Reversible switching of substrate activity of poly-*N*-isopropylacrylamide peptide conjugates†‡

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The activity of smart polymer peptide conjugates towards chymotrypsin catalyzed hydrolysis was reversibly switched on and off using temperature as the trigger.

The development of controlled free-radical polymerization techniques such as atom transfer radical polymerization (ATRP),[1] RAFT (reversible addition fragmentation),[2] and nitroxide-mediated free-radical polymerization (NMP)[3] has paved the way to the synthesis of complex polymer architectures. The synthesis of so-called smart polymers or stimuli responsive soft materials has recently become highly important.[4] Upon using an external stimulus such as temperature, pH, light *etc.*, the morphology (phase behavior) of a material can be selectively altered. The thermoresponsive poly-*N*-isopropylacrylamide (PNIPAM) has often been used in that regard.[5] An increase in the temperature to above 32 °C leads to a change in the morphology and hydrophilicity of the polymeric material in water. Below 32 °C, the polymer is soluble in water in the chain extended highly hydrated state. Above the so called lower critical solution temperature (LCST), water is expelled for entropic reasons and the polymer chains collapse and turn into their hydrophobic state. The highly aggregated polymer is then insoluble in water. This phase switch can be used to induce biological responses in PNIPAM biomolecule conjugates.[6,7] As recently shown by us, controlled polymerization of *N*-isopropylacrylamide (NIPAM) can be achieved *via* NMP using a sterically highly hindered nitroxide.[8] Herein, we present the synthesis of PNIPAM peptide conjugates. Moreover, we will show that a temperature-induced phase switch of the smart PNIPAM tail of these novel bioconjugates can be used to alter the substrate activity towards enzymatic hydrolysis of the PNIPAM peptide conjugates (Fig. 1).

Peptides have received growing attention as antibiotics and hence temperature-induced switching of their activity is important.[9] Moreover peptides are involved in key cell surface recognition events.[10]

Polymer peptide conjugates have been prepared by covalently binding a polymerization initiator to a peptide with subsequent polymerization (grafting from, Fig. 2). Alternatively, the conjugates can be synthesized by attaching a polymer to a peptide (grafting to).[11] Drawbacks of these two approaches are: (a) if the peptide bears more than one active side chain for covalent attachment of the initiator or the polymer, selectivity is a problem; and (b) if more than one initiator or polymer should be added to the peptide backbone, perfect initiator or polymer "loading" is difficult to achieve. Unreacted side chains may remain. As a novel approach, we have planned to synthesize unnatural amino acids bearing a radical initiator moiety. Importantly, this amino acid initiator has to be stable under standard peptide coupling procedures. This should allow the immobilization of the amino acid initiator at any site on the targeted peptide *without postmodification*. Polymerization will eventually lead to the conjugate. Due to the stability of the alkoxyamine functionality, NMP has been chosen as the polymerization technique.

We decided to use the side chain of L-serine to covalently bind the initiator moiety *via* ether bond formation. The bromide **1** was readily obtained from commercially available *p*-ethylbromobenzene *via* radical bromination. Alkoxyamine formation using 2,2,6,6-tetramethylpiperidine-*N*-oxyl radical (TEMPO, **2a**) or the sterically highly hindered nitroxide **2b**[12] and Cu-catalysis[13] afforded the alkoxyamines **3a** and **3b** (Scheme 1). Bromine–lithium exchange followed by trapping of the aryllithium compound with dimethylformamide (DMF) yielded the corresponding aldehydes. Lithium aluminium hydride (LAH) reduction and treatment of the corresponding benzylic alcohols with

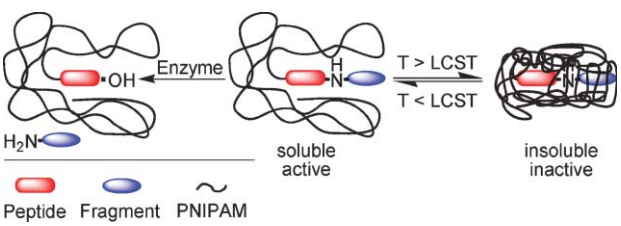

Fig. 1 Reversible switching of the activity in PNIPAM–peptide conjugates—the concept.

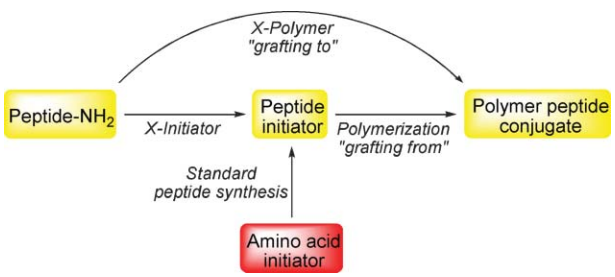

Fig. 2 Preparation of polymer peptide conjugates—different approaches.

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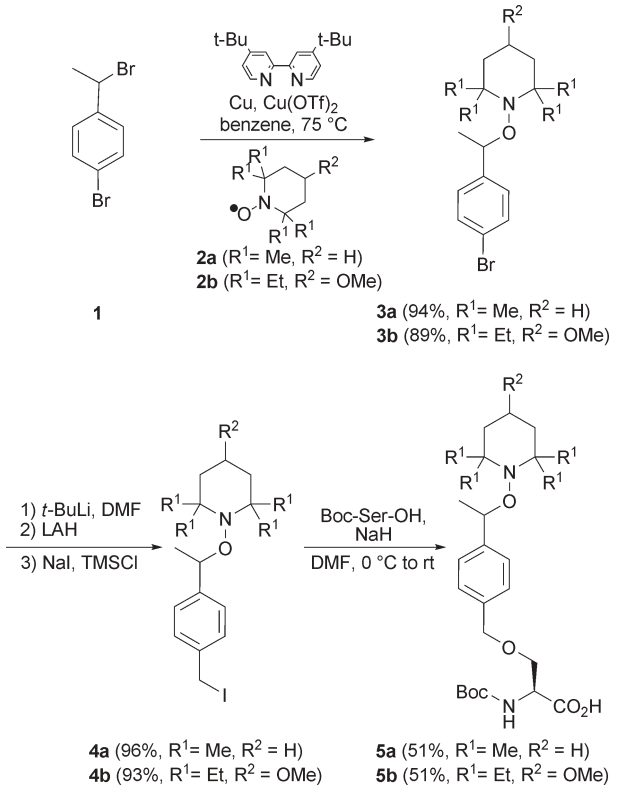

Scheme 1 Synthesis of alkoxyamines **5a** and **5b**.

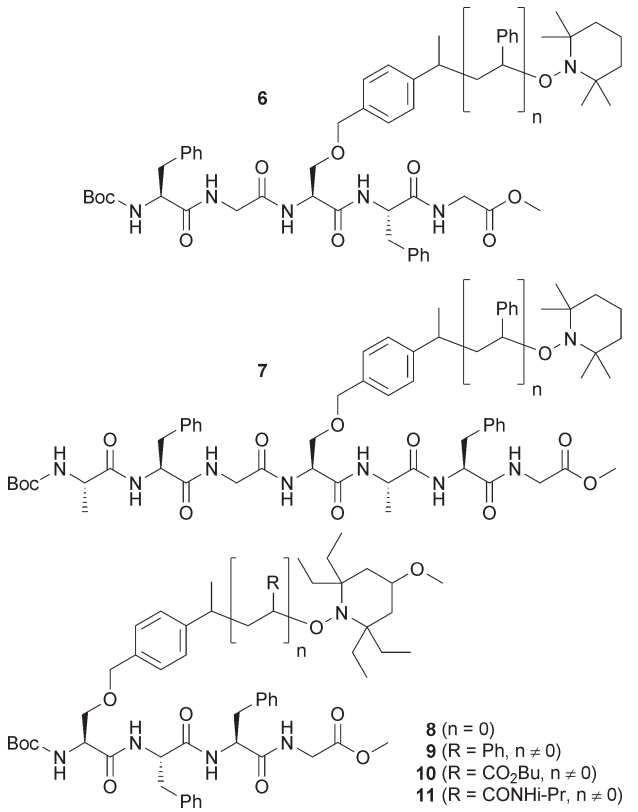

Fig. 3 NMP of styrene, butyl acrylate and *N*-isopropylacrylamide using alkoxyamines **6** ($n = 0$), **7** ($n = 0$) and **8** as initiators/regulators.

trimethylchlorosilane (TMSCl)–NaI in acetonitrile gave the iodides **4a** and **4b** in excellent overall yields. Side chain protection of Boc-Ser-OH with the benzylic iodides **4a** and **4b** was achieved in DMF using NaH to give the protected serines **5a** and **5b** containing alkoxyamine moieties able to initiate the NMP.

We successfully immobilized **5a** and **5b** into various peptides using standard solution phase peptide chemistry (see ESI‡). In Fig. 3, the modified peptides **6** ($n = 0$), **7** ($n = 0$) and **8** used for the initial polymerization studies are depicted. The peptides chosen are model compounds and have no particular biological relevance.

The controlled living radical polymerization of styrene with **6** ($n = 0$) or **7** ($n = 0$) was performed in neat styrene at 125 °C. The length of the polystyrene (PS) tail can be controlled by the amount of added alkoxyamine initiator. The polymerization for 24 h using 1 mol% of **6** ($n = 0$) provided the PS–peptide conjugate **6** in 72% conversion with a number average molecular weight (M_n) of 6100 g mol^{-1} and a polydispersity index (PDI) of 1.15 as determined from gel permeation chromatography (GPC). Reducing the alkoxyamine concentration to 0.5 mol% provided **6** with M_n = 11 700 g mol^{-1} (PDI = 1.15). Similarly, the PS–heptapeptide **7** bearing a PS-tail was successfully synthesized (0.5 mol% initiator, 18 h, 65% conversion, M_n = 12 200 g mol^{-1}, PDI = 1.16).

The alkoxyamines **6** ($n = 0$) and **7** ($n = 0$) contain the TEMPO moiety to control the radical polymerization. However, it is well known that controlled NMP using TEMPO as a regulator is restricted to the polymerization of styrene and styrene derivatives.[3] Therefore, we switched to **8** bearing a sterically highly hindered nitroxide.[12] As compared to the **6** ($n = 0$) and **7** ($n = 0$)-mediated polymerization of styrene, reaction with **8** was more efficient. The styrene polymerization using 0.5 mol% of **8** was conducted at 105 °C for 24 h to get **9** in 80% conversion (M_n = 16 700 g mol^{-1}, PDI = 1.10). Under the same conditions, polymerization with **6** ($n = 0$) or **7** ($n = 0$) delivered conjugate **6** in 45% conversion (PDI = 1.18) or **7** in only 41% conversion (PDI = 1.20), respectively. As expected, the controlled polymerization of butyl acrylate was readily achieved with **8** (1 mol%, 125 °C, 8 h) and **10** was obtained with a narrow molecular weight distribution (PDI = 1.13, M_n = 14 900 g mol^{-1}, 80% conversion). However, butyl acrylate polymerization with **6** ($n = 0$) did not work at all. Furthermore, **8** was chosen as a representative system to prove the control/"livingness" of the peptide initiator-mediated styrene and butyl acrylate polymerizations. We determined the conversion as a function of time and analyzed the molecular weight as a function of monomer conversion (see ESI, Fig. S1 to S4‡). The linear increase in $\ln([M]_0/[M])$ *versus* time and molecular weight *versus* monomer consumption proved the controlled character of the polymerizations.

We were also able to control the polymerization of NIPAM with **8**. The polymerization was conducted in C_6D_6 using 100 equiv. of NIPAM at 125 °C for 8 h to give **11**. The conversion (42%) was determined using ^{1}H NMR spectroscopy. M_n (6500 g mol^{-1}) and PDI (1.16) were determined from mass spectrometry.[8,14] Larger PNIPAM-tails were obtained upon increasing the reaction time and reducing the alkoxyamine concentration (0.5 mol%, 20 h, 71% conversion, M_w = 10 800 g mol^{-1}, PDI = 1.36). For the higher molecular weight conjugates, M_w was determined using multiple angle laser light scattering (M_w = weight average molecular weight).[8]

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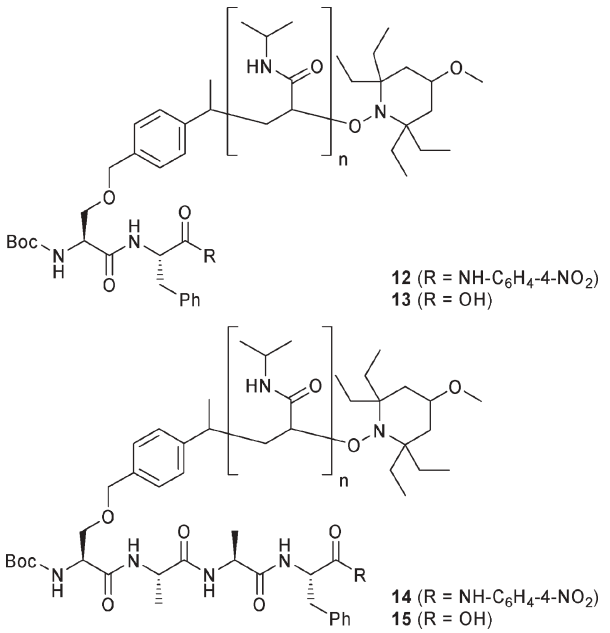

Fig. 4 Alkoxyamines **12** and **14** used in the enzymatic hydrolysis to yield **13** and **15**.

For enzymatic studies, we prepared the PNIPAM peptide conjugates **12** (M_w = 12 700 g mol^{-1}, PDI = 1.38) and **14** (M_w = 10 900 g mol^{-1}, PDI = 1.38, Fig. 4). The C-terminus of these peptides was protected as *p*-nitroanilide, which was known to undergo chymotrypsin-catalyzed hydrolysis to liberate the UV active *p*-nitroaniline and the conjugates **13** and **15**.

The temperature-induced phase transitions (LCST) of the PNIPAM peptide conjugates **11**, **12** and **14** were studied using ^{1}H NMR spectroscopy (see ESI‡).[15] Conjugate **11** showed a phase switch at around 31 °C. For **12** and **14**, the LCST was found at 29 °C. It has been known that low molecular weight PNIPAM conjugates bearing a hydrophobic moiety at the polymer chain end have LCSTs below 32 °C, in agreement with our results.[16]

The enzymatic reactions using **12** and **14** were conducted with commercially available chymotrypsin and were followed with UV spectroscopy (see ESI‡). At 37 °C, the enzymatic hydrolysis was completely suppressed for both substrates. At 22 °C, however, **12** and **14** are soluble in H_2O and the enzymatic reaction occurred. It is important to note that hydrolysis could be stopped by increasing the temperature to 37 °C. Renewed cooling to 22 °C reinstalled the active state of the system and the reaction went to completion (see Fig. S8 in the ESI‡).

In conclusion, we showed that serines bearing an alkoxyamine moiety able to initiate/regulate a controlled living radical polymerization could readily be synthesized and immobilized at any position of a peptide. NMP of various monomers delivered the corresponding polymer peptide conjugates. The activity of PNIPAM peptide conjugates towards enzymatic hydrolysis was readily switched by temperature control.§

Notes and references

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