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Thermoresponsive Hydrogels from Physical Mixtures of Self-Assembling Peptide and its Conjugate with PNIPAAm

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Summary: The phase behavior and rheology of a range of physical mixtures of self-assembling octapeptide FEFEFKFK and its conjugate with the thermoresponsive polymer poly(*N*-isopropylacrylamide) (PNIPAAM) have been studied. These systems typically combined the thermo-responsive character of PNIPAAM and the gelling property of octapeptides where both the elastic behavior and macroscopic melting of the system could be controlled by varying the ratio of pure peptide to peptide-polymer conjugate.

Keywords: bioconjugate; gels; peptide; poly(*N*-isopropylacrylamide); self-assembly; stimuli responsive polymer

Introduction

Stimuli-responsive materials typically change their characteristic properties in response to environmental cues such as temperature, pH, ionic strength or the presence of enzymes. Such materials have been studied widely in recent years due to their potential application as scaffolds for cell culture and tissue engineering or as drug delivery vehicles.[1,2] The prototypical polymer in this class is poly(*N*-isopropylacrylamide) (PNIPAAm) as its lower critical solution temperature (LCST) is observed in water around body temperature, i.e. 37 °C, and the transition can be triggered using a variety of external stimuli including temperature, pH and solvent composition.[3] In addition the transition can be tuned easily by incorporation of co-monomers[4]. More recently, however, significant effort has focussed on finding selective ways to conjugate PNIPAAm to biologically active molecules to create functional and/or dual responsive materials[5–8]. Such systems are attractive for biomaterials applications as they combine the controlled mechanical, thermal and electronic properties of polymers with the functionality of designed bioactive groups, such as peptides.

Herein we will focus on the synthesis and characterisation of a doubly thermosenstive PNIPAAm-peptide conjugate, and its mixtures with pure peptide, as a function of temperature. The peptide selected is the ionic-complementary peptide FEFEFKFK, where F represents phenylalanine, E is glutamic acid and K is lysine. It is well documented that this peptide self-assembles in water into β-sheet rich fibrillar hydrogels at $\sim 17\ mg\ ml^{-1}$ and its gelation is thermally reversible; the gel melts between ~45–85 °C depending on the concentration.[9] It is anticipated therefore that the conjugate material will not only self-assemble into a thermally reversible fibrillar hydrogel, but will also exhibit an LCST transition within the matrix at circa 32 °C. The macroscopic phase behaviour of these systems will be mapped out as a function of temperature before the thermal and mechanical characteristics of the resulting hydrogels will be discussed.

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Materials and Methods

Peptide Synthesis and Modification with SH Group

Both peptide and modified peptide were synthesized on a ChemTech ACT 90 peptide synthesizer (Advance ChemTech Ltd, Cambridgeshire, UK) using *N*-methyl-2-pyrrolidone (NMP) as a solvent and standard solid phase peptide synthesis protocols.[10] The side chain protected amino acids, the amino acid activator (HCTU) and the preloaded Fmoc-Lys(Boc)-Wang resin (beads size – 100 – 200 mesh, substitution – 0.70 mmol g^{-1}) were purchased from Novabiochem (Merck) and used as received. All other solvents were purchased from Aldrich and used without further purification. Functionalisation of the peptide with a thiol group was carried out as outlined in a previous publication and the material was treated as a standard peptide in subsequent purification.[8] Both peptides were cleaved from the resin and side chain protecting groups removed using a mixture of trifluroacetic acid (TFA) and anisole (95:5). The resin was removed by filtration and the peptide was precipitated in cold diethylether. The peptide was purified by dissolving in water and washing a further 4 times with diethylether before it was isolated by freeze-drying. The peptide yield ranged from 90–95%. Reverse-phase high performance liquid chromatography, mass spectrometry and ^{1}H NMR were used to confirm peptide structure and determine purity which was typically $> 90\%$.

Conjugate Synthesis by Free Radical Polymerization (FRP)

N-Isopropylacrylamide (NIPAAM) (Aldrich, 97%) and azo-iso-butyronitrile (AIBN) (Fisher Scientific, laboratory grade) were both recrystallized prior to use from hexane and methanol respectively. 3-Mercaptopropionic acid (Acros Organics) and dimethylsulfoxide (DMSO) (Aldrich) were both used as received. Typically, 0.0265 moles of NIPAAM, 2.44×10^{-4} moles of AIBN and 33.1×10^{-5} moles of SH-FEFEFKFK (acts as the chain transfer agent in the free radical polymerisation) were dissolved in 30 mL deoxygenated DMSO and the reaction mixture was stirred at 65 °C for 24 h under N_2. The reaction mixture was subsequently cooled to room temperature and the majority of solvent removed using a rotary evaporator. The remaining viscous liquid was diluted to 500 mL with deionised water and dialyzed against water for 5 days using tubing with 3500 g mol^{-1} molecular weight cut-off (Medicell Ltd., UK) to remove short polymer chains and any unreacted reagents. The resulting solution was lyophililyzed to give a white powder with a yield of 95%.

Phase Behaviour

A range of peptide/conjugate mixtures were prepared (molar ratio varied between 50 and 13 : 1 - see Table 1) by dissolving the desired quantities of material in 1 mL of deionised water, vortexing for 60 s and leaving to equilibrate at room temperature for 2 hrs prior to measurement. Macroscopic behaviour was studied at 20, 40 and 80 °C by placing the samples in a temperature controlled water bath and allowing 15 mins for equilibration before the state of the system was determined both visually and using the 'tilt tube test'.

Micro Differential Scanning Calorimetry (microDSC)

MicroDSC measurements were performed using a SETARAM microDSC III. The sample cell was filled with 0.5–0.6 mL solution using a micropipette and the reference cell filled with pure water and its weight adjusted using a micro balance to

Table 1.
Mass and molar ratios of the series of peptide:conjugate physical mixtures prepared.

Ratio of peptide:conjugate	
Mass ratio	Molar ratio
20-10	49-1
15-10	37-1
10-10	25-1
10-15	17-1
10-20	13-1

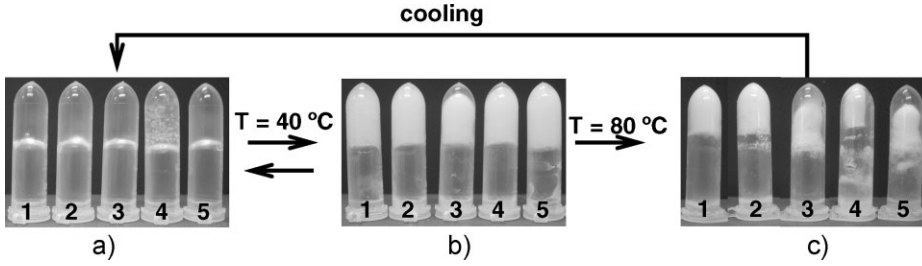

Figure 1.
Photographs of peptide:conjugate mixtures at the following molar ratios: 1) 49:1, 2) 37:1, 3) 25:1, 4) 17:1, 5) 13:1 at a) 20 °C b) 40 °C and c) 80 °C.

ensure an identical mass of solution was present in both sample and reference cells. MicroDSC thermographs were recorded using a 1.0 °C min^{-1} scanning rate in the temperature range 20 to 80 °C.

Oscillatory Rheology

Mechanical studies were performed on a stress-controlled Bohlin C-CVO rheometer, equipped with a Peltier device. Parallel plate geometry of 20 mm diameter and a 0.25 mm gap was used with a solvent trap to minimise evaporation.

The rheological experiments of all conjugates consisted of three stages for each sample. Firstly, a strain amplitude sweep was performed at constant frequency (20 °C, strain amplitude $\gamma = 0.001$–10, angular frequency $\omega = 1$ rad s^{-1}) to identify the linear viscoelastic region. Secondly, the frequency sweep at constant strain amplitude (20 °C, $\gamma = 0.01$, $\omega = 0.1$–10 rad s^{-1}) to determine the behaviour of the elastic (G') and viscous (G'') moduli. Finally temperature scans were performed by setting frequency and strain at 2π rad s^{-1} and 0.01 respectively. The changes in the G' and G'' were measured between 20 and 80 °C at 1 °C min^{-1}.

Results and Discussion

A range of peptide:conjugate mixtures were prepared (Table 1) where the molecular weight of the pure polymer and polymer in the conjugate was 28 kg mol^{-1}. The phase behavior of these mixtures at 20, 40 and 80 °C was monitored visually using the standard tilt test tube method. As Figure 1a reveals, self-supporting transparent gels were formed for all samples at 20 °C suggesting the peptide component had self-assembled, as expected, into β-sheet rich fibrils that self-associate when above a critical concentration, C^*. C^* for the pure FEFEFKFK is typically $\sim$17 mg mL^{-1}, therefore, it is postulated that the peptide from the conjugate is incorporated within the fibril as only sample 1 has a pure peptide concentration > 17 mg mL^{-1} (see Table 1).

As the samples were heated to 40 °C, the gels became opaque presumably due to the PNIPAAm polymer chains collapsing as they go through their LCST (Figure 1b). Interestingly the samples remained self-supporting and the transition was thermally reversible if the samples were subsequently cooled at this stage, indicating the conjugate remained trapped within the hydrogel structure. As the samples were heated to 80 °C the gel sample appeared to shrink while expelling water. Such behavior is typical of PNIPAAm solutions and has previously been related to the aggregation of the collapsed PNIPAAm chains[11]. This behavior became more pronounced, as expected, as the polymer conjugate, and hence polymer, concentration increased (moving from sample 1 to 5 in Figure 1c). Upon cooling the water was re-absorbed and the polymer re-dissolved to form clear hydrogels when cooled below 40 °C. Such temperature responsive behavior was reversible upon at least a further four thermal cycles.

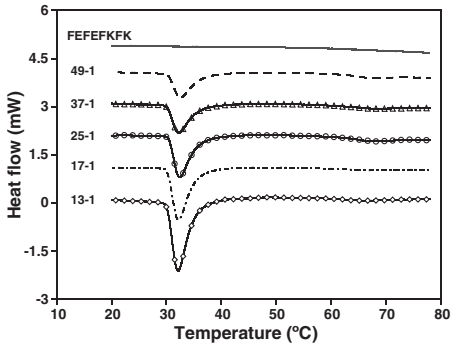

Figure 2.
Micro DSC thermographs for the peptide:conjugate mixtures at different molar ratios.

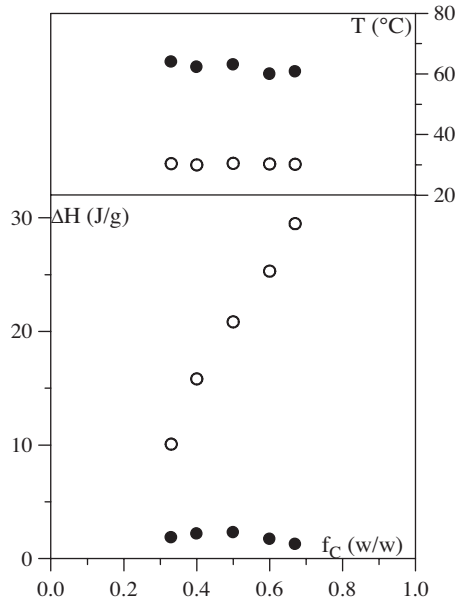

Figure 3.
Plot of the PNIPAAm LCST transition (○) and gel melting transition (●) (upper) and enthalpy per gram of peptide plus conjugate within the gel (lower) versus the mass fraction of conjugate, f_c.

Thermal Behavior of the Mixtures

The thermal behavior of each of the 5 samples with different molar ratios was monitored using microDSC in the temperature range of 20–80 °C and the results for the initial heating scan are given in Figure 2.

It is clear from Figure 2 that two endothermic transitions are observed for each sample; a sharp transition at ~31 °C and a second broader transition at ~61–65 °C. The first transition corresponds to the LCST of the PNIPAAm segment of the conjugate and agrees well with our visual observations. The temperature of this transition also agrees well with those reported in the literature for pure PNIPAAm with similar molecular weight[4]. The enthalpy associated with this transition increases linearly with the increase in the molar ratio of the conjugate, as expected, while the temperature of transition remains constant (Figure 3). The second peak is much broader in nature and corresponds to the macroscopic melting of the hydrogel mixture. Such a broad transition is typical of the melting of an entangled polymeric hydrogel[12]. The enthalpy associated with the gel melting transition seems to go through a maximum suggesting their might be the formation of a complex between the peptide and polymer (Figure 3). Further investigation into this phenomenon is required. It is interesting to note that a hydrogel composed of only 20 mg mL^{-1} pure FEFEFKFK melts at ~ 50 °C, in comparison to ~ 61–65 °C observed for the mixtures, suggesting the incorporation of the conjugate within the fibrillar structure is possibly enhancing fibril-fibril and/or peptide-peptide interactions.

Rheological Studies of the Gels

Figure 4a shows a mechanical spectrum performed at 20 °C for 5 peptide:conjugate mixtures. In each case the storage modulus (G') is greater that the loss modulus (G'') indicative of an elastic rather than viscous material. Both G' and G'' are essentially independent of frequency over the range 10^{-2} – 10^{1} rad s^{-1}, which indicates the hydrogels are relatively stable within this range. Such rheological behavior matches the characteristic signature of a solid-like gel. It is evident by comparing the trend of the G' values that as the quantity of either peptide or conjugate increases, so does G'. Interestingly all G' values are higher than those obtained for an equivalent concentration of pure FEFEFKFK

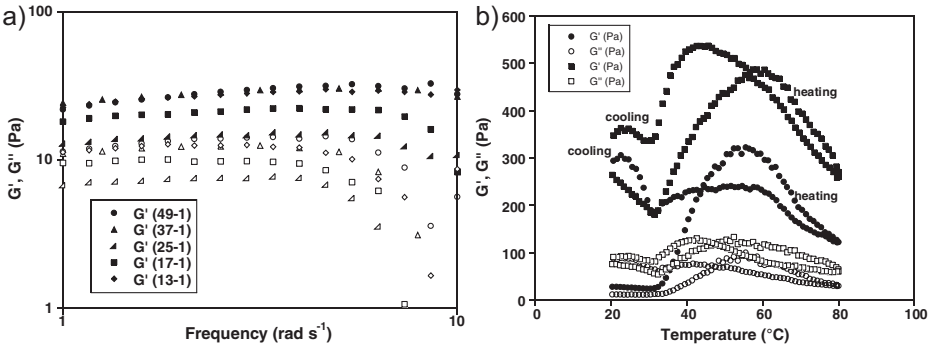

Figure 4.
Elastic, G' (solid symbols), and viscous, G'' (hollow symbols) moduli as a function of a) frequency for different molar ratios and b) temperature for the molar ratio of 49:1.

peptide (G' is only ~ 10 Pa for a 20 mg mL^{-1} sample). This supports the earlier postulation that the peptide segment from the conjugate actively participates in fibril, and consequently, gel formation.

Figure 4b shows the variation in elastic behavior as a function of temperature where a decrease in G' is observed at $\sim$ 31 °C, followed by a sharp increase to a much higher value of 300 Pa before a further decrease is observed at $\sim$ 62 °C. These changes in behavior correlate well with both the transitions observed in microDSC and those observed visually. The first transition leads to an initial decrease in G' presumably due to the collapse of the PNIPAAm polymer chains. The subsequent increase in G' could be due to a thickening in fibril diameter and enhancement of fibril-fibril interactions due to aggregation of the PNIPAAm chains, while the final decrease is due to the macroscopic melting of the sample. Such transitions are mirrored when the system is cooled where the gelation transition occurs circa 20°C lower than the melting transition. Such hysteresis was also observed in the microDSC scans (data not shown) and is typical of polymeric hydrogels[12]. Interestingly, the elasticity of the hydrogel is significantly greater after each subsequent heating cycle and the values of G' before and after 4 heating cycles are compared in Table 2. The mechanism behind such gel strengthening, as well as the rheology behavior as a function of temperature, is currently under investigation via scattering methods and will be the subject of a forthcoming article.

Table 2.
Elastic moduli values for the peptide:conjugate mixtures before and after the 4th heating/cooling cycle.

Ratio of FEFEFKFK:conjugate		G' (Pa)	
Mass ratio	Molar ratio	Before heating	After heating
20-10	49-1	30	200
15-10	37-1	20	180
10-10	25-1	15	130
10-15	17-1	20	160
10-20	13-1	30	200

Conclusion

Doubly thermoresponsive hydrogels with controllable thermal and mechanical properties have been prepared simply by physically mixing a peptide-polymer conjugate, FEFEFKFK-PNIPAAm, with its pure peptide, FEFEFKFK. Such gels exhibit a typical LCST at ~31 °C due to the PNIPAAm component within the molar ratio range studied here, and a gel melting transition at 61–65 °C, which increases with increasing peptide concentration. Such phase transitions are reversible upon cooling, however, oscillatory rheology revealed that the elasticity of the hydrogel network

increased by approximately an order of magnitude after 4 heating/cooling cycles. We have shown here that the hybrid materials prepared combine the responsive behavior of the polymer with the structuring properties of the peptide and as such could find important applications for the formation of 'smart' scaffolds for tissue engineering or for drug delivery devices.

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