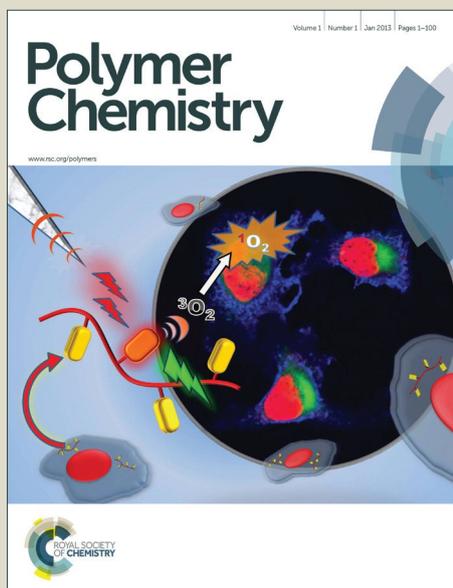


Polymer Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Zwitterionic Amphiphilic Homopolymer Assemblies

Rajasekhar. R. Ramireddy,[†] P. Prasad,[†] A. Finne, and S. Thayumanavan*

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Zwitterionic amphiphilic homopolymers can be conveniently prepared in one-pot using activated ester-based polymer precursors. We show that these zwitterionic polymers can (i) spontaneously self-assemble to form micelle-like and inverse micelle-like assemblies depending on the solvent environment; (ii) act as hydrophilic and hydrophobic nanocontainers in apolar and polar solvents respectively; (iii) undergo pH-responsive surface charge and size variations; (iv) exhibit least cytotoxicity compared to structurally analogous amphiphilic homopolymers.

Introduction

Supramolecular nanoassemblies capable of reducing non-specific interactions with complex biological milieu have attracted tremendous research interest, because of their potential in several nanomedicine applications.¹ Hence, recent efforts to design and develop versatile amphiphilic polymers that form stable nano-sized aggregates with effective non-fouling characteristics and hydrophobic guest encapsulation capabilities are on the rise.² Zwitterionic materials³ are replacing PEG and hydroxyl functional materials⁴ because of their high water solubility, charge neutrality and their ability to reduce non-specific interactions with biological media such as serum and blood platelets, attributed to electrostatically induced hydration layer formation.⁵ Incorporation of zwitterionic moieties as hydrophilic components in amphiphilic macromolecules could lead to a new class of nanoassemblies with anti-biofouling characteristics. In literature, there have been few reports on zwitterionic amphiphilic block copolymers that form self-assembled aggregates, because of the mutual immiscibility of the hydrophilic and hydrophobic blocks in the bulk solvent.^{2d,6} A useful complement to amphiphilic block copolymers involves random copolymers and homopolymers, because of the synthetically and functionally more accessible features.^{7,8}

In this article, we present a one-pot synthesis of acrylamide-based zwitterionic amphiphilic homopolymers with hydrophilic and hydrophobic components placed orthogonally in every repeat unit (Fig 1), where the hydrophilic part of the amphiphilic polymer is based on a zwitterionic moiety. Amphiphilic homopolymers often possess charged functionalities, such as the cationic tertiary ammonium or the anionic carboxylate moieties.^{7,9} Because of their ionic nature, the supramolecular assemblies from these polymers are prone to non-specific interactions with biological macromolecules. In fact, we have utilized this feature of the amphiphilic homopolymers in protein sensing and peptide detection.^{9,10} In this article, we introduce glycinebetaines as charge-

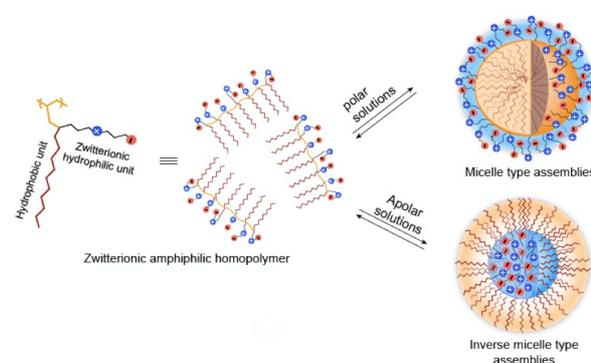


Figure 1. Schematic representation of solvent dependent self-assembled aggregate formation of zwitterionic amphiphilic polymers

neutral, zwitterionic, hydrophilic moieties in amphiphilic homopolymers. Further, we evaluate the self-assembly characteristics, pH-sensitivity, and the biocompatibility of the polymers relative to the corresponding polyelectrolyte nanoassemblies.

Experimental

Synthesis of zwitterionic and control polymers

Details on the syntheses and characterization of the zwitterionic homopolymer, and the corresponding anionic, cationic, and the charge-neutral oligoethyleneglycol polymers are provided in Electronic Supplementary Information (ESI).

Dynamic light scattering

The size distribution of the assemblies was determined by Nano series Nano-ZS (Malvern Instrument) Zetasizer. In a typical experiment, a stock solution of 0.1mg/mL polymer solution was

^a Department of Chemistry, University of Massachusetts, Amherst, MA 01003.

^b † Electronic Supplementary Information (ESI) available: [experimental and characterization details]. See DOI: 10.1039/x0xx00000x

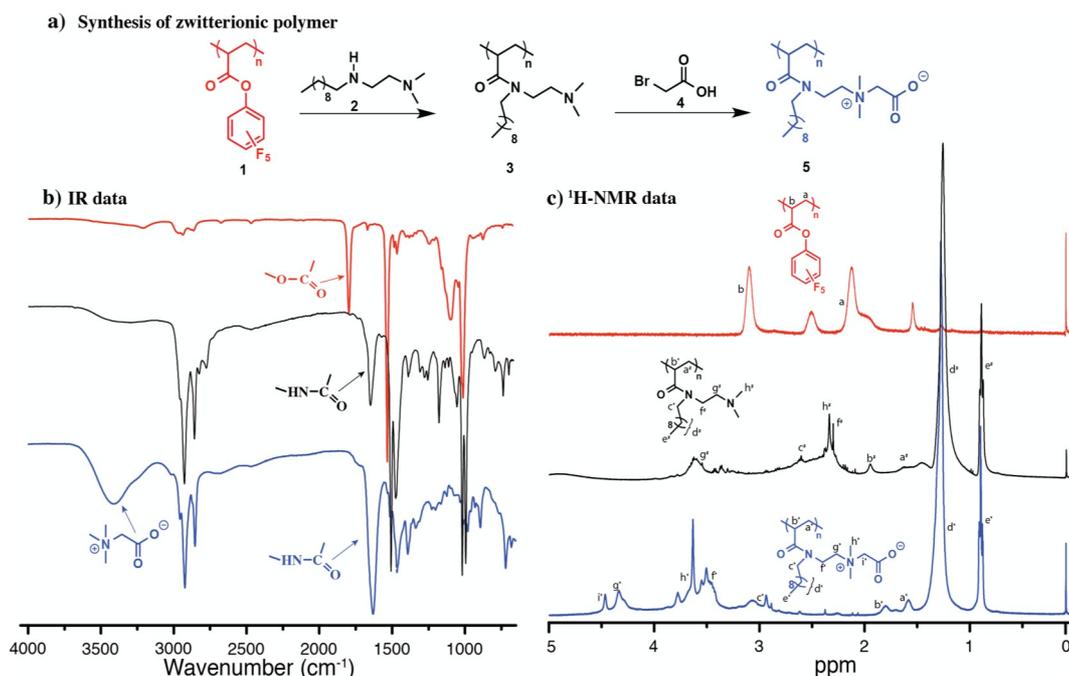


Figure 2. a) Conversion of reactive polymer to zwitterionic polymer; b) IR spectral data further confirms the zwitterionic polymer formation c) $^1\text{H-NMR}$ data confirms the conversion of reactive polymer to zwitterionic polymer.

prepared in milli-Q water by sonicating the solution for 2 hours at room temperature. The resultant solution was then filtered through 0.22 μm filters and then the size of the micellar solutions were measured by DLS.

Spectroscopic measurements

Fluorescence spectra were recorded on a JASCO (FP-6500) spectrofluorimeter, using 1 mL disposable fluorescence cuvettes. The emission spectra for Nile red and Rose Bengal were recorded by exciting at 550 nm and 525 nm respectively, with the excitation and emission bandwidths set at 3 and 5 nm respectively.

TEM measurements

TEM measurements were performed using a TEM JEOL 2000F. Samples were prepared by dipping copper EM grids in aqueous solution of mM concentrations and the excess of solvent was removed by placing the tissue in the bottom of the EM grid.

Cell culture

HeLa cells and HEK 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained at 37 $^\circ\text{C}$ and 5% CO_2 .

Cytotoxicity studies

In vitro cellular viability of the amphiphilic polymers was carried out using Alamar blue assay to compare the viability of zwitterionic polymer with cationic, anionic and the neutral analog of the polymer on Human Embryonic Kidney 293T cells and HeLa cells. The polymers with different concentrations in media were incubated with both the cell lines separately for 24 hours before performing the assay.

Results and Discussions

Synthesis of zwitterionic homopolymer

We first synthesized the reactive polymer, poly(pentafluorophenyl acrylate) **1**, via RAFT polymerization of pentafluorophenylacrylate¹¹ using cyanomethyl dodecyl trithiocarbonate, as the chain transfer agent (see Supporting Information for details). Polymer **1** was then treated with the secondary amine **2** in dry DCM in the presence of Hünig's base for 24 hours at room temperature. The tertiary amine moiety in the product polymer **3** was then converted to a zwitterionic moiety by treating this polymer with a slight excess of 2-bromoacetic acid (**4**). When the reaction mixture was precipitated in ethyl acetate, the zwitterionic polymer **5** was obtained as a white powder. The conversion of reactive polymer to zwitterionic polymer was confirmed by NMR and IR (Fig 2). The evolution of peaks corresponding to alkyl and zwitterionic components in the region of 1 to 5 ppm in $^1\text{H-NMR}$ (Fig 2c), and disappearance of the broad $^{19}\text{F-NMR}$ peaks corresponding to pentafluorophenyl groups of the activated ester-based precursor polymer in the region of -150 to -160 ppm (SI, Fig S1) indicates the conversion to the intermediate polymer **3**. Further, the disappearance of ester C=O stretching band corresponding to reactive polymer at 1780 cm^{-1} and evolution of amide C=O stretching band at 1640 cm^{-1} corresponding to polymer **3**. The evolution of carboxylate stretching band in the region of 3300 cm^{-1} (Fig 2b) in polymer **5** further confirms the zwitterionic polymer structure.

Self-assembly characteristics

Next, we investigated the solubility and self-assembly of **5** in aqueous phase. We were gratified to observe higher water

solubility for these polymers (>1 mg/mL) compared to structurally similar cationic or anionic polymers (~0.01 mg/mL). An attractive feature of amphiphilic homopolymers is that these are capable of providing nanoassemblies in both polar and apolar media, *i.e.* micelle-like assemblies in aqueous phase and inverse micelle-like assemblies in apolar organic solvents.^{7, 12} We were interested in investigating whether such possibility exists for the zwitterionic amphiphilic homopolymer as well. Therefore, we studied the solubility of these polymers in apolar solvents and we found these polymers to be quite soluble in apolar solvents as well. We then studied the self-assembly characteristics of these polymers in aqueous solutions by using 0.1 mg/mL solution. To assess whether these polymers self-assemble into nanostructures, we investigated these polymers using dynamic light scattering (DLS) and transmission electron microscopy (TEM). The sizes of the assemblies were found to be about 90 nm and 400 nm for assemblies formed in water and toluene respectively (Fig 3b). The sizes of these assemblies were found to be slightly smaller, when analyzed by TEM. This slight difference is understandable, as the sample is air-dried during the TEM measurements and since DLS measurement includes the hydration sphere of the assemblies. Nonetheless, TEM images suggest that these aggregates are likely to be spherical in morphology (Fig 3c,d).

If these assemblies were indeed micelle-like or inverse micelle-like from a host-guest perspective, then the hydrophobic interiors of these self-assembled aggregates in the aqueous phase should be able to encapsulate hydrophobic guest molecules. To test this possibility, we used hydrophobic small molecule Nile red as a spectroscopic probe. As such, Nile red is quite insoluble in aqueous solutions, however in the presence of polymer; it became soluble indicating its encapsulation in the hydrophobic pockets of the self-

assembled aggregates. We further confirmed the non-covalent encapsulation of Nile red *via* absorbance (SI, Fig S2) and fluorescence spectroscopy (Fig 3a). Similarly, the inverse micelle type aggregates should be able to encapsulate hydrophilic molecules such as Rose Bengal in organic solvents. Rose Bengal is insoluble in apolar solvents, but becomes soluble in apolar DCM solution, indicating the hydrophilic interior of inverse micellar assemblies (Fig 3a). To further confirm that this is indeed an encapsulation process in DCM, we compared the fluorescence of Rose Bengal inside the inverse micelle like assemblies and in water, where the concentration of the two solutions are identical (based on their solution absorbance). Although the global solution concentrations are identical in these two solutions, the local concentration of the dye inside the inverse micelles would be much higher as they are confined in these assemblies. This should manifest itself as much lower fluorescence in the inverse micellar solution, as Rose Bengal exhibits significant self-quenching properties. Overall, combination of these experiments confirms that amphiphilic zwitterionic homopolymers form micelle-like and inverse micelle-like assemblies depending on the polarity of the solvent.

Effect of pH

Next, we investigated the effect of pH on the surface charge of the polymeric assemblies.¹³ In the glycinebetaine moiety, one would expect the carboxylate moiety to be pH-sensitive as this functionality can be reversibly protonated upon reducing the pH. Although the pKa of the polycarboxylate moiety itself is around 4.5, it is interesting to investigate the effect of the proximal quaternary ammonium ion on the pKa of this functional group in the polymer. Therefore, we studied the variations in the zeta potential of the polymer assembly at different pH. We systematically varied the pH of the solution, using 0.01 M HCl or NaOH, from pH 2 to 10. We observed a gradual change in the zeta potential of the assembly from about +20 mV to about -20 mV, upon going from low pH to high pH (Fig 4a). The isoelectric point, *i.e.* the pH at which the overall surface charge of the assembly is zero, was found to be about 6.5. This is significantly higher than that observed for the carboxylate moiety by itself; the zeta potential of the carboxylate polymer **6** (one of the control ionic polymers) is close to zero at pH 2.1 (at pH below 2, the polymer precipitates out completely, SI, Fig S3a). This difference is understandable, because the quaternary ammonium moiety should stabilize the carboxylate counter ion, thus increasing the pKa of the proximal carboxylate moiety.

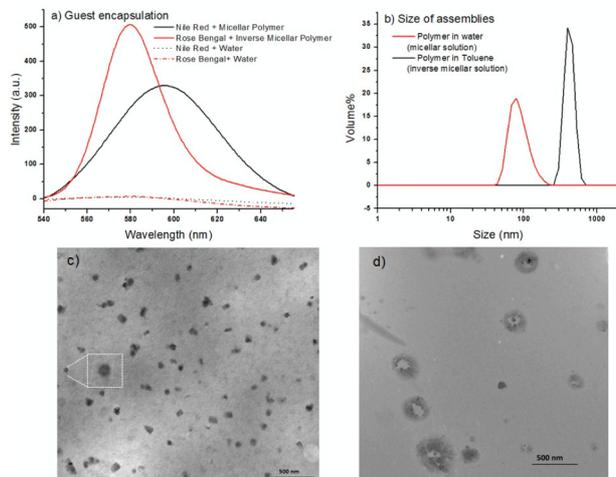


Figure 3. Self-assembly characteristics of zwitterionic polymers. a) Emission spectra of hydrophobic, Nile red ($\lambda_{\text{ex}} = 550$ nm) and hydrophilic, Rose Bengal ($\lambda_{\text{ex}} = 530$ nm) encapsulated in micellar and inverse micellar aggregates solutions. On the other hand, no fluorescence is observed in the corresponding solvents without polymers; b) Sizes of aggregates as determined by DLS; c, d) TEM images of the micellar and inverse micellar assemblies

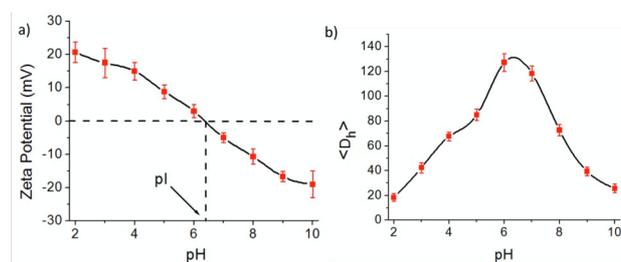


Figure 4. a) Plot of pH dependent zeta potential variations, pI = Isoelectric point at pH 6.5; b) pH dependent size variations of the micellar assemblies

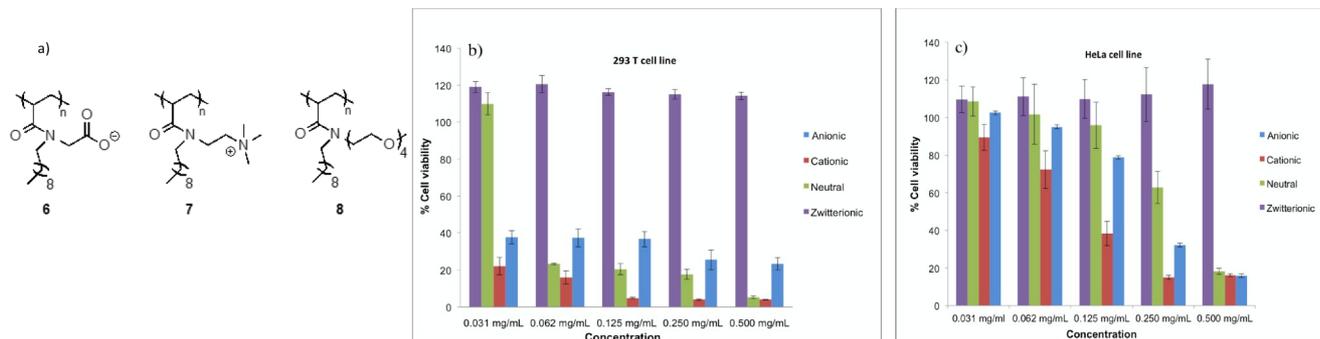


Figure 5. a) Structures of the control polymers; in vitro cellular viability of polymers on b) 293T cell line, and c) HeLa cell line.

On the other hand, this trend was surprising at pH above the isoelectric point. We anticipated that the cationic quaternary ammonium moiety could be considered to be a permanent charge and therefore at high pH (where all the carboxylates are deprotonated), there will be very little impact on the surface charge of the assembly. However, the surface charge continued to decrease. This is likely due to a stronger association of the tetraalkylammonium species with OH^- ions at high pH. To test this possibility, we investigated the pH dependence of the surface charge of cationic polyelectrolyte **7** (one of the control ionic polymers). We observed a modest decrease in zeta potential from +20 mV to +14 mV, indicating that complex with OH^- ions can play a role at high pH (SI, Fig S3b). However, the relatively modest decrease also suggests that this explanation is provisional at this time, in the absence of an alternate explanation.

We further studied the effect of pH on the size of the self-assembled aggregates. Here, we hypothesize that the nature of charge of the hydrophilic component of amphiphilic polymer would play an important role on the size of the resulting self-assembled aggregates due to electrostatics. To investigate this possibility we studied the variations in hydrodynamic size of the polymeric assemblies with changes in pH of the solution using DLS. We observed that the sizes of the assemblies are smaller at higher and lower pH, but the size significantly increased as we reached the isoelectric point (Fig. 4b, SI, Fig S4). This is attributed to the high density of charge on the pendant groups at low and high pH, which exhibits electrostatic repulsions between the pendent ionic groups in the polymer chains at microscopic level leading to formation of smaller aggregates. On the other hand, as we reach the isoelectric point, the reduced electrostatic repulsions result in the formation of the larger aggregates. This feature of the zwitterionic polymer could find use in pH-sensitive surface charge variations, which have been explored for use in drug delivery and biomedical diagnostics.¹⁴

In vitro cellular viability

Finally, considering the potential implications of a nanoassemblies with charge-neutral and pH-sensitive charge conversion characteristics in biological applications, we evaluated and compared the cytotoxicity of our zwitterionic polymeric assemblies to that of structurally similar ionic (**6**, **7**) and neutral PEG based amphiphilic polymers **8** (see SI for the synthesis and characterization). If surface functional groups are primary determinants of cytotoxicity, zwitterionic and the PEG-based polymeric assemblies should be less cytotoxic, because of their

charge neutral characteristics. Similarly, cationic polyelectrolyte assemblies should be more toxic compared to the anionic ones. To test this, we evaluated the in vitro cellular viability of all the polymers using Alamar blue assay on a healthy cell line (HEK-293T) and on a tumour cell line (HeLa). Indeed, we observed that zwitterionic polymers exhibit concentration independent, high cell viability with both HEK-293T and HeLa cell lines (Fig 5b, c). On the other hand, cationic polymers are the most toxic followed by anionic and charge-neutral PEG based polymers; their cytotoxicity was also found to be concentration-dependent. We were surprised to find that the PEG-based assemblies were more toxic than the zwitterionic polymer. The reason for this difference is not clear to us at this time and will be a subject of future investigations.

Conclusions

In summary, we present a one-pot synthesis of zwitterionic amphiphilic homopolymers using reactive polymer precursors. These polymers form self-assembled aggregates and stably encapsulate guest molecules both in polar and apolar solvents. Due to the presence of glycine betaine as the zwitterionic hydrophilic component of the polymer, it reversibly switches among cationic, zwitterionic, and anionic forms depending on the pH of the solution. These variations also afford pH-dependent size variations, which are attributed to electrostatic repulsion typical of polyionic assemblies. Cytotoxicity studies indicate that zwitterionic polymeric assemblies are least cytotoxic among structurally similar polymers. Facile preparation and robust guest encapsulation capabilities, combined with their biocompatibility, make them excellent candidates for further exploration in biomedical applications where anti-fouling characteristics is critical.

Acknowledgements

Supports from the National Science Foundation (CHE-1307118) NIGMS of the NIH (GM-065255) are gratefully acknowledged.

Notes and references

†R.R. Ramireddy, and P. Prasad contributed equally for this work.

- (1) (a) R. Langer, *Science.*, 2001, **293**, 58-59; (b) S. Salmaso and P.J. Caliceti, *J. Drug. Deliv.*, 2013, **2013**, 19; (c) S.M.Moghimi, A.C. Hunter and J.C. Murray, *Pharmacol. Rev.*, 2001, **53**, 283-318; (d) R. Duncan, *Nat. Rev. Drug Discov.*, 2003, **2**, 347-360.
- (2) (a) N. Kolishetti, S. Dhar, P.M. Valencia, L.Q. Lin, R. Karnik, S.J. Lippard, R. Langer and O.C. Farokhzad, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 17939-17944; (b) P.P. Ghoroghchian, G. Li, D.H. Levine, K.P. Davis, F.S. Bates, D.A. Hammer and M.J. Therien, *Macromolecules.*, 2006, **39**, 1673-1675; (c) J. Cheng, B.A. Teply, I. Sherifi, J. Sung, G. Luther, F.X. Gu, E. Levy-Nissenbaum, A.F. Radovic-Moreno, R. Langer and O.C. Farokhzad, *Biomaterials.*, 2007, **28**, 869-876; (d) Z. Cao, Q. Yu, H. Xue, G. Cheng and S. Jiang, *Angew. Chem. Int. Ed.*, 2010, **49**, 3771-3776; (e) B.M. Discher, Y.-Y. Won, D.S. Ege, J.C.M. Lee, F.S. Bates, D.E. Discher and D.A. Hammer, *Science.*, 1999, **284**, 1143-1146.
- (3) (a) A.B. Lowe and C.L. McCormick, *Chem. Rev.*, 2002, **102**, 4177-4189; (b) S. Jiang and Z. Cao, *Adv. Mater.*, 2010, **22**, 920-932.
- (4) (a) V. Gaberc-Porekar, I. Zore, B. Podobnik and V. Menart, *Curr. Opin. Drug Discov.*, 2008, **11**, 242-250; (b) J.M. Harris, *Poly (ethylene glycol) chemistry: biotechnical and biomedical applications*. Plenum Publishing Corporation: 1992. (c) M. J. Joralemon, S. McRae, and T. Emrick, *Chem. Commun.* **46**, 1377-1393 (2010)
- (5) (a) G.Z. Li, G. Cheng, H. Xue, S.F. Chen, F.B. Zhang and S.Y. Jiang, *Biomaterials.* 2008, **29**, 4592-4597; (b) G.Z. Li, H. Xue, G. Cheng, S.F. Chen, F.B. Zhang and S.Y. Jiang, *J Phys Chem B.*, 2008, **112**, 15269-15274; (c) Q. Tu, J.-C. Wang, R. Liu, Y. Zhang, J. Xu, J. Liu, M.-S. Yuan, W. Liu and J. Wang, *Colloids Surf., B.*, 2013, **102**, 331-340; (d) J. Ladd, Z. Zhang, S. Chen, J.C. Hower and S. Jiang, *Biomacromolecules.*, 2008, **9**, 1357-61; (e) C. Zhao, L. Li, Q. Wang, Q. Yu and J. Zheng, *Langmuir.*, 2011, **27**, 4906 - 4913; (f) S. Gam-Derouch, M. Gosecka, S. Lepinay, M. Turmine, B. Carbonnier, T. Basinska, S. Slomkowski, M.-C. Millot, A. Othmane, D. Ben Hassen-Chehimi and M. M. Chehimi, *Langmuir.*, 2011, **27**, 9285 - 9294; (g) S. Martwiset, A. E. Koh and W. Chen, *Langmuir.*, 2006, **22**, 8192 - 8196.
- (6) (a) Z.Q. Cao, L. Zhang and S.Y. Jiang, *Langmuir.*, 2012, **28**, 11625-11632; (b) S. Chen, L. Li, C. Zhao and J. Zheng, *Polymer.*, 2010, **51**, 5283-5293; (c) R.R. Maddikeri, S. Colak, S.P. Gido and G.N. Tew, *Biomacromolecules.*, 2011, **12**, 3412-3417; (d) F.C. Giacomelli, P. Stepanek, V. Schmidt, E. Jager, A. Jager and C. Giacomelli, *Nanoscale.*, 2012, **4**, 4504-4514.
- (7) (a) S. Basu, D.R. Vutukuri, S. Shyamroy, B.S. Sandanaraj and S. Thayumanavan, *J. Am. Chem. Soc.*, 2004, **126**, 9890-9891; (b) E.N. Savariar, S.V. Aathimanikandan and S. Thayumanavan, *J. Am. Chem. Soc.*, 2006, **128**, 16224-16230; (c) T.S. Kale, A. Klaiherd, B. Popere and S. Thayumanavan, *Langmuir.*, 2009, **25**, 9660-9670.
- (8) (a) L. Li, K. Raghupathi, C. Song, P. Prasad and S. Thayumanavan, *Chem. Commun.*, 2014, **50**, 13417-13432; (b) J. Du, H. Willcock, J.P. Patterson, I. Portman and R.K. O'Reilly, *Small.*, 2011, **7**, 2070-2080; (c) J.N. Cha, H. Birkedal, L.E. Euliss, M.H. Bartl, M.S. Wong, T.J. Deming and G.D. Stucky, *J. Am. Chem. Soc.*, 2003, **125**, 8285-8289; (d) S. Kumar, D-W. Kim, H-J. Lee, M. Changez, T-H. Yoon and J-S. Lee, *Macromolecules*, 2013, **46**, 7166.
- (9) (a) N. Rodthongkum, R. Ramireddy, S. Thayumanavan and R.W. Vachet, *Analyst.*, 2012, **137**, 1024-1030; (b) F. Wang, A. Gomez-Escudero, R.R. Ramireddy, G. Murage, S. Thayumanavan and R.W. Vachet, *J. Am. Chem. Soc.*, 2013, **135**, 14179-14188. (c) N. Rodthongkum, Y. Chen, S. Thayumanavan and R.W. Vachet, *Anal. Chem.*, 2010, **82**, 8686-8691.
- (10) (a) B.S. Sandanaraj, R. Demont and S. Thayumanavan, *J. Am. Chem. Soc.*, 2007, **129**, 3506-3507; (b) E.N. Savariar, S. Ghosh, D.C. Gonzalez and S. Thayumanavan, *J. Am. Chem. Soc.*, 2008, **130**, 5416-5417; (c) D.C. Gonzalez, E.N. Savariar and S. Thayumanavan, *J. Am. Chem. Soc.*, 2009, **131**, 7708-7716.
- (11) M. Eberhardt, R. Mruk, R. Zentel and P. Théato, *Eur. Polym. J.*, 2005, **41**, 1569-1575.
- (12) S. Basu, D.R. Vutukuri and S. Thayumanavan, *J. Am. Chem. Soc.*, 2005, **127**, 16794-16795.
- (13) (a) L. Wu, J. Jasinski and S. Krishnan, *J Appl Polym Sci.*, 2012, **124**, 2154-2170; (b) S. Liu and S.P. Armes, *Langmuir.* 2003, **19**, 4432-4438; (c) A. Niu, D.-J. Liaw, H.-C. Sang and C. Wu, *Macromolecules.*, 2000, **33**, 3492-3494.
- (14) (a) Y.-Y. Yuan, C.-Q. Mao, X.-J. Du, J.-Z. Du, F. Wang and J. Wang, *Adv. Mater.*, 2012, **24**, 5476-5480; (b) H.S. Sundaram, J.R. Ella-Menye, N.D. Brault, Q. Shao and S.Y. Jiang, *Chem Sci.*, 2014, **5**, 200-205.