pH-responsive chiral nanostructures

Jianzhong Du,[a,b] Helen Willcock,[a] Nga Sze Ieong[a] and Rachel K. O'Reilly[a,]*

[a]University of Warwick, Department of Chemistry, Gibbet Hill Road, Coventry, CV7 4AL, UK. Fax: +44 (024) 765 24112; Tel: +44 (0)247 652 3236; E-mail: R.K.O-Reilly@warwick.ac.uk.

[b]Current address: School of Materials Science and Engineering, Tongji University, 1239 Siping Road, Shanghai, China.

Abstract

There is great current interest in the design of robust synthetic polymers for the preparation of novel functional, well-defined, biocompatible and tailorable materials for a range of applications. In this work we have used reversible addition fragmentation chain transfer (RAFT) polymerization to prepare chiral and responsive amphiphilic block copolymers (based on polyphenylalanine acrylamide) which can be assembled at different pHs to form well-defined nanostructures, whose morphology and size were explored using TEM, DLS and SLS measurements and stability by fluorescence and NMR spectroscopy.

The application of these chiral and responsive nanostructures in the resolution of hydrophilic racemic amino acids has also been explored.

Introduction

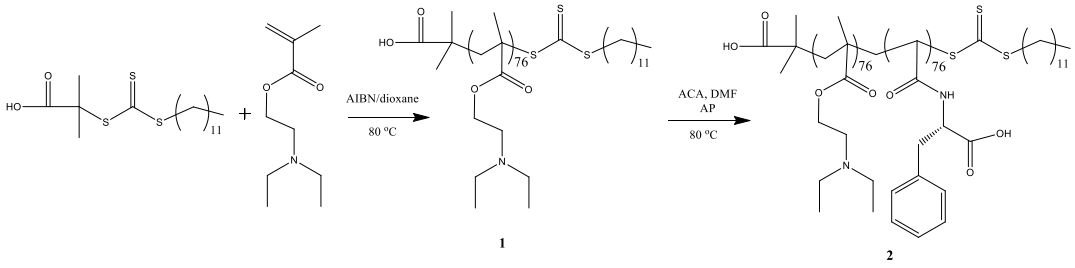
The preparation of responsive and functional nanostructures for applications as smart materials is an area of great interest in polymer science.(1-4) In particular recent advances in controlled radical polymerization (CRP) techniques have allowed for the preparation of a wide range of well-defined and functional polymers, thus affording readily tunable structural and chemical features.(5-10) Of particular interest is the development of responsive or smart materials that respond (for example through a change in solubility) to external stimuli.(11-13) There are a number of stimuli which can be used to afford a change in polymer properties including most commonly temperature, light, oxidation and pH.(14-18) A wide range of new polymers have been prepared and explored as responsive materials with particular interest in the development of block copolymers which display dual responsive properties.(19) Of further interest are schizophrenic systems which were first reported by Armes and co-workers.(20, 21) These systems offer an additional level of control and allow for the inversion of nanostructure domains and a change in overall nanostructure properties. For example, in 2009 we reported the preparation of a schizophrenic responsive diblock whose surface charge could be inverted by changing the pH of the solution.(22) It was proposed that this feature could be key in the preparation of functional nanostructures for selective encapsulation, interaction and release. Furthermore the introduction of additional functionality into these systems is of great interest as is the preparation of novel solution assembly morphologies to allow for access to highly complex nanostructures for utilization in a range of applications as nanoreactors and delivery vehicles. In recent years there has

been increasing interest in the development of new polymeric materials which can undergo selective assembly to form non-spherical morphologies including cylinders, toroids and discs.(23) The application of these non-spherical morphologies has not yet been fully realized, however the potential of cylindrical structures in advanced drug delivery applications has recently been reported. (24)

The groups of Endo, Mori and coworkers have pioneered the area of poly(amino acids) over the last 15 years.(25) This group have utilized both conventional radical techniques and also more recently CRP for the synthesis of a range of poly(amino acids). Compared to the sequential amidation reactions which are used in polypeptide formation often only the amino group is utilized in the coupling to the vinyl functionality leaving the acid (or often protected acid) group available along the polymer side chain. This can impart important solubility characteristics to the polymer and also provide a useful handle for further functionalization. We are interested in the preparation and properties of vinyl amino acid polymers which can possess a number of key features including chirality and catalytic activity which can exploited in the preparation of novel functional nanostructures. Furthermore we are interested in using readily accessible controlled radical techniques for the preparation of multiblocks and assemblies of these amino acid based polymers.(26-29) In particular our interest in this class of polymers was based on the elegant work of Endo and Sanda who have been demonstrated their temperature and pH responsivity.(26, 30) In this work we hoped to explore the further application of these chiral and pH responsive polymers for pH controlled self assembly to afford non-spherical responsive nanostructures that may find application as media for chiral separations.

Results and Discussion

To prepare poly (vinyl amino acid) block copolymers we utilized reversible addition fragmentation chain transfer (RAFT) polymerization given its tolerance to carboxylic acid functionality, ready end group modification and also precedent in the direct polymerization of acid functionalized amino acid monomers.(31) Hence, using established RAFT polymerization methods we prepared a chiral diblock copolymer by first polymerizing diaminoethyl methacrylate (DEA) using S'-1-dodecyl-(S')-(α,α'-dimethyl-α''-acetic acid) trithiocarbonate (DDMAT), as an initiator to afford PDEA, **1** (M_n = 14.1 kDa, M_w/M_n = 1.34). This macro-CTA could then be extended using L-phenylalanine acrylamide (PAP) in DMF (Scheme 1) using 4,4'-azobis(4-cyanovaleric acid) (ACA) as an initiator to afford block copolymer **2**. This initiator was chosen given its slower rate of decomposition compared to AIBN and this was found to allow for more efficient block copolymer synthesis. This diblock was characterized by both GPC and NMR analysis (M_n (GPC, THF) = 32.8 kDa, M_w/M_n = 1.19 and M_n (NMR) = 30.7 kDa) which confirm the preparation of the desired block copolymer, $PDEA_{76}$-b-PAP_{76}, with intact end groups.



Scheme 1 Synthesis of amphiphilic diblock **2** using RAFT polymerization.

This diblock could be directly dissolved (at 0.2 mg/ml) at both low (pH = 2) and high pH (pH = 12), however at neutral pH it was not fully soluble, to afford well-defined nanostructures. The selective solubility of both blocks at different pHs was confirmed by ^{1}H NMR spectroscopy (Figure 1) in a range of solvents. From Figure 1 it was confirmed as expected at low pH the PAP block will be hydrophobic (as determined by the absence of characteristic aromatic signals in the ^{1}H NMR spectrum) and the PDEA

block will be protonated and hence be hydrophilic. Given these observations at low pH and given the lengths of both blocks (with a hydrophilic weight fraction of 46%) a spherical or cylindrical morphology would be predicted based on established self assembly principles. At high pH given the length of both blocks and a hydrophilic weight fraction of 54% a spherical morphology would be predicted. However, we were interested to explore if the hydrophobic RAFT end group affected the assembly of these diblocks at different pH.

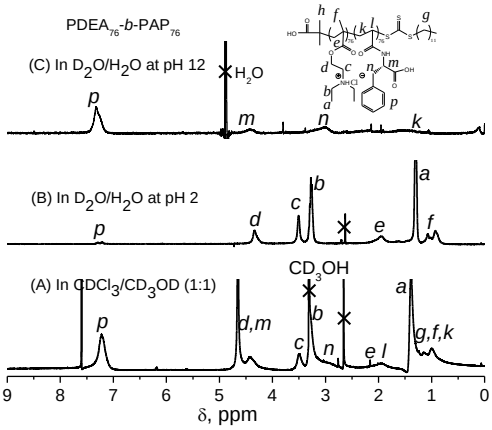


Fig. 1 . ^{1}H NMR spectra of $PDEA_{76}$-b-PAP_{76}, **2**: (A) in $CDCl_3/CD_3OD$ (1:1), a good solvent mixture for both blocks; (B) in D_2O/H_2O (1:9) at pH 2, the peaks from PDEA are visible whereas peaks assigned to PAP (such as p and n), and peaks assigned to DDMAT (g) are attenuated; (C) in D_2O/H_2O (1:9) at pH 12, peaks from PAP (such as p and n) are visible whereas peaks from PDEA (a, b, d, e, and f) are attenuated.

Dynamic light scattering (DLS) analysis indicated large relatively well-defined particles ca. 197 nm (PD 0.119), at low pH (solution concentration 0.2 mg/ml) which could not be spherical micelles based on the maximum stretching of the chains, hence TEM analysis was used to confirm the morphology of these nanostructures. Using TEM analysis (without staining) a cylindrical morphology was

confirmed, with diameters around 40 nm (Figure 2). Furthermore static light scattering (SLS) in combination with DLS data, using Zimm plot analysis confirmed a rod-like morphology with R_g/R_h = 1.93 which is close to the theoretical value of 2. At low pH we propose the PDEA forms the coronal domain and is protonated and this was confirmed by zeta potential analysis (+ 33 mV). From previous work we are confident that the trithiocarbonate hydrophobic end group does not undergo hydrolysis and is intact at the ω-end of the chains. Attempts to assemble this polymer at higher concentrations afforded similar results although the distribution of sizes as observed by DLS increased upon increasing polymer concentration.

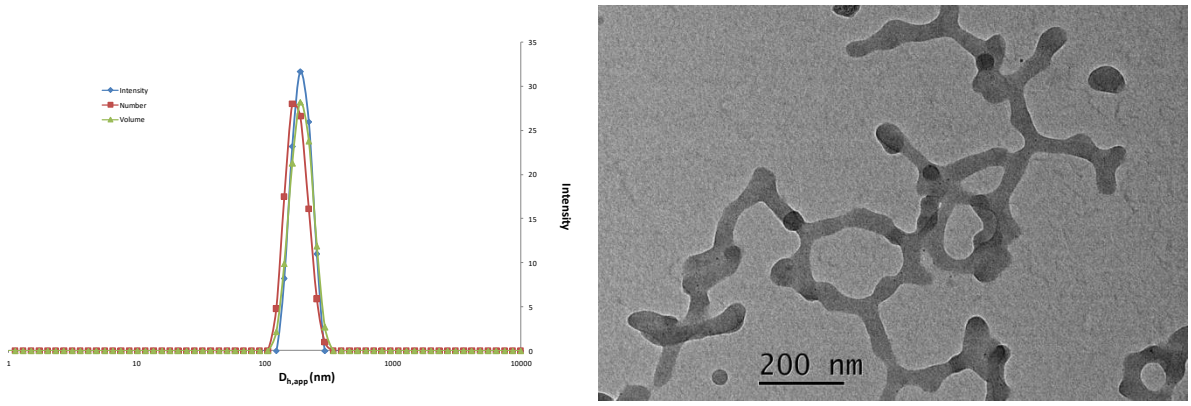


Fig. 2 DLS (LHS) and TEM (RHS) analysis (with no staining) of diblock **2** self-assembled at 0.2 mg/ml at low pH.

The assembly of this polymer **2** at high pH was also explored (at 0.2 mg/ml) and by DLS analysis nanostructures of ca. 57 nm (PD 0.219) were observed. Furthermore zeta potential analysis of this solution confirmed a negative surface charge (-40 mV) indicating a PAP corona, as expected based on the hydrophilicity of the PAP block. Further analysis by TEM (staining with uranyl acetate) indicated relatively ill-defined spherical particles were formed which agrees with the predicted structure based on

ca. 54 % hydrophilic weight fraction. Further analysis by cryo-TEM confirmed that spherical structures of ca. 26 nm were formed, which appeared to be micelles, although were rather disperse (Figure 3). We also investigated the angular dependence of the aggregate size of both solutions by DLS and as expected for non-spherical particles at low pH a strong dependence of diffusion coefficient was observed at a range of q values. Interestingly at high pH some dependence was observed with indicates that these particles are also anisotropic (Figure 4). SLS analysis of this assembly at high pH were inconclusive due to large errors in the Zimm plot analysis as a result of the broad dispersity of the assembly.

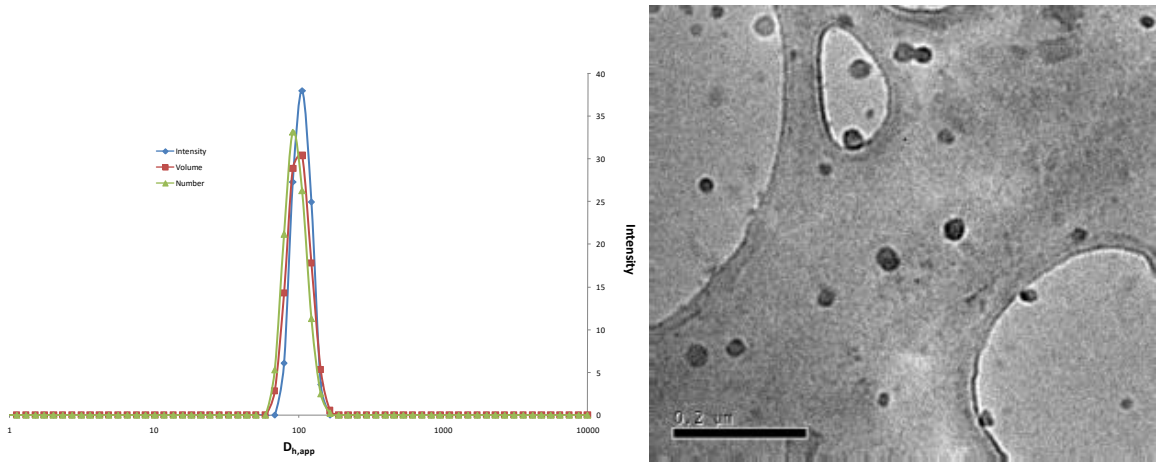


Fig. 3 DLS (LHS) and cryo-TEM (RHS) analysis of diblock **2** self-assembled at 0.2 mg/ml high pH.

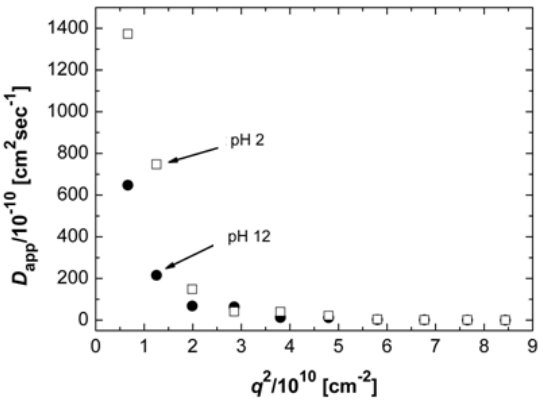


Fig. 4 Angular dependent size for polymer **2** assembled at pH =2 and pH = 12.

We also explored the solution properties of these polymers and determined the critical micelle concentration (cmc) of both nanostructures using *N*-phenyl-1-naphthylamine (PNA) as a probe. PNA has recently been proposed to be an improved probe for the cmc determination of amphiphiles given its robust nature and also stronger shift in absorbance compared to pyrene.(32) From this it was clear that the assembly at low pH had an order of magnitude higher cmc value compared to the assembly at high pH (3.16×10^{-3} compared to 1.78×10^{-2} g/L), although both values were close to reported cmc values for diblock copolymer assemblies. This provides an interesting responsive feature of these systems as altering the pH changes dramatically changes the cmc of the aggregate hence allowing for the tuned dissolution and reassembly at different pH and also concentrations. Indeed we observed by DLS after extended periods of time the samples prepared at low pH were stable, however at high pH the assemblies were not.

We explored increasing the solution pH of the aggregates formed at pH 2 through to pH 14 and through to pH 2 again, to examine the reversibility of the transition. We observed upon increasing the pH the size decreased slightly (to ca. 171 nm at pH 6) and at ca. pH 7-9 precipitation of the solution occurred, which prevented facile cycling between extremes of pH. However, the aggregates could be reformed by further increasing the solution pH, however a broad distribution of particles (PD ca. 0.41) were obtained, indicating less well-defined aggregates were formed.

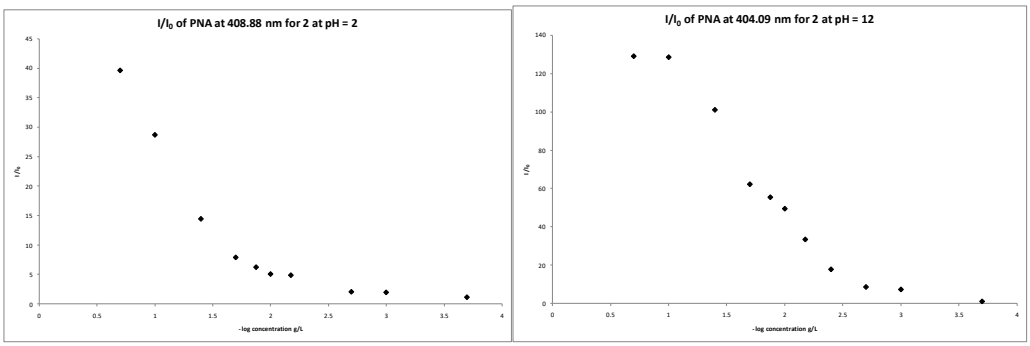


Fig 5 Plots to determine the cmc for polymer **2** assembled at pH =2 (LHS) and pH = 12 (RHS).

We propose at high pH it is known that the RAFT trithiocarbonate end group can be readily hydrolyzed and hence the assembly at high pH (and 0.2 mg/ml) was repeated with polymer **2** which had undergone end group removal using established methods.(33) Using this new polymer **2'** we observed very similar sized nanostructures, at low pH by DLS (174 nm, PD 0.126) as previously observed for polymer **2** which contained the RAFT end group. However, assembly of **2'** at high pH afforded much larger assemblies by DLS analysis (104 nm, PD 0.114). Furthermore upon ^{1}H NMR analysis of the polymer **2** after assembly at high pH after 1 month the end groups were no longer visible indicating that under these conditions the end groups are not stable.

We have recently reported that the phenylalanine methyl ester amino acid polymer can be utilized as a substrate for chiral enhancement due to the increased interaction between opposite enantiomers.(34) In this work we wanted to explore this interaction further using our new water soluble phenylalanine polymer (PAP). We proposed that at low pH when the PAP block is hydrophobic, there should be a stronger interaction with the opposite enantiomer compared to at high pH when the polymer is hydrophilic. Interestingly in this system we observed little recognition between enantiomeric pairs (L-PAP in our nanostructure and D-PAP in solution) compared to our previously reported system.

This may be in part due to the deprotonation of the PAP at low pH which prevent recognition and also highlights that a hydrophobic environment appears to be key to exploit recognition in the particles.

Experimental Section

Materials

All the reagents were purchased from Aldrich and used as received. Dialysis tubing was purchased from Medicell International Ltd with a molecular weight cut-off of 1 kDa. DDMAT (S'-1-dodecyl-(S')-(α,α'-dimethyl-α''-acetic acid) trithiocarbonate) was synthesized according to the reported procedures.(35)

Characterization

The molecular weight distributions of polymers were assessed at 40 °C using a Polymer Laboratories PL-GPC50 Integrated GPC system equipped with a Polymer Laboratories micro-volume double piston pump (approx. 10 µL per stoke), a PLgel 5 µm MIXED-D column (300 × 7.5 mm), and a refractive index detector. The calibration was carried out using ten polystyrene standards with M_p values ranging from 580 to 377,400 Da. The eluent was THF containing 2.0% (v/v) TEA and 0.05% (w/v) BHT and the flow rate was 1.0 mL/min. The data were processed using Cirrus GPC offline GPC/SEC software. ^{1}H NMR spectra were recorded using a Bruker AV 400 (400 MHz) spectrometer at ambient temperature using either $CDCl_3$, CD_3OD, $CD_3OD/CDCl_3$, D_2O/H_2O or D_2O as solvents. TEM images were obtained using a JEOL electron microscope operating at 200 kV equipped with a LaB6 gun and a Gatan digital camera. Freshly prepared aqueous 1.0 % uranyl acetate solution was used to stain the nanostructures. The averaged size of vesicles was calculated based on examining at least fifty particles from at least six different TEM images. Cryo-TEM was performed on a Jeol 2010F TEM (200 kV FEG) with 4K Gatan Ultrascan camera

and cryo capability. Zeta potential and DLS studies were conducted at 25°C using a Zetasizer Nano series instrument (Malvern Instruments). DLS studies of aqueous aggregates were conducted over a range of solution pHs at a fixed scattering angle of 173°. The data were processed by Cumulants analysis of the experimental correlation function and aggregate diameters were calculated from the computed diffusion coefficients using the Stokes-Einstein equation. Each reported measurement was the average of three runs. SLS studies were performed at 25°C using a Malvern Autosizer 4800 Instrument. To produce the Zimm plot 20 angles between 30 to 150 ^{o}C and 5 different concentrations of micelle solution were scanned and included in the calculation.

Synthesis of CTA and Polymers

Polymer **1** ($PDEA_{76}$) was synthesized to afford the macro-CTA for polymer **2**. In a typical RAFT protocol, a flask with a magnetic stirrer bar and a rubber septum was charged with AIBN radical initiator (3.0 mg; 3.3×10^{-2} mmol), DDMAT CTA (94.0 mg; 0.162 mmol), DEA monomer (3.04 g; 16.2 mmol) and dioxane (8.12 mL). This solution was deoxygenated using a N_2 purge for 30 minutes before heating at 80 ^{o}C under a nitrogen atmosphere. The [DEA]: [DDMAT]: [AIBN] relative molar ratios were 100:1:0.2. After 4.5 h the monomer conversion was calculated to be 76% by ^{1}H NMR spectroscopy. The final polymerization solution was diluted with acetone and 0.01 M HCl aqueous solution and then purified by dialysis against water at pH 2-4. A fine white powder was obtained after freeze-drying. ^{1}H NMR spectra are shown in the manuscript (M_n (NMR) = 14.7 kDa). THF GPC: M_n = 16.5 kDa; M_w/M_n = 1.7.

In a typical RAFT protocol, a flask with a magnetic stirrer bar and a rubber septum was charged with ACA radical initiator (2 mg; 7.0×10^{-3} mmol), protonated $PDEA_{76}$ macro-CTA (244 mg; 1.4×10^{-2} mmol), AP monomer (316 mg; 1.4 mmol) and DMF (8 mL). This solution was deoxygenated using a N_2

sparge for 30 minutes before heating at 80 ^{o}C under a nitrogen atmosphere. The [AP]: [PDEA76]: [ACA] relative molar ratios were 100:1:0.5. After 27 h reaction was purified by precipitation in ethyl acetate to form a white precipitate. THF GPC: M_n = 19.7 kDa; M_w/M_n = 1.27. By ^{1}H NMR, M_n = 27.1 kDa.

Preparation and characterization of aggregates

Block copolymer **2** (100 mg) was dissolved into 100 g pure water at pH 2 at 80 ^{o}C for several minutes to form aggregates directly, then stirred at room temperature. At pH 12, the solution needed to be stirred for several hours to afford a clear blue nanostructure solution. The assemblies were characterized by ^{1}H NMR spectroscopy. After four months, the initial nanostructures still exists at pH 2 but do not exist at pH 12, as a result of different stabilities of trithiocarbonate groups in the vesicle membrane at low (stable) and high pH (unstable). The resultant aggregates were characterized by DLS at different pH values and the correlation functions fit well with the Cumulants analysis and the intensity-averaged vesicle size distributions are reasonably consistent with number and volume averaged size distributions. The resultant vesicles were stained and viewed by TEM and cryo TEM.

Chiral resolution studies

Using the procedures described previously,(36) we explored the chiral recognition of our phenylalanine nanostructures at both low and high pH. The nanostructures (2 ml of solution of concentration 0. 2 mg/ml) were incubated overnight with D-phenylalanine. After this time the micelle solution was transferred to an ultrafiltration cell (MWCO 3.5 kDa) and *ca*. 0.5 mL of solute was collected. The

resulting solutions were analyzed by polarimetry and the reaction had a specific rotation that was close to zero which corresponds to the original racemic solution.

Conclusions

We have shown a new class of amphiphilic and pH responsive polymers based on chiral vinyl amino acids, prepared by RAFT polymerization which can assemble into well-defined nanostructures. We have highlighted the versatility of this diblock system towards the assembly of non-spherical morphologies and also the importance of end group on the resultant morphology. Disappointingly these chiral nanostructures did not display chiral resolution abilities, however further work is expanding the scope of these systems towards the design of functional and responsive nanostructures.

Acknowledgements

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