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ARTICLE

pH Responsive Highly Branched Poly(N-isopropylacrylamide) with Trihistidine or Acid Chain Ends

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Thermally responsive highly branched poly(N-*iso*propyl acrylamide)s (HB-PNIPAM) were prepared and end-functionalised to give polymers with acid or trihistidine end groups. These polymers exhibit a broad coil-to-globule transition across a wide temperature range which can be measured using covalently attached fluorescent tags. The acid chain ends provided a material with a distinct change in solution behaviour at pH close to the pKa of the carboxylate group. At pH 11 this polymer did not show a cloud point up to 50 °C but fluorescence measurements on the labelled polymers showed that a coil to glubule transition did take place. The globular state, above the LCST, appeared to be more swollen if the end group carried charge then when it was uncharged. A polymer with trihistidine and free carboxylate chain ends, which contained multiple charges at various pH, did show LCSTs at all pH and the polymer globule was shown to be swollen at each pH.

Introduction

Study of polymer materials in recent years have focused on the development of advanced polymer architectures with tuneable materials properties[1-5]. Branched polymers are interesting because their solution[6, 7], rheological[8-10] and surface properties[10, 11] are different to their linear analogues. Branching provides polymers with many chain ends that can be used to provide functionality that is often more accessible than similar pendant functionality. In aqueous media branching affects the critical solution behaviour of water soluble polymers, such as polyacrylamides. Poly(N-isopropyl acrylamide) is one of the most extensively studied water soluble polymers because it desolvates at temperatures above a lower critical solution temperature (LCST)[12, 13]. The LCST is a desolvation event that progresses the polymer chain from an open solvated coil to a desolvated (but not hydrophobic) globule[6, 14]. The LCST of PNIPAM can be modified by adding surfactants to the solution[15, 16] or incorporating comonomers. However, much of the current literature shows that segmented PNIPAM-based materials (block and graft copolymers) provide materials in which the segments behave independently[17, 18], and materials with tunable properties can more easily be created by altering the feed ratio of statistical or alternating copolymers[19-22]. Production of these materials depends on employing suitable comonomers that polymerise with comparable reaction rates.

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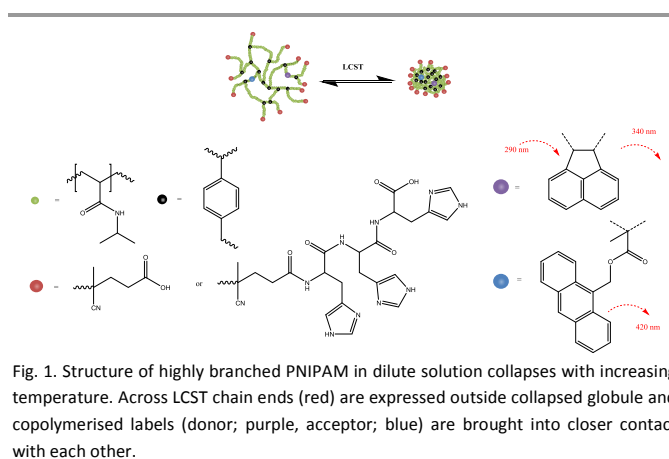†* Author to whom correspondence should be addressed

Fig. 1. Structure of highly branched PNIPAM in dilute solution collapses with increasing temperature. Across LCST chain ends (red) are expressed outside collapsed globule and copolymerised labels (donor; purple, acceptor; blue) are brought into closer contact with each other.

Thus, with linear polymers of moderate molar mass additional blocks or changing chain ends do not significantly affect the LCST of the bulk material[1, 2, 23] (but may trigger supramolecular association[3, 4, 23-26]). However, in highly branched water soluble polymers this is not the case and the alteration of end groups can change solution properties. For example, incorporation of peptides can be used to tune the critical solution properties of hydrogels[27-30] as well as imbuing biofunctional properties[31-34]. In linear polymers changes in critical solution behaviour are produced by adding comonomers and in PNIPAM based statistical copolymers acid or basic monomers provide materials that respond to pH[19-22].

In general highly branched (HB) polymers have a decreased LCST compared to linear analogues[6, 35-37] Chain segments are clustered into dense regions in a highly branched polymer[38, 39] sometime the role of acidic/basic end groups in the formation of the collapsed globule is largely unstudied. In recent studies we have shown that changing the

solubility[27, 32] a desolation transition from coil to globule. A wide range of peptides with tunable properties can be easily prepared via solid stage synthesis and attached to polymer chain ends of HB polymers[5, 27, 31, 34] promising and largely untapped area of research.promising and largely untapped area of research.

This paper concerns the study of HB-PNIPAM polymers with charged chain end functionality via labelling with luminescent groups (Fig. 1). Previously we reported the synthesis of these polymers and we showed how they form gels on heating.[40] The behaviour of two HB-PNIPAMs was examined with either carboxylic acid or His-His-His-COOH (trihistidine) end groups. For this purpose two covalently bound fluorescent labels were used; acenaphthylene (ACE) and 9-anthryl methyl methacrylate (AMMA). The labels had good spectral overlap and a known critical Förster distance[41]. Low loadings of these labels are randomly distributed throughout the polymer chain and so they could be used to report on structural changes to the polymer in solution as it undergoes its LCST. This system allows for study of ionisable cyanopentanoic acid and trihistidine end groups on large branched polymers. The former deprotonates around pH 5, affecting the switch between globule and solvated forms, whilst trihistidine end groups provide a means to alter the pH behaviour as the chain ends can carry multiple charges depending on the pH of the solvent. Current theories about branched polymers suggest that the chain ends of the polymer cannot penetrate the globule however results in this publication suggest that the ionisation of the chain end can fundamentally alter the swelling of the polymer globule.

Results

Polymer design

Highly branched polymers were prepared via reversible addition-fragmentation transfer (RAFT) self-condensing vinyl polymerisation. 4-Vinylbenzyl pyrrolecarbodithioate (**1**) was used to create highly branched polymers containing fluorescent labels. The ratio of NIPAM to **1** was 25:**1**, which gave a degree of branching (branch points per repeat unit of 0.036.[6] The properties of these materials has already been reported and the molar mass distributions and averages are provided along with details of the synthesis in the electronic supporting information. Low concentrations (<0.5 mol %) of donor label (ACE) and acceptor (AMMA) were copolymerised to allow fluorescence interrogation of the polymers.

Ionisation of End Groups and Turbidity

The states of ionisation of chain ends of functionalised polymers (cyanopentanonic acid and trihistidine) were estimated using the Chemaxon Marvin pKa program. As expected this indicated that the carboxylic acid chain end was largely uncharged in solution at pH < 4.5 and predominantly negatively charged at pH > 4.5. The trihistidine group consisted of a distribution of charge states, with three dominant

microspecies; < pH 2 (three positive charges), pH 2 – 6 (three positive charges and one negative resulting in overall two positive charges) and pH 7 – 12 (a single negative charge) (see supporting information for full details).

The different ionisation on the polymer chain end led to changes of the cloud point of these materials in dilute solution (0.5 mg ml^{-1}) at various pHs as desribed previously.[40] The turbimetric data are included in ESI for completeness. In the previous work we showed that the LCST of the acid ended polymer was 20 °C at low pH (pH 2) and produced a sharp increase in turbidity. At pH 5.5 the LCST increased due to partial ionisation of the end group, and at pH 11 complete ionisation of the end group meant no turbidity was observed up to 60 °C. In the trihistidine functional polymer, containing three basic groups and one acid group, at pH 2 the protonation of the imidazoles led to a higher cloud point, than in the carboxylate polymer (35 °C) and at pH 5 this decreased to 25 °C. At pH 11 (net negatively charged) no cloud point was observed.

Fluorescence Analysis

Energy Transfer measurements were carried out by both static and dynamic fluorescent methods. The fluorescence emission of the donor and acceptor labels were compared. The self-quenching and radiative energy transfer is represented by the ratio of the peaks. This was calculated by comparing the peak emission of the ACE at 345 nm (I_A), and the AMMA at 420 nm (I_D) following excitation of just the ACE at 295 nm.

Alternatively the amount of non-radiative energy transfer occurring in the system can be determined by measuring the rate with which the acceptor moiety quenches the donor. By measuring the fluorescence lifetime the amount of quenching ($E_{D\tau}$) can be represented by the difference between the lifetime of a sample in the absence (τ_D) / presence (τ_{ET}) of energy transfer acceptor.

$$E_{D\tau} = 1 - \frac{<\tau_{ET}>}{<\tau_D>}$$

Eq 1

The average distance between donor and acceptor species (r) can be resolved using the critical (Förster) distance (R_o) between these labels. This is known as the spectroscopic ruler technique.

$$E_{D\tau} = \frac{R_0{}^6}{R_0{}^6 + r^6}$$

Eq. 2

The fluorescence lifetimes used in these equations were determined using the fluorescence decay observed following excitation at 295 nm and fitting it to a triple exponential function. The form of a multi-exponential function takes the form

$$I(t) = A + B_1 exp\frac{-t}{\tau_{f1}} + B_2 exp\frac{-t}{\tau_{f2}} + B_3 exp\frac{-t}{\tau_{f3}}$$

Eq. 3

where the absolute lifetime can be expressed as

$$<\tau> = \frac{\sum B_i \tau_i^2}{\sum B_i \tau_i}$$

Eq. 4

Utilising both techniques (I_A/I_D, $E_{D\tau}$) is advantageous as one measures total fluorescence yield of the system, whilst the second gives spatial information about the relative position of labels (under a 10 nm separation limit), and they report on independent kinetic processes from the absorption / emission excited states.

Static Energy Transfer

Static energy transfer measurements were carried out following excitation of the polymer at 295 nm. AMMA does not adsorb at 295 nm so that in the absence of the fluorescence donor, ACE, the fluorophore AMMA will not fluoresce. Therefore, emission at 420 nm, from AMMA, is only possible via the FRET mechanism. Example emission spectra of HB-P(NIPAM-co-ACE) and HB-P(NIPAM-co-ACE-co-AMMA) are shown in Fig. 2. Distinct emission peaks corresponding to the donor (340 nm) and acceptor (420 nm) wavelengths can be identified. The copolymerised ACE label is not a solvatochromic dye and it has fixed absorbance and emission at a range of pH and temperatures. However it was found that the emission intensity of the ACE label decreased as the temperature was increased and the highly branched polymer was driven through the LCST. This effect was not observed for similar linear polymers (see electronic supporting information)[42-44]. It is likely that the effect is due to the highly branched architecture, which brings multiple segments into closer proximity than in the linear analogues. The data indicated that the ACE labels were being brought close enough together to be self-quenched. However despite this potential interference by comparing the spectra, in Fig 4b, at 10 and 50 °C it was possible to discern the transfer of energy to the acceptor label; the intensity of the AMMA increased as the polymer collapsed across the LCST.
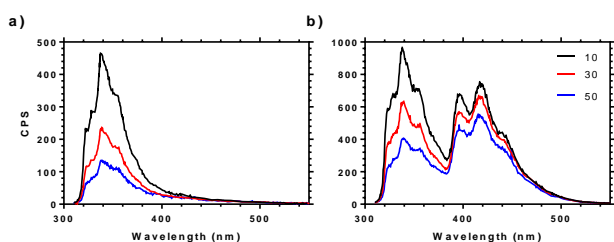

Fig. 2 - Emission Spectra of highly-branched a) HB-P(NIPAM-co-ACE) and b) HB-P(NIPAM-co-ACE-co-AMMA) polymers at pH 6, 10 (black), 30 (red) and 50 (blue) °C.

These measurements were repeated for both acid and trihistidine functionalised polymers, and the I_A/I_D was calculated for each (Fig. 3). Overall I_A/I_D was greater in the carboxylic acid functional polymers than in the His-His-His polymers and in each polymer the highest values of I_A/I_D were observed at pH 2. In the carboxylate-functional polymers there was a step change in I_A/I_D around the LCST. Interestingly, although the turbimetric data showed that there was no cloud point in the polymer at pH 11 the data did exhibit a step change and I_A/I_D continued to increase above this step change. The data from the trihistidine functional polymers was different, showing a gradual increase in I_A/I_D as the temperature was increased. These data indicated that the nature of the end groups had a large effect on the swelling of the polymer coil; at all pH and temperatures (above and below the LCST) I_A/I_D was greater for the carboxylate-functional polymer than the His-His-His polymer.
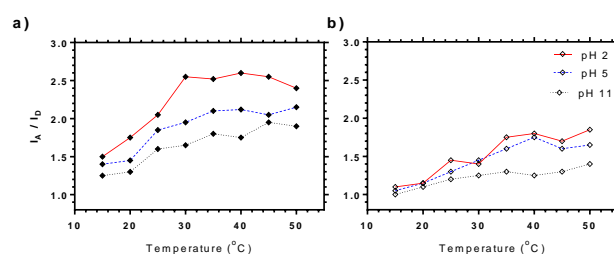

Fig. 3 - Change in fluorescence peak ratio in Highly-branched Polymers as a Function of Temperature and pH for a) 4-cyanovaleric acid ends (◆) and b) trihistidine ended polymers (◇), at pH 2 (——), 5.5 (– – –) and 11 (-----).

Dynamic energy transfer measurements

Dynamic energy transfer measurements, which additionally record quenching by non-radiative effects, were carried out on these samples. The fluorescence lifetime of the ACE donor label was recorded over a range of temperatures and pH.

P(NIPAM-co-ACE) undergoes a fast transition between a swollen and desolvated structure, with the transition occurring across the LCST over only a few °C[12]. In the singly (ACE) labelled system self-quenching was increased (the lifetime reduced) by the collapse of the solvated polymer to a globule. However branched polymers undergo a more gradual collapse. The lifetime of the ACE label in highly branched polymers was compared at various temperatures both in the absence and presence of AMMA. The lifetime of singly and doubly labelled polymers are shown in Fig. 4 for both the carboxylate and His-His-His functional materials. For all polymers the fluorescence lifetime of the ACE label gradually became shorter as the temperature of the solution was increased. This was evidence of a change in the local environment of the labels across the polymer LCST, and the highly branched polymers showed increased quenching compared to similar linear polymers[12].
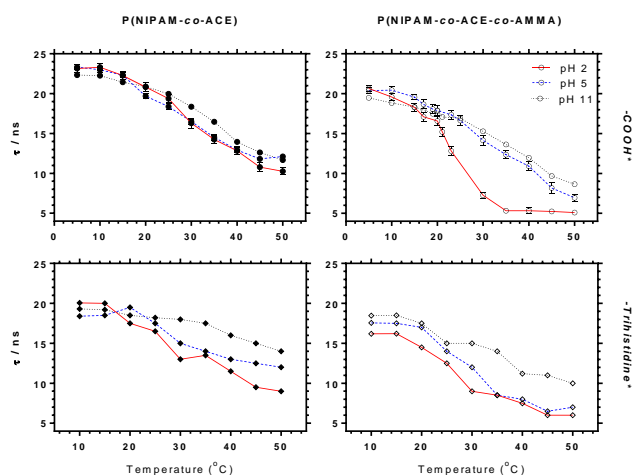
Fig. 4 - Fluorescence lifetimes for highly branched acid (top, ● / ○) and trihistidine (bottom, ◆ / ◇) functionalised Polymers. Filled (left) = HB-P(NIPAM-*co*-ACE), Empty (right) = HB-(PNIPAM-*co*-ACE-*co*-AMMA), at pH 2 (———), 5.5 (– – –) and 11 (-----).

In the acid polymer single label system only small changes were observed with pH, whilst the trihistidine functional polymer responded to a greater degree. Again this indicated that the ionised chain ends were swelling the globule and preventing effective clustering. On addition of the second label (AMMA) the largest response was seen for the acid-end material at pH 2 (protonated) which has both the highest and lowest observed lifetime (τ = 22.6 − 5.1 ns) suggesting that whilst it initially exists in an expanded form as this polymer collapses the labels are brought closest together when the polymer chain ends are not deprotonated. This quenching by the AMMA label is not as apparent at higher pH suggesting that the negative charges on the chain end are preventing effective collapse. Furthermore the trihistidine chain end material, which is charged at all pHs studied, exhibits a more gradual increase in quenching.

Figure 5 shows that the inter-label distances calculated from Eq 3. These calculations allow a direct estimation of changes in swelling of the coil. In the carboxylate polymer there is little change in inter-label distances between pH 11 and 5.5 up to 40 ° C. At pH 11 there was no evidence for a cloud point in the turbimetric data but there was small step change in the inter-label distance at 40 ° C. In the turbimetric data a clear cloud point occurred at 40 ° C at pH 5.5 and the data in Figure 7 showed that the cloud point was associated with a step change decrease in the inter-label distance. At pH 2 a step change in the non-ionised polymer was observed showing a sharp decrease in inter-label distance. Interestingly at 50 ° C the inter-label distances at pH 2 and 5.5 are similar and this indicates that the globular state is similar at these two pHs despite the different charge states.

The trihistidine polymer behaved differently. Sharp decreases in inter-label distances were observed at both pH 11 and 5.5. This polymer at pH 11 did not exhibit a cloud point. However a clear step decrease in the interlabel distance was observed

with onset at approximately 18 °C. At pH 5.5 the onset of this transition was also at approximately 18 °C, which was lower than the cloud point at this pH (25 °C). At pH 2 the change in inter-label distance was more gradual and the onset was at a higher temperature (20 °C). The cloud point at pH 2 was also higher that at pH 5.5 for this polymer. As in the carboxylate polymers the final inter-label distance was similar at each pH at 50 °C.
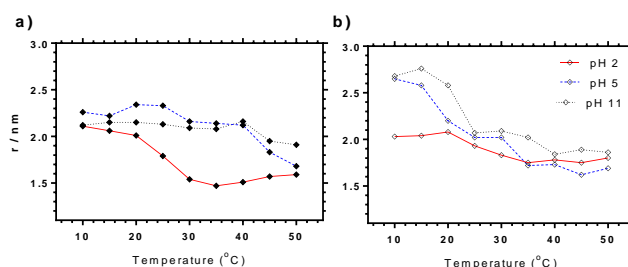

Fig. 5 - Inter-label distances (r) for a) 4-cyanovaleric acid ends (◆) and b) trihistidine (◇) chain end polymers as a function of temperature at pH 2 (———), 5.5 (– – –) and 11 (-----).

The particle sizes of the aggregates are shown in Table 1. The data show that above the LCST each of the polymers formed colloidal dispersions. The data show clearly that the ionisation of the carboxylate increased the particle size of the aggregates above the LCST; the effect is a consequence of increased osmotic swelling as counter ions are associated with the ionised chain ends. The same osmotic swelling effect is observed in the polymer with the trihistidine end group. In this case ionisation occurs at all pH so that a change in particle diameter with pH was not observed.

Table. 1 Particle diameter of aggregates determined at 50 °C (PALS)

pH	HB-PNPAM-COOH/nm	HB-PNIPAM-His-His-His/nm
2	96	163
5.5	140	170
11	156	157

Discussion

This work demonstrates that for highly branched polymers charged end groups alter the solution properties of the polymer. A carboxylate-functional HB-PNIPAM was compared to a polymer with chain ends composed of a trihistidine peptide linked be the N terminus to the polymer and with a free carboxylate group. Turbimetric determination of the LCST[40] showed that both polymers behaved very differently as the pH was altered and more detailed information was obtained by considering the inter-label distances of fluorescent donor and acceptor labels.

The solution behaviour of linear PNIPAM materials can be modified by copolymerisation and modification results in loss of the cloud point behaviour at pH above the pK$_a$.[45] Previously

we showed that a similar effect was observed in these branched polymers with carboxylic end groups at pH 11; i.e. both cloud points indicated that the coil-to-globule transition did not occur up to 50 ° C. However, in this work consideration of the inter-label distances in FRET measurements showed that the polymers did pass through a coil to globule transition. An important finding in this work was that addition of the trihistidine group at the chain ends provided a hydrophobic moiety that shifted the LCST at pH 11 in these experiments. In many systems the addition of segments other than PNIPAM, as blocks or grafts, does not affect the LCST of PNIPAM segments. In this respect the addition of a trihistidine segments at the chain ends would not be expected to influence the LCST in the manner that was observed. At pH 2 the trihistidine groups were highly ionised and the increased osmotic potential derived from the counter ions clearly had a small effect on the temperature of the LCST but a larger effect was observed on the nature of the coil; the decrease in inter-label distance indicated that the coil was more compact than when the carboxylate was ionised even though the trihistidine group provided three positive charges in the dominant ionised species. At all pH the trihistidine polymer was ionised but the carboxylate polymer was unionised at pH 2. In the globular state measurements of inter-label distance, at 50 ° C, and particle diameters obtained by light scattering indicated that that ionisation had the effect of swelling the globular aggregates; in the carboxylate polymer at pH 2 the inter-label distance was lower than at pH 5.5 or 11 and the particle diameter was smaller than in the ionised states at pH 5.5 and 11. On the other hand the trihistidine polymer was ionised at all pH and the swelling of the globular state produces colloidal particles in which the inter-label distance and particle diameter was relatively unchanged with pH.

Conclusion

Modification of HB-PNIPAM end groups to give either carboxylate end groups or trihistidine with carboxylate end groups alters the critical solution behaviour. Further work with other peptide end groups can be used to finely tune the behaviour of PNIPAM. The ionisation of the end groups increases the temperature of the LCST and increases swelling of the globular state.

Experimental

Instrumentation

Fluorescence steady state measurements were made on a Perkin-Elmer LS-50 Luminescence Spectrometer with a xenon discharge lamp and Monk-Gillieson type monochromators with a wavelength accuracy of ±1.0 nm. A Haake K20 water bath and DC30 temperature controller were used to vary the temperature of the sample. A concentration of 10^{-3} wt% in buffer or water was used for all polymers. Emission and excitation slits were set between 2.5 and 5 nm depending

upon the sample and ten accumulation scans were made for each reading to ensure smooth spectra. The data was collected using FL Winlab software. The ACE label was excited at 290 nm with an emission range of 310-550 nm to show emission from both ACE and AMMA labels in doubly labelled samples. The AMMA label was excited at 370 nm with an emission range of 380-550 nm. Excitation scans were carried out with emission wavelengths of 340 and 420 nm for ACE and AMMA respectively with excitation wavelengths of 200-330 and 200-410 nm. Lifetime measurements were made using a System 5000 monochromator with a Pulsed Diode Nano LED source. The temperature of the sample was controlled using a Haake D water bath and a Haake G1 temperature controller. Sample concentrations were 10^{-3} wt% for all polymer solutions with 30,000 counts being collected for each sample. A Ludox prompt was run after each sample to take into account scattered light. Data was analysed using IBH version 4.2 software. The ACE label was excited at 295 nm and the emission detected at 340 nm. The AMMA label was excited at 370 nm and the emission detected at 420 nm.

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