Supplementary Information

Fluorescently tagged polymer bioconjugates from proteins derived macroinitiators

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

[a] *Department of Chemistry, University of Warwick, Coventry CV4 7AL, United Kingdom. Fax: +44 24 7652 4112; Tel: +44 24 7652 3256; E-mail: D.M.Haddleton@warwick.ac.uk*

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

Materials

Albumin from bovine serum (BSA, molecular weight ~66 kDa, V fraction), lysozyme (molecular weight ~14.3 kDa), poly(ethylene glycol) methyl ether methacrylate ($PEGMA_{475}$, average M_n = 475 g.mol^{-1}, Aldrich), 2-(dimethylamino)ethyl methacrylate (DMAEMA, Aldrich, 99 %) and methacryloyl chloride (Aldrich, 97 %) were used as received without further purification. Copper bromide (Cu(I)Br) was purified by stirring in glacial acetic acid and rinsing with ethanol and diethyl ether. Anhydrous dimethyl sulfoxyde (DMSO, Aldrich, 99 %) and triethylamine (TEA, Fischer, 99 %, stored over potassium hydroxide pellets), dichloromethane (DCM, Fischer, >99 %), acetonitrile (BDH, 99.9 %, HPLC grade) and trifluoroacetic acid (TFA, Aldrich, 99 %) were used as received. *N*-(Ethyl)-2-pyridylmethanimine was prepared as described earlier.[1] Hostasol fluorescent monomer was synthesised as previously describe.[2] Maleimide and *N*-succinimidyl 2-bromo-2-methylpropionate initiators were prepared as described elsewhere.[3,4]

Analytical methods

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX400 spectrometer using $CDCl_3$ (Aldrich) as the solvent. Fluorescence spectroscopy analysis was performed on a Perkin Elmer LS 50B spectrofluorometer with 1 cm quartz cuvette, 200 nm.min^{-1} scan speed, 2.5 nm excitation and emission slit widths, ambient temperature in phosphate buffer (10 mM, pH = 7.1). UV-Visible spectroscopy analysis was performed on a Jasco V-550 spectrophotometer with 1 cm quartz cuvette, 0.5 nm data pitch, 200 nm.min^{-1} scan speed, ambient temperature

in phosphate buffer (10 mM, pH = 7.1). Circular Dichroism (CD) analysis was performed on a Jasco J715 spectropolarimeter with 0.1 cm quartz cuvette, 0.2 nm data pitch, 100 $nm.min^{-1}$ scan speed, 8 accumulations, ambient temperature in phosphate buffer (10 mM, pH = 7.1) for a concentration of 0.1 $mg.mL^{-1}$. The RP-HPLC system was fitted with a Phenomenex column (Jupiter, C_{18}, 4.6 x 250 mm, 5 μm, 300 Å) and a UV detector (Gilson, UV-VIS-155) at 280 nm and 0.001 sensitivity under a flow rate of 1 $mL.min^{-1}$. The separation was performed using a gradient between mobile phase A (water/acetonitrile, 90/10, v/v and 0.05 % TFA) and mobile phase B (100 % acetonitrile, 0.04 % TFA) (Figure S1).

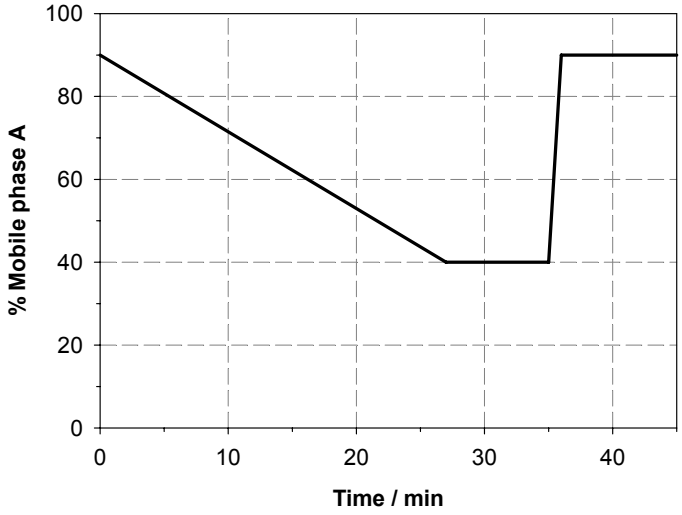
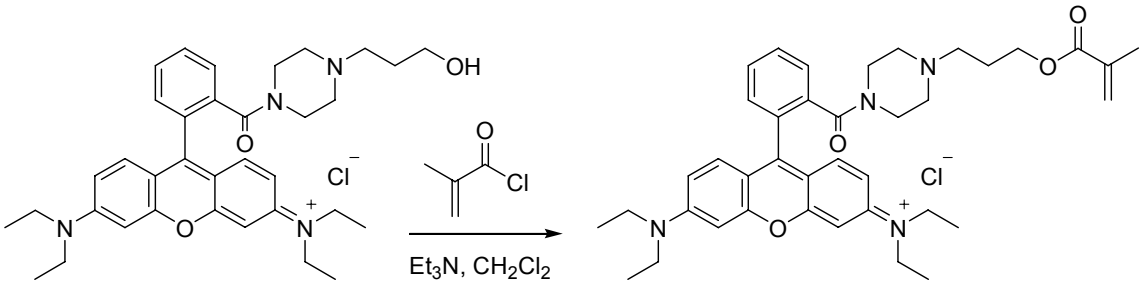


Figure S1: RP-HPLC gradient used in this study.

The SEC-FL-HPLC system was fitted with two BioSep-SEC-S3000 columns and a fluorescent detector (Hitachi, L-7480) continuously measuring the relative intensity of the mobile phase. The system was eluted with 0.1 vol.% trifluoroacetic acid solution in water and acetonitrile (69/31, v/v) at a flow rate of 0.5 $mL.min^{-1}$. SDS-PAGE was conducted using a stacking polyacrylamide gel of 5 % cross-linking and a resolving polyacrylamide gel (8 % cross-linking for BSA conjugates; 12 % cross-linking for lysozyme conjugate) and a running buffer consisting of 25 mM of TRIS base, 250 mM of glycine and 0.1 % of SDS at pH 8.7.

Synthesis of rhodamine B methacrylate fluorescent monomer



To a solution of suitable rhodamine B alcohol[5] (5.93 g, 9.80 mmol) in DCM (100 mL), TEA (1.44 g, 14.3 mmol) and methacryloyl chloride (0.970 g, 10.7 mmol) were sequentially added at 0 ºC. After 1 h, the temperature was raised to ambient temperature for a further 12 h. The volatiles were then removed under reduced pressure and the residue was purified by a pre-eluted flash chromatography (SiO_2) using methanol/triethylamine (95/5), to give the rhodamine B methacrylate as purple glassy solid in 72 % yield.

^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K): δ = 1.20 (t, *J* = 7.0 Hz, 12H), 1.67–1.76 (q, *J* = 6.8 Hz, 2H), 1.78 (s, 3H), 2.24 (br s, 4H), 2.31 (t, *J* = 7.2 Hz, 2H), 3.30 (br s, 4H), 3.50–3.55 (q, *J* = 7.3 Hz, 8H), 4.02 (t, *J* = 6.4 Hz, 2H), 5.38–5.45 (m, 1H), 5.91–5.96 (m, 1H), 6.63–6.68 (d, *J* = 2.5 Hz, 2H), 6.80–6.91 (dd, *J* = 2.3, 9.5 Hz, 2H), 7.07–7.15 (d, *J* = 9.5 Hz, 2H), 7.19–7.25 (m, 1H), 7.41–7.49 (m, 1H), 7.51–7.61 (m, 2H). ^{13}C NMR (100.59 MHz, $CDCl_3$, 298 K): δ = 12.50, 18.12, 25.51, 41.34, 46.01, 47.17, 52.19, 52.88, 54.48, 62.52, 96.11, 113.52, 114.32, 125.40, 127.51, 129.77, 129.99, 130.06, 130.46, 131.91, 135.17, 136.02, 155.44, 155.70, 157.54, 167.12, 167.22. IR (solid, ATR cell): ν (cm^{-1}) 3070 (=CH_2), 1709 (C=O ester carbonyl), 1635 (C=O amide), 1582 (C=C aromatic), 1460 (C=C aromatic), 1250 (C-O ester), 1169 (C-O ester), 1070 (C-O ether). High resolution MS-ES calcd. for $C_{39}H_{49}N_4O_4$ (M+): 637.3754; found: 637.3760.

UV-VIS spectrometry of a rhodamine B monomer solution in phosphate buffer (10 mM, pH = 7.1) was performed to obtain the absorption spectrum (it was determined: λ_{max} = 566 nm). Then the solution was analysed by fluorescence spectrometry to obtain the emission spectrum using $\lambda_{excitation}$ = 566 nm. Spectral properties of the rhodamine B monomer are represented in Figure S2.

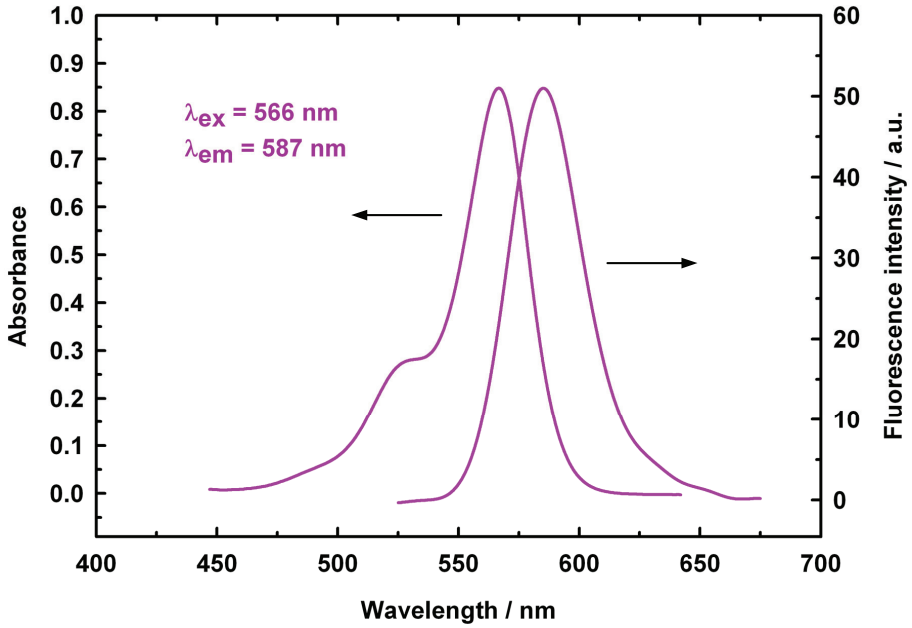


Figure S2: Absorption and emission spectra of the rhodamine B methacrylate.

Preparation of the BSA-macroinitiator

BSA (34 mg, 0.52 μmol) was dissolved in 4.6 mL of 100 mM phosphate buffer (pH = 7.0). A solution of 15 mg of maleimide initiator in 0.2 mL of DMSO was added to this slowly. The mixture was gently stirred during 12 h at ambient temperature and the solid residue was then removed by centrifugation. The supernatant was diluted with deionised water and dialysed with a 12,000-14,000 molecular weight cut-off (MWCO) membrane against deionised water for several days. The solution was then lyophilised to isolate the BSA-macroinitiator. RP-HPLC chromatograms of the native BSA, the BSA-macroinitiator and the control experiment are shown in Figure S3.

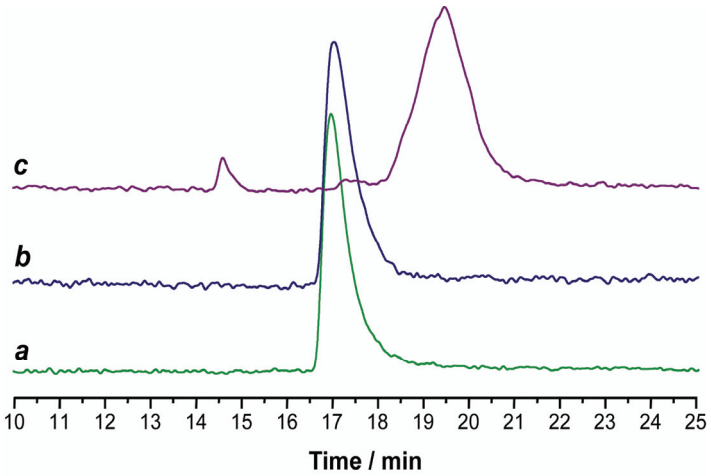


Figure S3: RP-HPLC of native BSA (a), control experiment (b) and BSA-macroinitiator (c). Control experiment: BSA in PBS (100 mM, pH = 7.0) with DMSO, stirred overnight at ambient temperature.

Preparation of the lysozyme-macroinitiator

N-Hydroxysuccinimide-2-bromo-2-methylpropionate (37 mg, 140 μmol) in 400 μL of DMSO was added to lysozyme (100 mg, 7 μmol of protein) dissolved in 20 mL of phosphate buffer (100 mM, pH = 7.0). To this, 50 μL of a TEA solution of 2.26 mM, prepared by dissolving 50 μL of TEA in 0.16 mL of phosphate buffer (100 mM, pH = 7.0), was added. The mixture was incubated at ambient temperature during 48 h under gentle stirring. The suspension was diluted with water and the solid residue was removed by centrifugation. The supernatant was dialysed using a 6,000-8,000 MWCO membrane against deionised water for several days. The solvent was removed by lyophilisation to isolate the lysozyme-macroinitiator. The native lysozyme and the lysozyme-macroinitiator were then analysed by RP-HPLC (Figure S4).

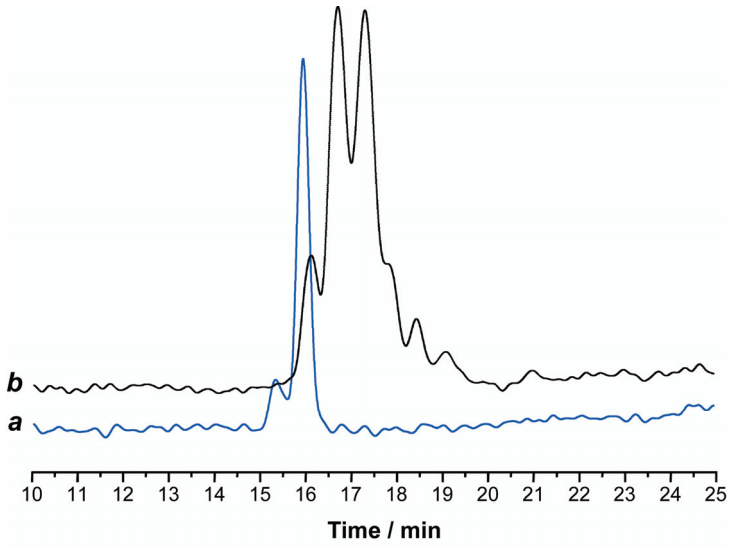


Figure S4: RP-HPLC of the native lysozyme (a) and of lysozyme-macroinitiator (b).

Synthesis of the BSA-poly(PEGMA$_{475}$-*co*-rhodamine) conjugate.

BSA-macroinitiator (100 mg, 1.51 μmol), PEGMA$_{475}$ (0.11 g, 0.38 mmol) and Cu(I)Br (32 mg, 0.23 mmol) were placed in a Schlenk tube and were dissolved in 23.8 mL of deionised water. 200 μL of a stock solution of rhodamine B methacrylate (3.8 μmol), previously prepared by dissolving 25 mg of the rhodamine B methacrylate in 2 mL of deionised water, was added. The solution was then deoxygenated by three freeze-pump-thaw cycles. After this, three vacuum-nitrogen cycles were applied and the Schlenk was placed in an oil bath at 25 ºC. Then, *N*-(Ethyl)-2-pyridylmethanimide (59 μL, 0.48 mmol) was added and the reaction mixture immediately turned dark brown/red, triggering the beginning of the polymerisation. After 48 h under nitrogen atmosphere, the reaction medium was diluted with deionised water and bubbled with air overnight. The solid residue was removed by

centrifugation and the supernatant was dialysed using a 12,000-14,000 MWCO membrane against deionised water. The solution was lyophilised and the BSA-poly(PEGMA$_{475}$-*co*-rhodamine) conjugate was obtained as a fluorescent purple solid.

Synthesis of the BSA-poly(PEGMA$_{475}$-*co*-hostasol) conjugate.

BSA-macroinitiator (75 mg, 1.1 μmol), PEGMA$_{475}$ (85 mg, 0.28 mmol) and Cu(I)Br (24 mg, 0.167 mmol) were placed in a Schlenk tube and dissolved in DMSO (17.8 mL). The hostasol monomer (1.3 mg, 2.83 μmol) was added. The mixture was deoxygenated by three freeze-pump-thaw cycles. The Schlenk tube was placed in an oil bath at 25 ºC and *N*-(Ethyl)-2-pyridylmethanimide (44 μL, 0.357 mmol) was added, which triggered the beginning of the polymerisation (a dark brown/orange colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 48 h. The reaction was then diluted with DMSO and bubbled with air overnight. The suspension was centrifuged and the supernatant was then diluted with deionised water (where unreacted hostasol monomer precipitated). The solid residue was removed by centrifugation and the supernatant was dialysed using a 12,000-14,000 MWCO membrane against deionised water. The solvent was removed by lyophilisation in order to isolate a fluorescent yellow solid.

Synthesis of the BSA-poly(DMAEMA-*co*-rhodamine) conjugate.

BSA macroinitiator (100 mg, 1.5 μmol) was dissolved in 23.6 mL of degassed water. It was added 200 μL of a solution of DMAEMA (0.377 mmol), prepared by dissolving 630 μL of DMAEMA in 2 mL of deionised water and adding some drops of HCl until pH 6.6. To this, 200 μL of a rhodamine B methacrylate solution (3.8 μmol), obtained by dissolving 25 mg of the fluorescent monomer in 2 mL of deionised water, was added. The mixture was deoxygenated with three freeze-pump-thaw cycles. Cu(I)Br (32 mg, 0.23 mmol) was then added to the frozen mixture and three vacuum-nitrogen cycles were applied. Then, the Schlenk tube was placed in an oil bath at 25 ºC under a nitrogen atmosphere. *N*-(Ethyl)-2-pyridylmethanimide (59 μL, 0.48 mmol) added and the reaction mixture exhibited a dark brown/red colour. After 17 hours, the polymerisation medium was diluted with deionised water and air was bubbled into the resulting solution overnight. The solid residue was removed by centrifugation and the supernatant was dialysed using a 12,000-14,000 MWCO membrane against deionised water during several days. The solution was lyophilised to give the conjugate as a purple solid. The SEC-FL-

HPLC spectra of the BSA-poly(DMAEMA-*co*-rhodamine) bioconjugate and of the rhodamine B monomer are shown in Figure S5.

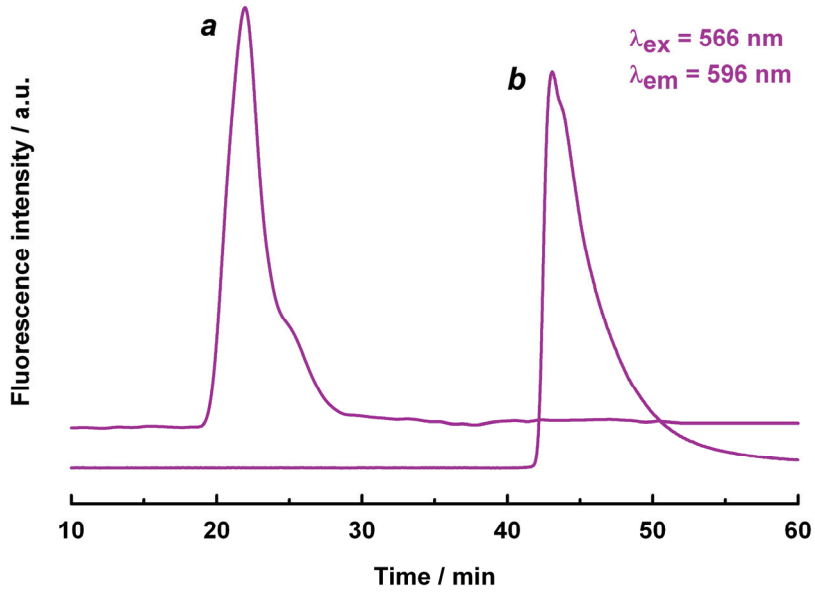


Figure S5: SEC-FL-HPLC of BSA-poly(DMAEMA-*co*-rhodamine) bioconjugate (a) and rhodamine B methacrylate monomer (b).

Synthesis of the lysozyme-poly(PEGMA$_{475}$-*co*-rhodamine) conjugate.

Lysozyme-macroinitiator (18 mg, 1.11 μmol), PEGMA$_{475}$ (83 mg, 0.278 mmol) and Cu(I)Br (24 mg, 0.167 mmol) were dissolved in 15 mL of deionised water. To this, 150 μL of a rhodamine B methacrylate solution (2.78 μmol), prepared by dissolving 19 mg of the fluorescent monomer in 2 mL of deionised water, was added. Then the solution was deoxygenated by three freeze-pump-thaw cycles. The Schlenk was then placed in an oil bath at 25 ºC. *N*-(Ethyl)-2-pyridylmethanimide (44 μL, 0.351 mmol) was added and the reaction mixture immediately turned dark brown/red. The mixture was stirred during 48 hours under nitrogen. The reaction medium was then diluted with deionised water and bubbled with air in order to stop the polymerisation. The mixture was centrifuged to remove the solid residue and the supernatant was dialysed using a 12,000-14,000 MWCO membrane against deionised water. The solution was then lyophilised to isolate a fluorescent purple solid.

Determination of the spectral properties of BSA-poly(PEGMA$_{475}$-*co*-rhodamine) and BSA-poly(DMAEMA-*co*-rhodamine) bioconjugates

The same experimental procedure used with the fluorescent rhodamine B methacrylate was applied for the BSA-poly(PEGMA$_{475}$-*co*-rhodamine), BSA-poly(DMAEMA-*co*-rhodamine) and lysozyme-poly(PEGMA$_{475}$–*co*-rhodamine) fluorescent bioconjugates (see Figure S6 and Figure S7 respectively). With the BSA-poly(PEGMA$_{475}$-*co*-hostasol) bioconjugate, we followed the same procedure and found $\lambda_{excitation}$ = 461 nm and $\lambda_{emission}$ = 528 nm in very good agreement with the spectral properties of the starting hostasol methacrylate monomer.[6]

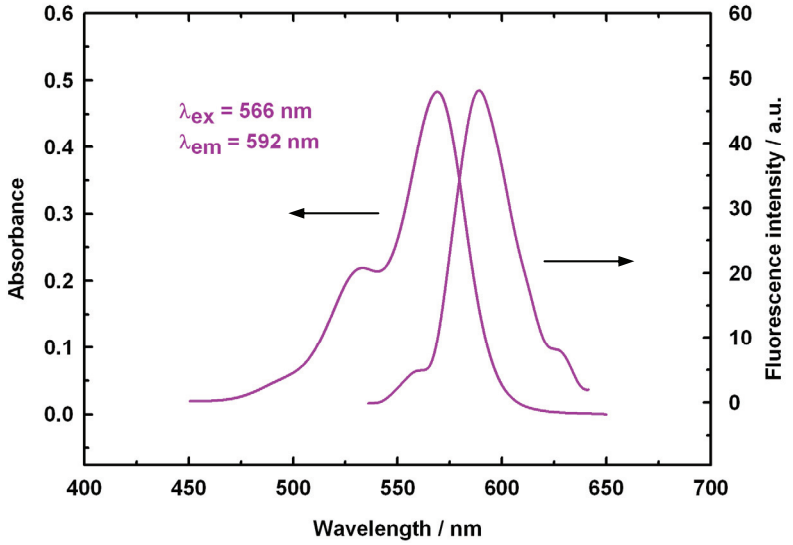


Figure S6: Absorption and emission spectra of BSA-poly(DMAEMA–*co*-rhodamine) fluorescent bioconjugate.

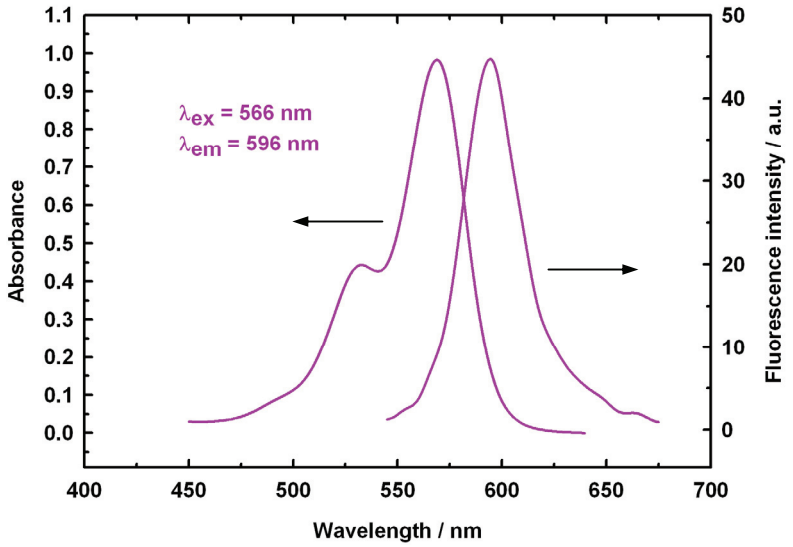


Figure S7: Absorption and emission spectra of lysozyme-poly(PEGMA$_{475}$–*co*-rhodamine) fluorescent bioconjugate.

Circular Dichroism analysis of native BSA and BSA-macroinitiator

We performed CD analysis to investigate any changes on the secondary structure between native BSA and BSA-macroinitiator. Native BSA and BSA-macroinitiator concentrations (0.1 $mg.mL^{-1}$ in 10 mM phosphate buffer at pH = 7.1) were checked by U.V. absorption at 280 nm under denaturating condition (6 mM GdmCl) using Beer's law. Percentages of α-helices and β-sheets were determined by applying the protein CD structure-fitting program, CDSSTR.[7] Overlayed CD spectra of native BSA and BSA-macroinitiator indeed showed a slight difference in the 195 – 210 nm region, demonstrating only a slight change in the secondary structure (Figure S8). The protein CD structure-fitting program (CDSSTR) gave the following compositions: i) native BSA (54 % α-helix; 1% β-sheet); ii) BSA-macroinitiator (43 % α-helix; 8 % β-sheet).

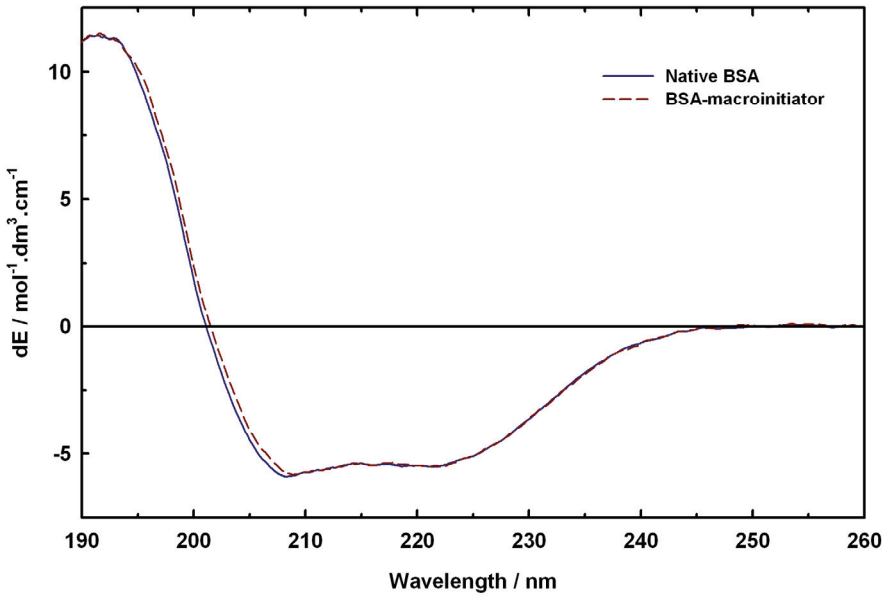


Figure S8: Circular Dichroism spectra of the native BSA and of the BSA-macroinitiator.

1 D. M. Haddleton, M. C. Crossman, B. H. Dana, D. J. Duncalf, A. M. Heming, D. Kukulj, A. J. Shooter, *Macromolecules* 1999, **32**, 2110.

2 A. J. Limer, A. K. Rullay, V. San Miguel, C. Peinado, S. Kelly, E. Fitzpatrick, S. D. Carrington, D. Brayden, D. M. Haddleton, *React. Funct. Polym.* 2006, **66**, 51.

3 G. Mantovani, F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. L. M. Cornelissen, K. Velonia, *J. Am. Chem. Soc.* 2005, **127**, 2966.

4 F. Lecolley, L. Tao, G. Mantovani, I. Durkin, S. Lautru, D. M. Haddleton, *Chem. Commun.* 2004, 2026.

5 T. Nguyen, M. B. Francis, *Org. Lett.* 2003, **5**, 3245.

6 A. J. Limer, A. K. Rullay, V. San Miguel, C. Peinado, S. Kelly, E. Fitzpatrick, S. D. Carrington, D. Brayden, D. M. Haddleton, *React. Funct. Polym.* 2006, **66**, 51.

7 W. C. Johnson, *Proteins Struct. Funct. Genet.* 1999, **35**, 307.