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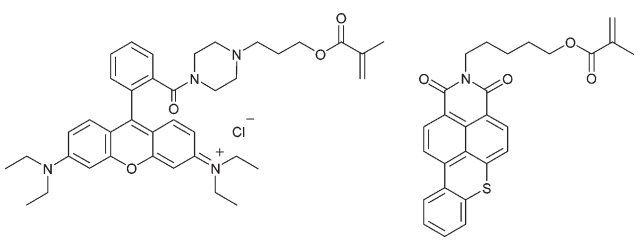
Fluorescently tagged polymer bioconjugates from protein derived macroinitiators†

Julien Nicolas,[a] Veronica San Miguel,[b] Giuseppe Mantovani[a] and David M. Haddleton*[a]

BSA and lysozyme have been transformed into macroinitiators for living radical polymerisation and used to produce well-defined bioconjugates which can be fluorescently labelled providing a versatile strategy for the preparation of bioconjugates which is complementary to traditional PEGylation.

Protein–polymer conjugation provides an established method to develop protein and peptide therapeutics. The most significant example is undoubtedly the covalent modification of proteins with poly(ethylene glycol) (PEG), "PEGylation".[1] The resulting conjugates exhibit a number of advantages including improved biodistribution and pharmacokinetics, reduced immunogenicity and longer plasma half-life from both reduced renal filtration and proteolysis, when compared to non-PEGylated analogues.[1] The emergence of living radical polymerisation (LRP)[2] has provided an efficient tool to finely tune the nature and the characteristics of many synthetic polymers. We have recently reported works involving the "*grafting to*" strategy for the synthesis of bioconjugates, the traditional way to bioconjugates *via* PEGylation. α-Functional well-defined polymers were shown as alternative PEGylation agents and conjugated with residual amine (lysine and *N*-terminal α-amino residues) and thiol (cysteine residues) functionalities present at the surface of the proteins.[3] In contrast, the "*grafting from*" synthetic route, which consists of transforming a protein into a macroinitiator for subsequent polymerisation, has not been as deeply investigated.[4] This is interesting as this is the method commonly used from non-biological surfaces.[5]

Herein, we report a strategy for the synthesis of fluorescent bioconjugates by LRP from protein macroinitiators. Materials containing fluorescent tags are useful allowing observation *via* fluorescence microscopy and in particular laser scanning confocal microscopy. This is crucial for tracing in biological systems during biomedical assays as the location of the material can be finely observed. Our idea was to incorporate a fluorescent probe *in situ* during the conjugate synthesis using fluorescent monomers under copolymerisation conditions with poly(ethylene glycol) methyl ether methacrylate ($PEGMA_{475}$) or dimethylaminoethyl methacrylate (DMAEMA). For this purpose, we used a hostasol methacrylate monomer[6] and synthesized a new methacrylate monomer derived from Rhodamine B (Scheme 1). The method described by Francis *et al.*[7] demonstrated the preparation of a Rhodamine B-based alcohol which was transformed into the corresponding methacrylate monomer.† This synthetic route was chosen for three main reasons: (i) contrary to the rhodamine-secondary amides, the tertiary amides avoid intramolecular cyclisation which would result in a loss of fluorescence; (ii) this compound is freely soluble in aqueous solutions whilst retaining its fluorescence emission under a broad range of pH and (iii) it allows enlargement of our range of fluorescent starting materials. The resulting Rhodamine B monomer exhibited a $\lambda_{ex.}$ = 566 nm and a $\lambda_{em.}$ = 587 nm, with a Stokes shift of 21 nm, in agreement with the spectral properties previously reported for the Rhodamine B-tertiary amide derivatives.[7]



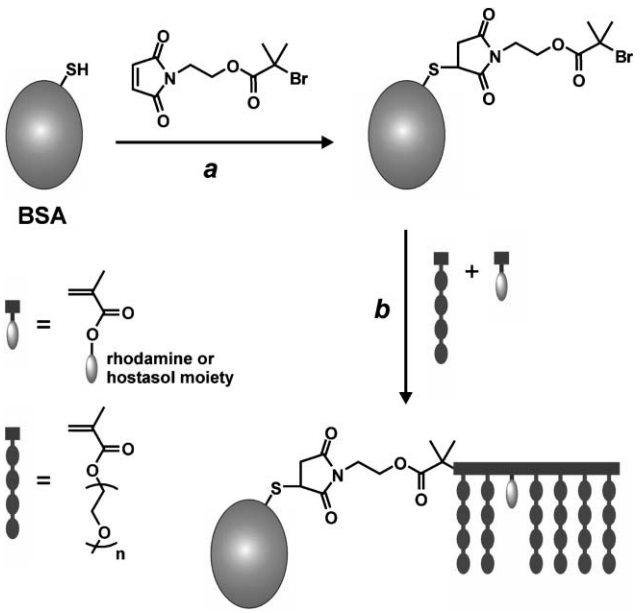
Scheme 1 Rhodamine B (left) and hostasol (right) methacrylate fluorescent monomers used in this work.

Bovine serum albumin (BSA) is a ~66 kDa commercially available protein selected as a model protein to demonstrate this approach. The synthetic route for the synthesis of a fluorescent bioconjugate is shown in Scheme 2. The synthesis of the BSA–protein macroinitiator targeted the free cysteine residue (Cys-34) as reactive towards the maleimide. Although only approximately 40–60% of free cysteine was available due to partial oxidation,[8] this route allowed the conjugation of one BSA protein per polymer chain as opposed to a statistical multi-site attachment as previously reported.[4]

All synthetic steps to the fluorescent bioconjugate were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The BSA macroinitiator exhibited two bands: the most significant being slightly greater than 66 kDa and a broad band ranging from 200 kDa to higher molecular weight. This is explained by aggregation of unreduced BSA caused by intermolecular thiol–disulfide interchange reaction, which induces the formation of BSA oligomers and thus of higher molecular weight compounds.[9]

Aggregation of BSA may also induce some conformational changes, especially accompanying the decrease of the α-helix and an increase of β-sheet content.[9] Circular dichroism (CD) analysis of native BSA and BSA-macroinitiator indicated that the α-helix

[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]*Instituto de Ciencia y Tecnologia de Polimeros, CSIC, Juan de la Cierva 3, Madrid, 28006, Spain*



Scheme 2 Synthetic strategy for the BSA–maleimide macroinitiator and subsequent living radical polymerisation of $PEGMA_{475}$ and fluorescent monomers. Reagents and conditions: (a) phosphate buffer (100 mM, pH = 7.0)/DMSO (96/4; v/v), 25 °C, 12 h; (b) Cu(I)Br/*N*-(ethyl)-2-pyridylmethanimine, $PEGMA_{475}$, Rhodamine B methacrylate, H_2O, 25 °C, N_2, 48 h.

content decreased from 54 to 43% whilst the β-sheet content increased from 1 to 8%, respectively.† This confirmed a conformational difference between native BSA and BSA-macroinitiator due to the presence of BSA oligomers, which may also be ascribed to the attachment of the initiator group to the BSA.

Differentiation between native BSA and BSA-macroinitiator was achieved by RP-HPLC with a linear gradient.† Complete disappearance of the native BSA peak (t_R = 17.0 min) was observed together with the appearance of the peak of the BSA-macroinitiator (t_R = 19.5 min). A very small peak observed at t_R = 14.6 min may probably be due to traces of BSA oligomers. The isolated BSA-macroinitiator was used to initiate the polymerisation of both $PEGMA_{475}$ and DMAEMA using copper-mediated living radical copolymerisation with either hostasol or rhodamine fluorescent monomers. The bioconjugates were purified and isolated by dialysis and lyophilisation. Bioconjugation was monitored by SDS-PAGE (with both white light and UV) where a complete shift of the BSA-macroinitiator to higher molecular weight was observed with all monomers (Fig. 1). As expected, all bioconjugates exhibited intense fluorescence† in accordance with the respective excitation/emission spectra of the rhodamine and hostasol fluorescent monomers (Fig. 2).

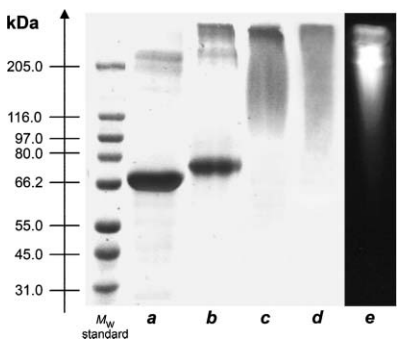


Fig. 1 SDS-PAGE of native BSA (a), BSA-macroinitiator (b), BSA-poly(DMAEMA-*co*-rhodamine) (c), BSA-poly($PEGMA_{475}$-*co*-rhodamine) (d) and BSA-poly($PEGMA_{475}$-*co*-rhodamine) under UV at λ = 302 nm (e).

For both monomers ($PEGMA_{475}$ and DMAEMA‡†), the fluorescent bioconjugates obtained could be detected by SEC-HPLC equipped with a fluorescence detector (at appropriate $\lambda_{ex.}$ and $\lambda_{em.}$ for each monomer) (Fig. 3). This confirmed that the fluorescent probes were incorporated into the conjugates during the polymerisation process.

To demonstrate the flexibility of this approach, a similar synthetic route was investigated with lysozyme. In this case, the free amine groups were targeted for multi-site attachment with *N*-succinimidyl 2-bromo-2-methylpropionate (Scheme 3).

The lysozyme-macroinitiator was isolated, characterised† and used to initiate the copolymerisation of $PEGMA_{475}$ in aqueous solution with rhodamine monomer using living radical polymerisation. The bioconjugation was monitored by SDS-PAGE (Fig. 4) and the recovered fluorescent bioconjugate, lysozyme-poly($PEGMA_{475}$-*co*-rhodamine), was observed by SEC-HPLC *via* fluorescence detection ($\lambda_{ex.}$ = 566 nm and $\lambda_{em.}$ = 596 nm),

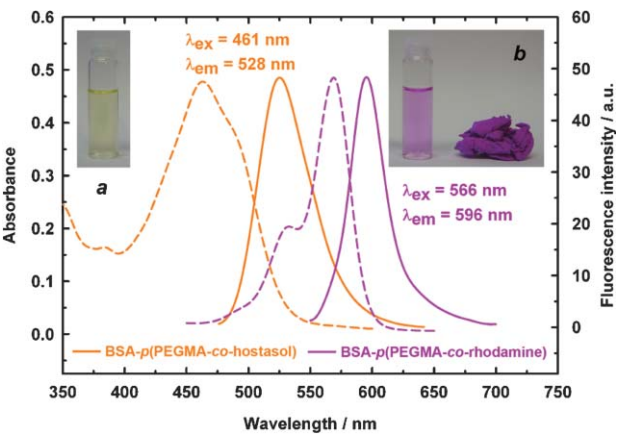


Fig. 2 Absorption (dashed line) and emission (solid line) spectra of BSA-poly($PEGMA_{475}$-*co*-hostasol) and BSA-poly($PEGMA_{475}$-*co*-rhodamine) fluorescent bioconjugates. Inserts: pictures of BSA-poly($PEGMA_{475}$-*co*-hostasol) (a) and BSA-poly($PEGMA_{475}$-*co*-rhodamine) (b) bioconjugates in phosphate buffer solution (10 mM, pH = 7.1).

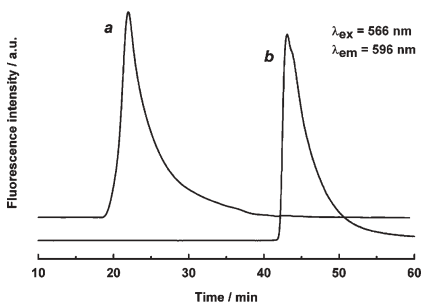
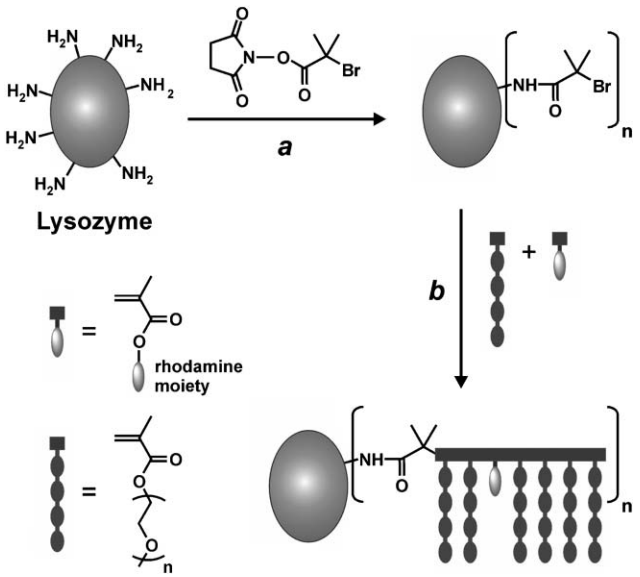


Fig. 3 SEC-FL-HPLC of BSA-poly($PEGMA_{475}$-*co*-rhodamine) bioconjugate (a) and Rhodamine B methacrylate (b).



Scheme 3 Synthetic strategy for the lysozyme-*N*-hydroxysuccinimide initiator and subsequent living radical polymerisation of $PEGMA_{475}$ and rhodamine monomer. Reagents and conditions: (a) Phosphate buffer (100 mM, pH = 7.0), DMSO/TEA (95/5; v/v), 25 °C, 48 h; (b) Cu(I)Br/*N*-(Ethyl)-2-pyridylmethanimine, $PEGMA_{475}$, Rhodamine B methacrylate, H_2O, 25 °C, N_2, 48 h.

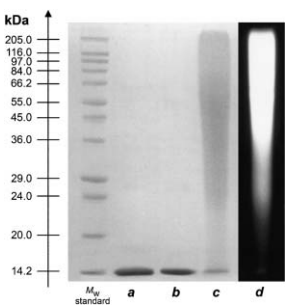


Fig. 4 SDS-PAGE of native lysozyme (a), lysozyme-macroinitiator (b), lysozyme-poly($PEGMA_{475}$-*co*-rhodamine) (c), and lysozyme-poly($PEGMA_{475}$-*co*-rhodamine) under UV at λ = 302 nm (d).

which demonstrated the incorporation of the fluorescent probe *in situ* during polymerisation (Fig. 5).

Contrary to the BSA bioconjugates, *via* SEC-FL-HPLC were seen: (i) the higher retention time of the lysozyme bioconjugate ($t_{R,BSA}$ = 22 min and $t_{R,lysozyme}$ = 32 min) due to an initial difference of molecular weight (~66 kDa for BSA and ~14.3 kDa for lysozyme); (ii) the broad peak from the lysozyme bioconjugate, probably due to the multi-site attachment of the lysozyme-macroinitiator which induced a broader molar mass distribution as regularly observed in living radical polymerisation involving multifunctional initiator.

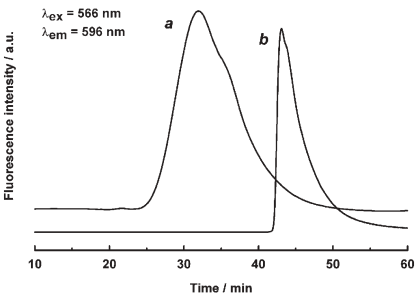


Fig. 5 SEC-FL-HPLC of lysozyme-poly($PEGMA_{475}$-*co*-rhodamine) fluorescent bioconjugate (a) and Rhodamine B methacrylate (b).

In summary, both bovine serum albumin (BSA) and lysozyme proteins were transformed into efficient macroinitiators for the synthesis of fluorescent bioconjugates from living radical polymerisation. A new fluorescent methacrylate monomer based on Rhodamine B was designed and synthesised. These bioconjugates were observed by fluorescence detection SEC-HPLC; allowing for *in situ* observation of the products.

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Notes

‡ It is noted that DMAEMA hydrolyses rapidly in pure water to methacrylic acid and the amino alcohol, however, this is suppressed by buffering at pH = 6.6 such that <1% hydrolysis occurs after 93 h, detectable by 400 MHz ^{1}H NMR.

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