross section.

the major role during the bioconjugation reaction excluding the possibility of adsorption of the fluorescent PEGMA-based copolymer.

The most intense fluorescence intensity was obtained from the outer layer of the hair. In contrast, when focusing beneath the hair surface, fluorescence was only observed on the edges whereas the core did not fluoresce (Fig. 4). These observations showed the outer surface of the hair indeed reacted and there was little penetration inside. Thus, when hair is treated with NHS-poly(PEGMA$_{475}$-*co*-HMA) copolymers, a minor part of the fluorescence arises from copolymer adsorption but the major part of the fluorescence is due to a covalent link between the fluorescent copolymer and the hair.

Hair fibers can easily suffer from damage from a number of environmental and chemical sources such as exposure to UV or chlorine, bleaching, straightening, perming and frequent washing with harsh surfactant-based cleansing shampoo compositions. Thus, we decided to use the NHS α-functional copolymers as a potential shield to enhance the properties of damaged hair fibers. Differential scanning calorimetry (DSC) under aqueous conditions[7] was conducted onto bleached hair (control) and bleached hair treated with NHS α-functional copolymers P1 and P3 to evaluate the denaturation temperature (T_d) of the α-helical material of the hair fibers (Fig. 5).[7] Bleached hair exhibited a T_d of 144.2 ± 1.5 °C whereas bleached hair treated with copolymer P1 and P3 exhibited a T_d of 151.5 ± 1.1 and 151.9 ± 1.2, respectively (B2 and B3, Fig. 5), which is relatively close to the T_d of undamaged hair under identical conditions.[7b] More interestingly, no significant change of the denaturation temperature occurred after a standard shampooing procedure; T_d = 153.2 ± 0.4 and 152.7 ± 1.1 for hair fibers treated with copolymer P1 and P3, respectively (B4 and B5, Fig. 5). This significant, permanent increase (~7–9 °C) of the denaturating temperature demonstrated, in addition to being a further evidence of the bioconjugation reaction, that damaged hair fibers can be efficiently repaired using NHS α-functional copolymers *via* the enhancement of their thermal properties.

Direct covalent conjugation of synthetic polymers onto a biological surface has been demonstrated. Fluorescently tagged poly(PEGMA$_{475}$) polymers synthesised by ATRP with NHS

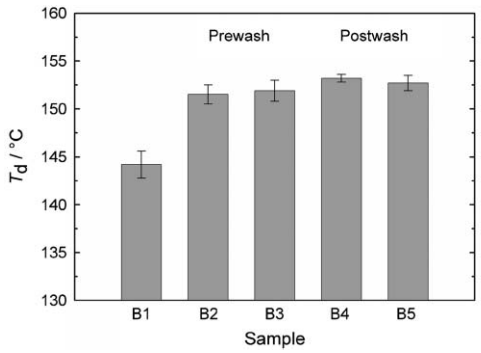

Fig. 5 Denaturation temperature, T_d, of the α-helical material of bleached hair fibers (B1), bleached hair fibers after treatments with copolymers P1 and P3 before washing (B2 and B3) and after washing (B4 and B5).

α-functionality were successfully grafted onto hair by an amidation reaction with amino groups from the keratin. Hair treated in this way exhibited strong fluorescence, as observed by confocal laser scanning microscopy. Damaged hair fibers were subsequently treated with these NHS α-functional copolymers and it was shown that the denaturation temperature of the α-helical materials increased substantially. This synthetic route is versatile and adaptable to other polymers with variable architectures as well as to other reaction media (alcohol, water *etc*), which allows a new and simple strategy in cosmetic research and related areas.

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Notes and references

§ Poly(methyl methacrylate) standard calibration is not appropriate for poly(PEGMA)-based polymers as higher molecular weights than expected are usually obtained due to differences in hydrodynamic volume. These plots were reported to show that M_n increased linearly with monomer conversion and that polydispersity indexes were low. The M_n could have been determined by ^{1}H NMR comparing the peak related to the succinimidyl ring (δ = 2.8 ppm) to the peak corresponding to the methyl ether (δ = 3.3 ppm), however, partial hydrolysis of the NHS moiety may lead to inaccurate values.

¶ Hair exhibits autofluorescence and the confocal fluorescence image of untreated hairs served as a blank experiment.

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