Selective synthesis of double temperature-sensitive polymer–peptide conjugates†

Florentina Stoica,[a] Cameron Alexander,[b] Nicola Tirelli,[c] Aline F. Miller[a] and Alberto Saiani*[d]

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A novel synthetic route has been developed to couple selectively a modified octa-peptide, that is able to gel at low temperature, to the prototypical thermoresponsive polymer poly(*N*-isopropylacrylamide) to give a bioconjugate that exhibits double thermoresponsiveness.

Recently, significant research effort has focussed on exploring new ways to prepare bioconjugates for cell culture,[1–3] tissue engineering[4–7] and drug delivery[8–10] applications. Bioconjugate materials are attractive for such applications as they may combine the controlled mechanical, thermal and electronic properties of synthetic polymers with the functionality of designed bioactive groups, such as peptides. There have been various synthetic routes[11,12] proposed for the preparation of polymer–peptide conjugates, including solid-phase procedures that have been used to synthesise a peptide where the last coupling step involved the linking of a polymer initiator. This was subsequently used to grow the polymer segment *via* either nitroxide mediated polymerisation[13,14] or atom transfer radical polymerisation[15,16] (ATRP) while still attached to the resin. One other route to prepare polymer–peptide conjugates involved the attachment of amine functionalised polymers to the solid resin which were subsequently used to synthesise the peptide.[17] In all cases the methodology used is exclusive to the specific polymer and peptide. The present challenge, therefore, is to find a general and very selective strategy to synthesise such hybrids with controlled properties or functions.

Herein we set out a versatile way to prepare peptide–polymer bioconjugates, and demonstrate the feasibility of the method by combining a polymer and peptide structure that are both thermoresponsive. The prototypical thermoresponsive polymer of choice is poly(*N*-isopropylacrylamide) (PNIPAAm) which has a lower critical solution temperature (LCST) around physiological temperature (~37 °C) in aqueous solution.[18–21] In addition the LCST of PNIPAAm can be tuned easily to a desired temperature by incorporating a suitable comonomer.[22–25] The peptide selected is the ionic complementary octa-peptide FEFEFKFK, where F is phenylalanine, E is glutamic acid and K is lysine. This peptide is known to form a three-dimensional beta-sheet rich fibrillar hydrogel at 30 g L^{-1} in water, at room temperature and its gelation is reversible; the gel melting at ~75 °C.[26,27]

The strategy for the synthesis of PNIPAAm-FEFEFKFK conjugate was to initially synthesise the peptide, modify it at one end with a free thiol and subsequently use it as a chain transfer agent in the free radical polymerisation of NIPAAm. This versatile method allows most polymer chains to be initiated at and propagated from one specific position on the peptide, thus giving the desired conjugate *via* a selective synthesis route which is shown schematically in Fig. 1. The peptide (**1**) was synthesised using Fmoc solid phase procedures using Fmoc-Lys(Boc)-Wang resin (mesh = 400, loading = 0.66 mmol g^{-1}) and coupling the amino acids using standard peptide synthesis protocols.[28] Each reaction step took ~4 h and the attachment of each amino-acid was confirmed by the Kaiser test.[29] In the last step the –COOH end of the transfer agent, 3-mercaptopropionic acid, was activated with 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylamonium hexafluorophosphate (HCTU) and coupled to the $-NH_2$ end group of the peptide over 6 h to give (**2**). After deprotection and cleavage of the modified peptide from the resin with trifluoroacetic acid–anisole (95 : 5), (**3**) was recovered by precipitation in cold diethyl ether, centrifugation (6000 rpm, 5 min) and lyophilisation for 3 days. The purity of the modified peptide (**3**) was found to be 76 wt% by HPLC and the modification was confirmed by ^{1}H NMR and MALDI-Tof mass spectrometry.

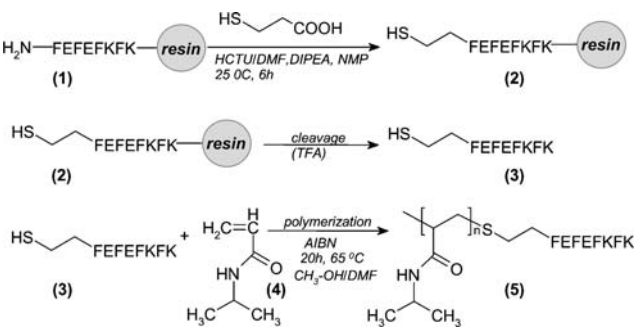

Fig. 1 Polymer–peptide conjugates were accomplished by modification of an octapeptide end group that was subsequently cleaved from its solid support before being used as the transfer agent in the free radical polymerisation of NIPAAm.

[a] *School of Chemical Engineering and Analytical Science, University of Manchester, Manchester, UK M60 1QD*
[b] *School of Pharmacy, University of Nottingham, Nottingham, UK NG7 2RD*
[c] *School of Pharmacy, University of Manchester, Manchester, UK M13 9PT*
[d] *School of Materials, University of Manchester, Manchester, UK M1 7HS. E-mail: a.saiani@manchester.ac.uk; Fax: +44 161 306 3586; Tel: +44 161 3065981*

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The presence of free –SH groups was confirmed by Ellman UV test at 412 nm[30] and the presence of sulfur was detected by elemental analysis. Subsequently, this intermediate product (**3**) (0.165 mmol) was used as the transfer agent in the polymerisation of NIPAAm (**4**) (26.5 mmol). The reaction was undertaken under an inert atmosphere, at 65 °C for 20 hours in the presence of AIBN (0.245 mmol) as an initiator and DMF (40 ml) as solvent. Several steps were involved in conjugate (**5**) purification; first the majority of the solvent was removed *via* freeze-drying. Second, the concentrated solution was precipitated in cold diethyl ether and the solid was recovered by centrifugation (6000 rpm, 5 min) and lyophilisation (5 days). This allowed removal of unreacted monomer, initiator and any residual solvent. Third, the product (**5**) was re-dissolved in pure water at room temperature, re-precipitated in water at 50 °C and centrifuged (6000 rpm, 5 min) to remove any unmodified peptide. This step was repeated three times before recovering the conjugate by freeze-drying for 5 days. Finally, the conjugate (**5**) was dialysed against water for 3–4 days using dialysis tubing with a cut-off of 3500 Da, before being recovered by lyophilisation for 4 days. Conjugation (reaction yield 53 wt% after last dialysis) was confirmed by solid-state ^{1}H NMR and by the Ninhydrin test. The molecular weight of conjugate was found to be 18.3 kDa with a polydispersity of 1.9. The approximate concentration of peptide in the conjugate was deduced from its UV spectrum at 258 nm and confirmed by ^{1}H NMR and was found to be 16 g of peptide per 100 g of conjugate (1.5 mol% peptide, 98.5 mol% NIPAAm).

Simple visual observations of a 200 g L^{-1} conjugate–water mixture as a function of temperature proved very intriguing (Fig. 2): at 20 °C, the conjugate formed a self-supporting, semi-transparent gel (pH ~ 6.3) with time, while the polymer (molecular weight 6.5 kDa; polydispersity 1.7) synthesised in the absence of the peptide was a transparent solution. The gelation effect was attributed to the behaviour of the peptide, which is known to form a strong gel at 30 g L^{-1}.[26,27] At ~30 °C the conjugate exhibited a phase transition; it switched from a semi-transparent to a turbid gel, indicating the occurrence of PNIPAAm LCST. The non-conjugated polymer exhibited a transition from solution to a self-supporting "paste" at the higher temperature of ~35 °C. If the maximum temperature used was kept below ~75 °C then on cooling the conjugate was found to revert back from a turbid to a semi-transparent gel. On the other hand if the temperature of the conjugate was increased above ~75 °C the sample remained self-supporting and on cooling the conjugate returned to its original semi-transparent liquid state.

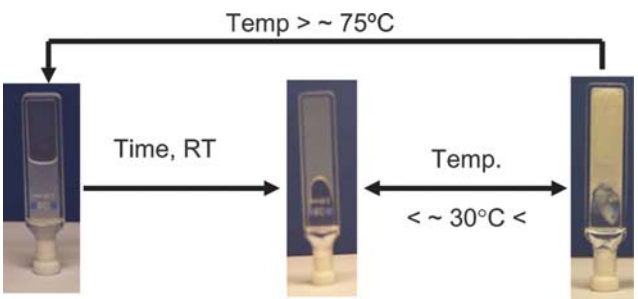

Fig. 2 200 g L^{-1} PNIPAAm–FEFEFKFK conjugate behaviour during a heat–cool cycle.

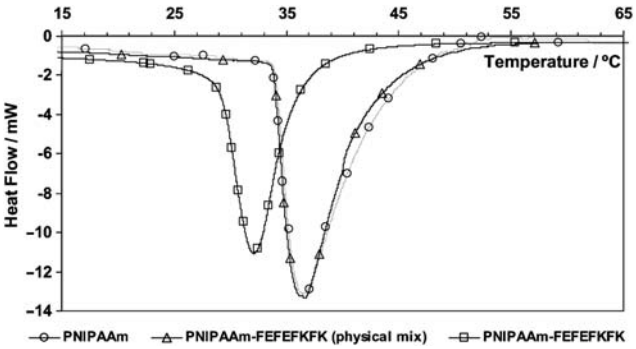

Fig. 3 MicroDSC curves for PNIPAAm (160 g L^{-1}), a physical mixture of PNIPAAm (170 g L^{-1}) and FEFEFKFK (30 g L^{-1}), and PNIPAAm–FEFEFKFK conjugate (200 g L^{-1}). Heating rate: 1.0 °C min^{-1}.

After *ca.* 15 min at room temperature it began to reform a gel. This behaviour indicates that above ~75 °C melting of the gel occurs and the conjugate becomes a self-supporting "paste" in the same way that the non-conjugated polymer behaves owing to the relatively high concentration of PNIPAAm used. Similar behaviour was observed when the sample was subjected to a further four heat–cool cycles. These observations suggest that our conjugates are doubly thermo-sensitive and have reversible behaviour. This was confirmed using microDSC where three samples were considered for calorimetric analyses: the non-conjugated polymer (160 g L^{-1}), the conjugate (200 g L^{-1}, corresponding to 168 g L^{-1} of PNIPAAm and 32 g L^{-1} of peptide) and the physical mixture of non-conjugated polymer plus peptide (170 g polymer + 30 g peptide L^{-1}). It is immediately apparent from Fig. 3 that all three samples exhibit an exothermic transition upon heating that coincides with the LCST transition observed visually. The pure polymer and the physical mixture both show a sharp peak with an onset at 34 °C indicating that the physically mixed free peptide, even at high concentration, has a limited influence on the LCST transition of the polymer *i.e.* the physical mixture behaves as two distinctive systems (it should be noted that the physical mixture also forms a semi-transparent gel at room temperature). In the case of the conjugate a narrow exothermic peak was also observed, but at the lower temperature with an onset of 29 °C. This indicates the PNIPAAm behaviour to be influenced by the covalently bound peptide despite the relative molar concentration of peptide in the conjugate being small (1.5 mol%).

In conclusion we have shown a novel, selective synthesis route for the preparation of polymer–peptide conjugates utilising a modified peptide with a thiol terminus as a transfer agent in the free radical polymerisation of PNIPAAm. This synthesis route has the advantage of being site specific and avoids any complications arising from the inherent interactions between metal catalysts and the peptide, which are often unaccounted for in ATRP synthesis.[31,32] Moreover, the conjugate PNIPAAm–FEFEFKFK exhibits double thermoresponsive behaviour, showing both an LCST phase transition at ~30 °C and a gel melting transition at ~75 °C. Both were found to be fully reversible. This strategy opens up the possibility of combining a variety of peptides with different responsive polymers to give new thermoresponsive macromolecules with diverse and controllable functions for biomedical

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applications. It should also be mentioned that the peptides used in this work are pH responsive (*i.e.* form gels at pH < 8 and solution at pH > 8) and therefore these conjugates are expected to display additional acid–base responses.

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