Polymersomes

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One-Pot Synthesis of an Acid-Labile Amphiphilic Triblock Copolymer and its pH-Responsive Vesicular Assembly**

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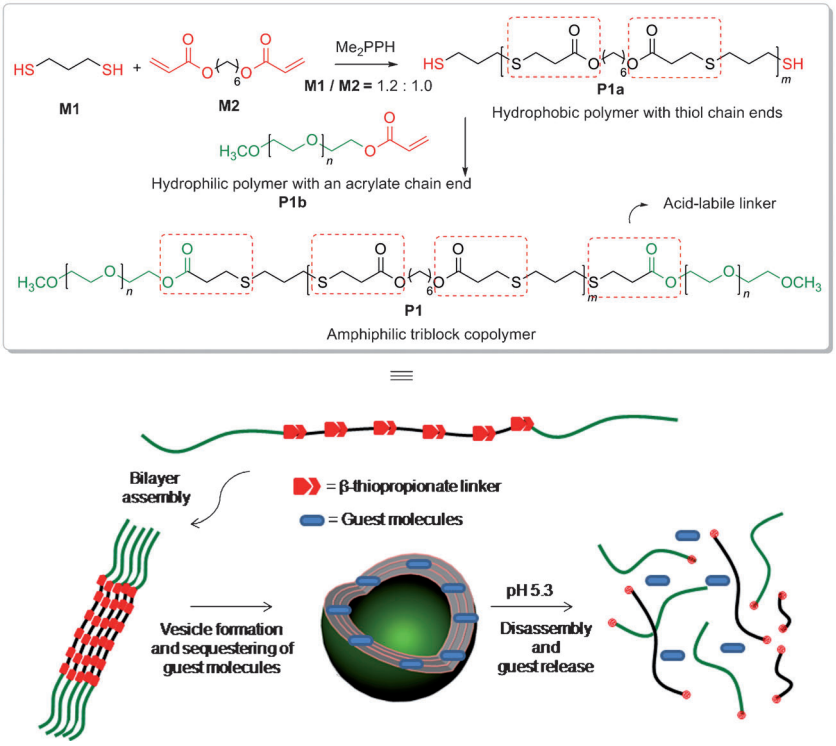
Various "click" reactions[1] have made tremendous impact in polymer synthesis[2] during the last decade. This area has been expanded significantly by including versatile sulfhydryl chemistry owing to high-fidelity reactions of the thiol group with many functional groups such as isocyanates, alkenes, maleimides, alkynes, acrylates, disulfides, halogenated alkanes and so forth.[3] The utility of these extremely efficient click reactions in enriching the structural diversity of macromolecules will be more appealing in the research area of functional polymers, if the resulting linker is sensitive to an external stimulus, particularly a biologically relevant one.[4] In this context the thiol–acrylate Michael addition reaction is highly attractive, because it produces a β-thiopropionate linker, which can be hydrolyzed selectively under mild acidic conditions (pH ≈ 5.5) at a very slow rate[5] unlike other acid-labile functional groups, such as acetal, ketal, and hydrazone groups, which show much faster degradation kinetics.[6] This feature of the β-thiopropionate group is highly relevant in the sustained release of drug molecules selectively in tumor cells, which have a more acidic environment than healthy cells.[7] Polymersomes[8] have been studied in this context because of their ability to sequester both hydrophobic and hydrophilic guest molecules, high kinetic stability, and appropriate particle size to utilize the enhanced permeability and retention (EPR) effect.[9] Polymersomes are formed mainly by amphiphilic triblock or diblock copolymers, which are synthesized in a stepwise manner by various controlled polymerization techniques. The structural diversity in such elegant macromolecular scaffolds has been explored to a great extent by generating free thiol groups as well as various thiol-reactive functional groups either in the chain end or as pendant.[10] In contrast similar strategies have been rarely reported for step-growth polymerizations[11] in which, unlike in chain polymerizations, opportunities are galore for structural engineering in the polymer backbone. Herein we have revealed an extremely simple synthetic approach (Scheme 1) for the synthesis of an ABA-type amphiphilic triblock copolymer (P1) by using a sequential thiol–acrylate Michael addition reaction in one pot.[12] Further we show its vesicular assembly, guest encapsulation, and pH-specific sustained-release properties.



Scheme 1. One-pot synthesis of amphiphilic triblock copolymer P1, vesicular assembly, and pH-responsive disassembly

For synthesizing the polymer, a dithiol (M1) and diacrylate (M2) monomer were mixed with stoichiometric imbalance (M1/M2 = 1.2:1.0) and condensed in the presence of a catalytic amount of Me_2PPh to generate a telechelic hydrophobic polymer P1a, which is periodically segmented by an acid-labile β-thiopropionate functional group and should contain free sulfhydryl groups at both ends, since M1 was used in excess.

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An aliquot was collected from the polymerization mixture after 20 min for structural analysis. FTIR analysis of the sample showed (Figure S1 in the Supporting Information) complete disappearance of the peak at 810 cm^{-1} owing to the C=C bond of the acrylate group of the limiting monomer (M2), thus suggesting 100% conversion. Subsequently acrylate-functionalized PEO-2000 (P1b) was added to the polymerization mixture and stirring was continued for additional 2 h. The resulting mixture was precipitated out from diethyl ether and the obtained solid was dissolved in water and subjected to extensive dialysis to remove unreacted P1b, if present. The desired polymer was isolated by freeze drying. At 100% conversion and at a 1.2:1.0 stoichiometric ratio, the molecular weight of P1a could be theoretically calculated to be 3593 $g\,mol^{-1}$ by using the following formula.[13]

Degree of polymerization $(DP) = (1+r)/(1-r)$, where $r = [M2]/[M1]$.

The molecular weight of P1a was estimated to be 3400 $g\,mol^{-1}$ (PDI = 1.8) by gel permeation chromatography (GPC; Figure S2a in the Supporting Information) and was in perfect agreement with the theoretically calculated value.

Furthermore, the molecular weight of the block copolymer P1 was found to be 8000 $g\,mol^{-1}$ (PDI = 1.27; Figure S2a), which was also in agreement with the theoretically predicted $[M(P1) = M(P1a) + 2M(P1b)]$ value of approximately 7700 $g\,mol^{-1}$, thus indicating structural purity of the triblock copolymer P1. To confirm that P1 was not contaminated with trace amounts of P1a or a hypothetical diblock copolymer (which can be formed by PEO attachment to only one end of P1a), Ellman's assay[14] was performed to detect trace amount of free sulfhydryl group. Firstly P1a was treated with Ellman's reagent while a distinct peak appeared with $\lambda_{max} = 510$ nm (Figure S2b), indicating the presence of free sulfhydryl groups at the chain ends. But when a similar test was performed with P1, no such peak was noticed, thereby confirming the absence of any thiol-terminated polymer contamination in P1. All polymers were further characterized by ^{1}H NMR spectroscopy (Figure 1, and Figure S3 in the Supporting Information). For P1b, peaks appeared at $\delta = 6.6$–5.6 ppm corresponding to the terminal acrylate group, at $\delta =$ 3.6–3.4 ppm corresponding to the methylene units of the polymer backbone, at $\delta = 4.30$ ppm corresponding to the terminal OCH_2 group adjacent to the acrylate ester, and a singlet at $\delta = 3.3$ ppm representing the terminal -OCH_3 protons. For polymer P1a also similarly all peaks could be assigned according to the proposed repeat unit structure. For the final polymer P1, no peak was noticed around $\delta = 6.6$–5.6 ppm and $\delta = 4.30$ ppm, thus confirming no P1b contamination in the block copolymer. Interestingly, a new peak appeared at $\delta = 4.25$ ppm corresponding to the -OCH_2- group (H_g) adjacent to the ester group in the junction of hydrophobic and hydrophilic segments. Other than those, all other peaks corresponding to both the constituent polymers appeared around the same range, thereby suggesting structural integration of P1a and P1b in P1. Since the molecular weight of the PEO block is known, the integration ratio of the peaks at $\delta = 3.6$–3.4 ppm corresponding to -OCH_2CH_2O- (H_i) protons of the PEO block and at $\delta = 4.11$–4.06 ppm corresponding to the H_a protons of the hydrophobic block suggested the molecular weight of P1 was approximately 7200 $g\,mol^{-1}$, which is in close agreement with the theoretically estimated value. This is further indicative of quantitative chain-end functionalization.

Self-assembly of P1 was tested by its guest (Figure 2a) encapsulation ability. A hydrophobic probe pyrene was treated with an aqueous P1 solution, and the resulting solution showed intense absorption and emission bands (Figure 2b), suggesting that P1 acts as container for hydrophobic molecules. Note that pyrene on its own is poorly soluble in water and thus almost no absorption signals could be seen in the absence of P1. The ratio of the first and third vibrational peaks (I1/I3) of the pyrene emission spectrum has been used as an indicator of the local dielectric properties of the probe.[15] In this case the value was found to be 1.26, which closely matched that for $CHCl_3$ (1.28),[15] thus suggesting the dielectric properties of the environment where pyrene is located are similar to those of $CHCl_3$ (4.8069 ε_0 at 20 °C). It may be a coincidence but worth noting that the value also matches with reports of pyrene encapsulated in a vesicular assembly.[16] To estimate the encapsulation efficacy, an aqueous solution of P1 (1.0 $mg\,mL^{-1}$) was treated with an excess of pyrene, and from the absorption spectrum the concentration of encapsulated dye was estimated (for details see Figure S4 in the Supporting Information) to be 0.1 mg/1.0 mg of P1. Furthermore, the container properties of P1 were tested by Förster resonance energy transfer (FRET)[17] between a hydrophobic donor DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) and acceptor DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), which were co-encapsulated in the aggregates of P1. Excitation at the DiO absorption (450 nm) resulted in a weak donor emission at 510 nm but in a strong acceptor emission at 575 nm (Figure 2c; FRET ratio = 0.7)

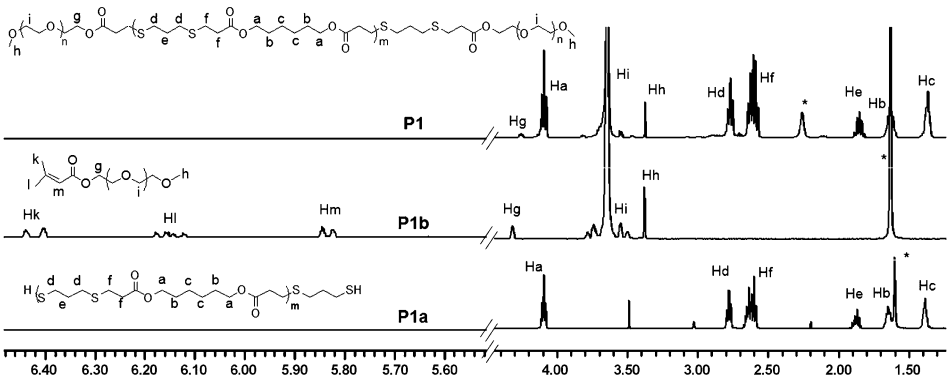

Figure 1. ^{1}H NMR spectra of P1a, P1b, and P1 in $CDCl_3$; * indicates solvents peaks. The chemical shift values in ppm are shown on the *x* axis.

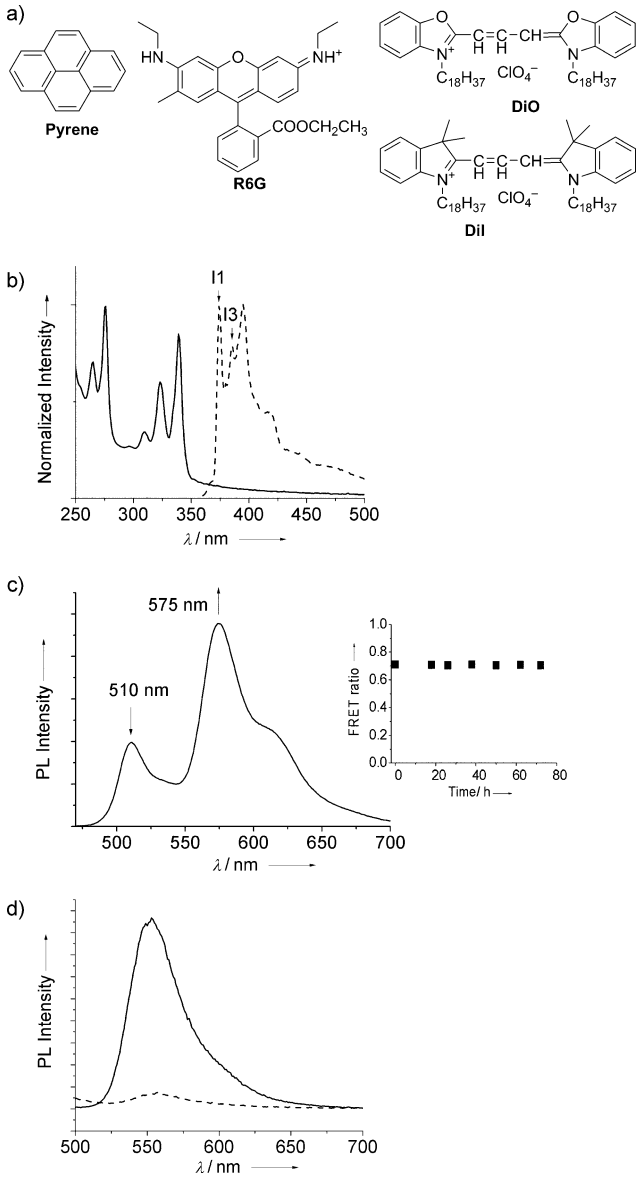

Figure 2. a) Structures of various encapsulated dye molecules. b) Absorption (——) and emission (-----; $\lambda_{ex} = 337$ nm) spectra of pyrene (10^{-6} M) encapsulated in P1. c) Emission spectra ($\lambda_{ex} = 450$ nm) of DiO and DiI (0.05 mg mL^{-1}) co-encapsulated in P1; inset: variation of the FRET ratio [$I_{575}/(I_{510}+I_{575})$] with time. d) Emission spectra ($\lambda_{ex} = 450$ nm) of R6G encapsulated in P1 (-----) and in polymer-free aqueous solution (——). PL = photoluminescence.

suggesting effective co-encapsulation and energy transfer inside the aggregates. Note that under identical excitation conditions ($\lambda_{ex} = 450$ nm), the emission from the acceptor (DiI) alone was negligible owing to lack of any significant absorption at this wavelength (See Figure S5 in the Supporting Information for absorption and emission spectra of the individual donor and acceptor chromophores).

Time-dependent studies indicated very minor changes in the FRET ratio (inset in Figure 2c) even after 72 h, thus suggesting a high kinetic stability of the aggregates.[18] These data certainly indicate no disassembly; however, they do not provide conclusive evidence that no minor morphological changes of the vesicular aggregates occur during the time span of the experiment. Furthermore we tested the ability of P1 to encapsulate a hydrophilic dye R6G, which was treated with P1 and the solution was subjected to extensive dialysis for removal of any nonencapsulated dye. The emission spectrum of the resulting solution showed an emission band at $\lambda_{max} = 557$ nm (Figure 2d) suggesting encapsulation of R6G. The ability of P1 to encapsulate both hydrophobic and hydrophilic dyes as well as the exceptionally high kinetic stability indicates vesicular assembly. Further the emission properties of the free dye were compared with those of the polymer-encapsulated dye (the emission intensity was normalized by the absorption intensity of the corresponding sample), which showed significantly reduced intensity in the latter case (Figure 2d), suggesting self-quenching as expected in the confined water pool inside vesicles.[16] Vesicles with encapsulated R6G could also be observed by using a fluorescent microscope as red-emitting spherical particles (Figure S6 in the Supporting Information).

The morphology of the aggregates was tested by high-resolution TEM images (Figure 3 and Figure S7 in the Supporting Information), which showed hollow spherical particles with diameters in the range of 50–70 nm, suggesting vesicular assembly. In few places, we also noticed a signature of vesicle fusion (Figure S7, middle panel). The average hydrodynamic diameter (D_h) of the aggregates was determined to be 80 nm from dynamic light scattering (DLS) measurements (Figure S8 in the Supporting Information); this diameter was slightly higher than that observed by TEM owing to shrinkage of the membrane in the dry state, where the TEM images were recorded; this phenomenon was also observed by others.[8]

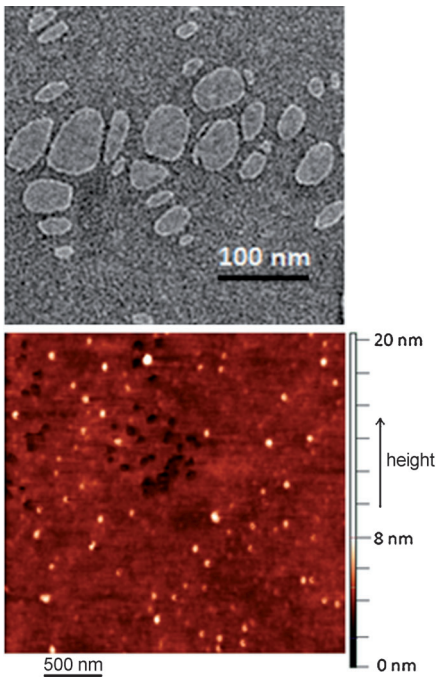

Figure 3. HRTEM (top) and AFM (bottom) images of P1 aggregates showing vesicular morphology.

The vesicular morphology could be further supported by AFM images (Figure 3 and Figure S9 in the Supporting Information), which showed near-spherical structures with average height and width in the range of 8–10 nm and 60–70 nm, respectively. The significantly lower height compared to the width is indicative of a hollow soft vesicular morphology, which is expected to be flattened (particularly at low concentration) when the structures are adsorbed on a surface.[19]

To examine the possibility of pH-responsive guest release by acid-induced cleavage of the β-thiopropionate linker, vesicles with encapsulated pyrene were prepared in a Tris buffer (10 mM, pH 5.3), and the absorption spectra of pyrene was monitored as a function of time (Figure 4a). During the

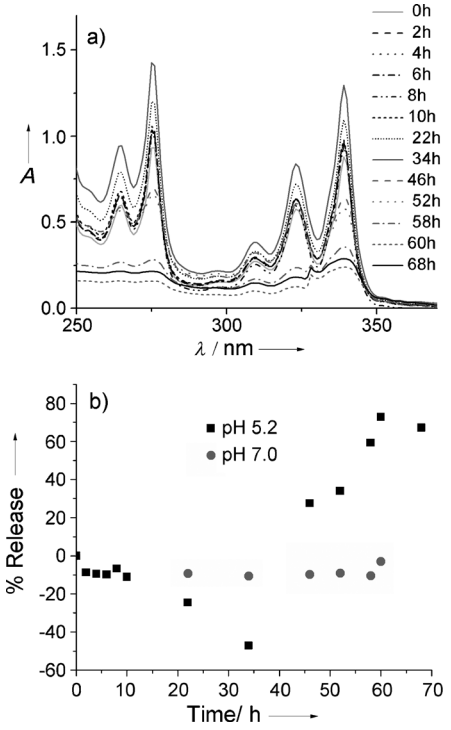

Figure 4. a) Time-dependent variation of absorption spectra of pyrene encapsulated in P1 at pH 5.3. The absorption at 400 nm was subtracted from each spectrum to reduce the effect of scattering on data analysis. For raw data see Figure S10 in the Supporting Information. b) Plot of release in % as a function of time (calculated by monitoring the absorbance at 338 nm).

first 10 h almost no change was observed. Subsequently the absorption intensity increased to some extent followed by a significant decrease indicating release of encapsulated guest. The observed increase in absorption intensity (see curves at 22 and 34 h in Figure 4a) was rather surprising. We noticed during that time the baseline intensity at 400 nm, where no absorption is expected, also increased (Figure S10), suggesting contribution from scattering owing to the turbidity arising from cleavage-induced hydrophobic product. The scattering intensity does not remain constant but rather increases exponentially with decreasing wavelength.[20] Thus the contribution to the absorption value from the increase owing to scattering is higher than that of the decrease owing to dye release, thus resulting in an overall increase of the absorption value. But at a later stage when the cleavage happens to a greater extent, thereby leading to the release of a significant amount of pyrene, the scattering effect on the absorption is compensated, and one observes a decrease in the absorption intensity. The pyrene release kinetics (Figure 4b) clearly show almost 80% dye was released in a sustained manner over a period of 60–70 h. As a control, the same experiment was carried out at pH 7.0 where almost no change was observed (Figures S12 and Figure 4b), thereby indicating release was selectively happening under mild acidic condition.

To examine the relationship between the dye release and acid-induced structural disintegration of the polymer, an aqueous P1 solution (1 mg mL^{-1}) was stirred at the same pH value as in the pyrene release experiment and aliquots were taken at regular time interval, freeze-dried, redissolved in DMF, and analyzed by GPC (Figure 5a). With increasing time, the peak was shifting towards higher elution times with concomitant appearance of a shoulder at 17–19 min, indicating the generation of low-molecular-weight products. After 120 h, more distinct multiple peaks were observed, suggesting disintegration of the block copolymer to the constituent blocks and other smaller fragments owing to cleavage of the ester group in the junction of two blocks and also in other places in the hydrophobic block. However, we noticed the pyrene release (Figure 4a) was faster than the actual disintegration of the polymer. This apparent anomaly can be explained by the proposed model depicted in Figure 5b.

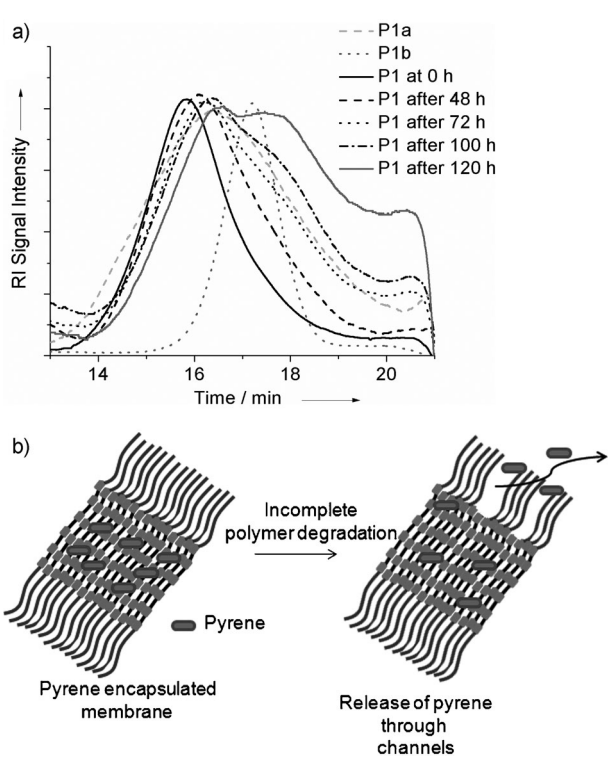

Figure 5. a) GPC traces of acid(pH 5.3)-treated P1 after various time interval. b) Possible mechanism of pyrene release even at partial disintegration of vesicular membrane. RI = refractive index.

Even when a small fraction of the hydrophilic PEO chains are detached, it generates channels in the membrane through which pyrene can be released by diffusion. Thus for pyrene release complete degradation of the polymer chains is not essential.

It is evident that even after 120 h, the degradation is not complete and thus the polymer was treated with excess trifluoroacetic acid (TFA) and the mixture was freeze-dried and analyzed by GPC and NMR spectroscopy. In this case, (Figure S13a in the Supporting Information) the GPC trace showed a new broad peak, part of which matched with PEG-2000 and the rest corresponded to lower-molecular-weight products. This suggests under relatively acidic treatment (pH ≈ 2), the extent of degradation was higher. Furthermore, in the ^{1}H NMR spectrum (Figure S13b), the multiplet at $\delta =$ 4.25 ppm corresponding to Hg of P1 was absent, indicating the PEO block was not linked to the hydrophobic block. In turn a new multiplet arose at $\delta = 4.4$ ppm, which matched with a peak at the same position in the NMR spectrum of commercially available PEO-OH (molecular weight ca. 2000 g mol^{-1}; Figure S14 in the Supporting Information), corresponding to the terminal -CH_2-OH protons. This also confirmed the presence of PEO-OH owing to disintegration of the block copolymer. But even in this acid-digested product, most peaks were matching with those for P1a apart from the appearance of a few multiplets with low intensity at various places (indicated by arrows in Figure S13b). This possibly suggests, that although the P1b block is made out of the same β-thiopropionate linkers it did not undergo extensive hydrolysis at each linker. It is important to understand, even if a single ester linker is cleaved in the middle of the hydrophobic block, it will reduce the molecular weight to approximately half, while not much change is expected to be seen in the NMR spectrum. This can be attributed to the fact that after the PEO blocks were detached, the hydrophobic block precipitated out (which was also visible as precipitation), and thus further hydrolysis under heterogeneous conditions became extremely sluggish.[21]

In conclusion we have shown the synthesis of an amphiphilic triblock copolymer by sequential thiol–acrylate Michael addition reaction in one pot. In aqueous medium the resulting polymer showed spontaneous vesicular assembly, which disassembled selectively under mild acidic conditions resulting in sustained release of encapsulated guest molecules. Considering the enormous difficulties one encounters in synthesizing triblock copolymers, the present one-pot approach appears to be extremely interesting and offers an exciting opportunity to explore various thiol-mediated click reactions at the chain end of a degradable polymer backbone. In addition, the stimuli-responsive disassembly and sustained release is highly relevant in targeted drug delivery[4] to tumor cells by exploiting their difference in pH value compared to healthy cells. Currently investigations in these directions are underway.

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Keywords: block copolymers · Michael addition · polymersomes · polymer synthesis · sustained release

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[20] To further confirm this, we examined (Figure S11 in the Supporting Information) absorption spectra of the hydrophilic dye R6G in aqueous solution containing poly(*N*-isopropylacrylamide) (PNIPAM), which is known to exhibit a lower critical solution temperature (LCST) at approximately 37 °C. The absorption intensities for R6G (at 526 nm) at 25 °C (below LCST) and 40 °C (above LCST) were found to be 0.35 and 0.80, respectively, thereby clearly revealing the apparent increase above LCST is an artefact arising owing to a contribution from scattering, which is larger for a turbid solution. Now by manual subtraction, the intensities at 650 nm were made to be zero for both the plots; still the absorption intensity 40 °C was significantly higher than that at 25 °C, because the scattering intensity as evident from the plot is not a constant as a function of wavelength, but rather increases exponentially with decreasing wavelength.
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