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Reversible pH- and Lipid-Sensitive Vesicles from Amphiphilic Norbornene-Derived Thiobarbiturate Homopolymers

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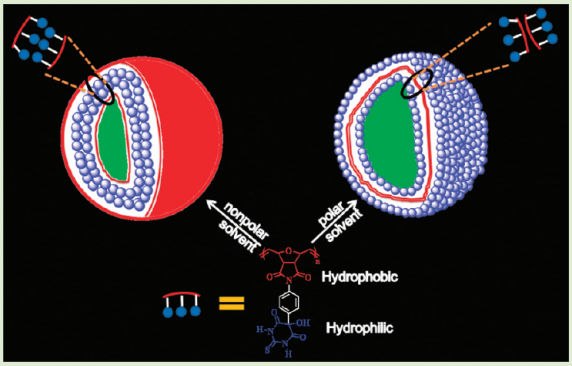
Supporting Information

ABSTRACT: Synthesis of a new molecular architecture, an amphiphilic, norbornene-derived thiobarbiturate homopolymer (**NTBH**), by ring-opening metathesis polymerization (ROMP) and its characterization is discussed. The newly designed hompolymer shows a self-assembled vesicle formation in aqueous solution. Dynamic light scattering and critical aggregation concentration studies confirm the aggregate formation in solution while atomic force microscopy and transmission electron microscopy of the dried sample on the silicon substrate further confirm the vesicular morphologies of these amphiphilic homopolymers. Encapsulation studies of hydrophilic doxorubicin and hydrophobic Nile red suggest the reversible nature of the **NTBH** vesicles. Dye release studies in acidic and lipophilic environment demonstrate the stimuli-responsive nature of the novel systems. The results demonstrate that these self-assembled **NTBH** vesicles have great scope in the field of medicine as they symbolize themselves as promising carriers for the stimuli-triggered intracellular delivery of hydrophobic drugs.



Supramolecular architectures are known to form usually by the natural process of "self-assembly".[1a,b] Inspired by nature, researchers try to exploit this process for the synthesis of entirely novel materials.[2a,b] Especially the specific feature of "amphiphilic molecules self-assembling into an amazing variety of structures" is always a source of attraction for them to explore further in this area.[3a−f] Self-assembling systems have a remarkable property of promptly responding to external stimuli, such as pH, solvent polarity, and temperature.[4a,b] Moreover, these systems show long-term stability and enhanced loading capacity for guest molecules.[5a−f] Specifically structures like vesicles (enclosed spherical bilayer assemblies) that are formed by the self-assembly of amphiphilic homopolymers are fundamentally important.[6a−d] From their fundamental perspective, they have rich potential applications in drug or gene delivery, nanotechnology, and are even considered as model systems of biomembranes.[7a−i]

Pyrimidine-based structures are of specific interest in the field of supramolecular chemistry due to their strong hydrogen bonding nature.[8] Especially the functionality of barbiturate and thiobarbiturate is of particular interest, not only due to their strong ability to make noncovalent interactions but also for their therapeutic nature.[9a−c] Herein, we report a pH- and lipid-sensitive bilayer vesicle formation from a new molecular architecture, an amphiphilic, norbornene-derived thiobarbiturate homopolymer (**NTBH**), by utilizing the ring-opening metathesis polymerization (ROMP).[10] The molecular orientation of **NTBH** is systematically modified based on the hydrophobicity and hydrophilicity of the solvent. The thiobarbiturate functionality of each monomer unit in **NTBH** can act as a hydrophilic headgroup, whereas the norbornene backbone can behave as a hydrophobic moiety.

To our knowledge, this is the first example of a vesicle formed from an amphiphilic **NTBH**. Previous literature mostly reports on the amphiphilicity of the side chain, which means that both hydrophobic and hydrophilic groups are functionalized as a pendent group. In our design, the norbornene backbone itself provides the hydrophobicity, while the barbiturate motif is responsible for the hydrophilicity. This is interesting because the hydrophilic head groups are collectively attached to a flexible hydrophobic coil, which in turn increases the whole amphiphilicity of the polymer, thus, producing interesting vesicles. It is hypothesized that hydrophilic thiobarbiturate head groups, attached to each repeating unit of the hydrophobic norbornene backbone, can form bilayer vesicles similar to lipids, achieving a greater stability and exerting the capability to spontaneously respond to their environmental conditions, such as lipophilicity and pH. **NTBH** vesicles demonstrate the versatility in serving as a nanoreservoir for both hydrophobic and hydrophilic molecules.

The synthesis of exo-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride (**1**) has been previously reported in literature.[10e] From the ^{1}H and ^{13}C NMR spectroscopy data, the new signal at δ 3.20 ppm and at δ 49.1 ppm respectively confirmed the formation of compound **1** (Figures S1 and S2).

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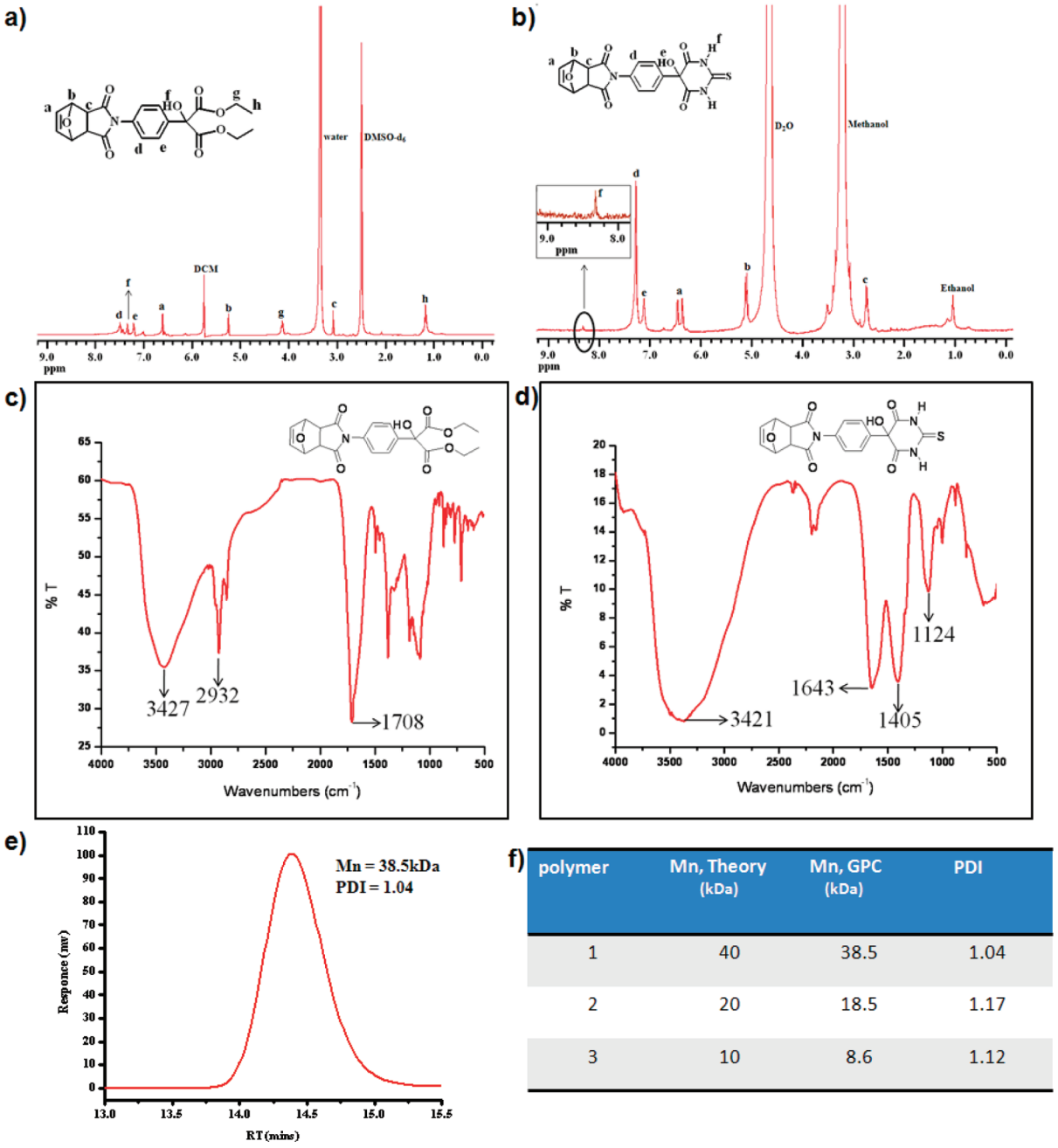

polymer	Mn, Theory (kDa)	Mn, GPC (kDa)	PDI
1	40	38.5	1.04
2	20	18.5	1.17
3	10	8.6	1.12

Figure 1. (a) ^{1}H NMR of compound **3**; (b) ^{1}H NMR of compound **NTB**; (c) FT-IR spectrum of compound **3**; (d) FT-IR spectrum of compound **NTB**; (e) GPC traces of **NTBH**; (f) molecular weights and PDIs of **NTBH**.

The compound **2** was synthesized by the coupling reaction of compound **1** with aniline, which was confirmed by ^{1}H NMR in which the aromatic proton signal of aniline was observed at δ 7.20 to δ 7.60 ppm. Also, in ^{13}C NMR, the signal at δ 176 ppm was observed due to the formation of cyclic amide (Figures S3 and S4). The compound **3** was synthesized by Friedel−Crafts acylation reaction between compound **2** and diethyl ketomalonate by using stannous chloride as a catalyst. The formation of this product was confirmed by ^{1}H NMR. The signal at δ 7.39 ppm was responsible for the newly formed hydroxyl group whereas the signal at δ 4.19 ppm and δ 1.20 ppm was responsible for OCH_2 and CH_3, respectively. The ^{13}C NMR signal at δ 92 ppm was due to the newly formed hydroxyl group attached to carbon (Figures 1a and S5). Formation of compound **3** was also confirmed by FT-IR spectroscopy, where the stretching frequency at 1708 cm^{-1} was due to an ester carbonyl (Figure 1c). Finally, the norbornene-derived thiobarbiturate (**NTB**) was prepared by coupling compound **3** with thiourea, and its synthesis was confirmed by ^{1}H NMR and FT-IR spectroscopy. The ^{1}H NMR spectrum in D_2O conveyed the absence of ester proton's signal at δ 4.19 ppm for the ester group and one new signal at δ 8.45 ppm, which was responsible for the amide proton (Figure 1b). The complete disappearance of the ester carbonyl at 1708 cm^{-1} of compound **3** and the appearance of a new stretching frequency at 1643 cm^{-1} for the cyclic amide in the FT-IR spectroscopy confirmed the formation of **NTB** (Figure 1d).

Next, the homopolymerization of **NTB** was carried out by using second generation Grubbs' catalyst at room temperature in dry dichloromethane and methanol (9:1 v/v %) solvent

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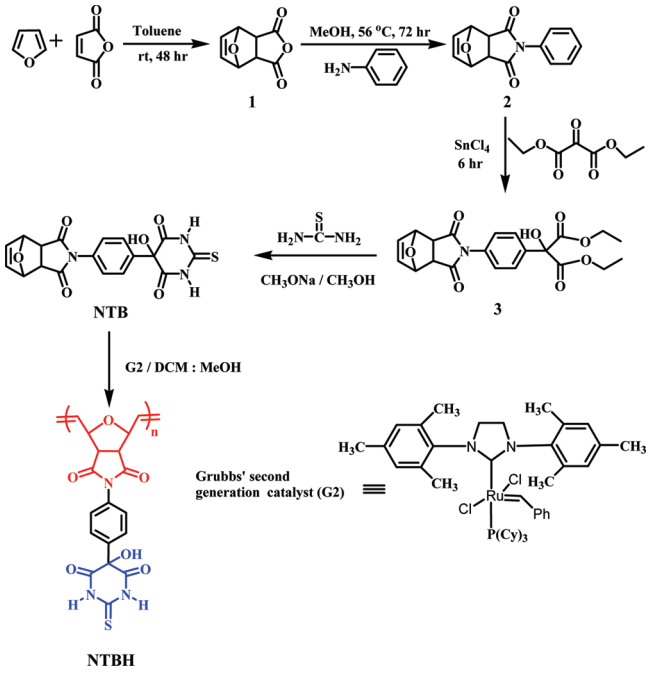
Scheme 1. Synthesis of Norbornene-Derived Polythiobarbiturate Homopolymer (NTBH)



system and was monitored by ^{1}H NMR. New signals were observed at δ 5.4 and δ 4.9 ppm, indicating the formation of **NTBH** (Figure S6). The molecular weight of **NTBH** (M_n = 38500 with PDI 1.04) was determined by the gel permeation chromatography (Figure 1**e and f**). The absence of stretching frequency at 1567 cm^{-1} for norbornene olefinic bond (C=C) confirmed the formation of **NTBH** (**Figure S7**).

The aggregation behavior of these polymers was studied using transmission electron microscopy (TEM), dynamic light scattering (DLS), atomic force microscope (AFM), and a scanning electron microscope (SEM), whereas their reservoir properties were evaluated by dye/drug-encapsulation studies. The critical aggregation concentration (CAC) was measured by employing pyrene as an extrinsic probe.[11,12] The ratio of the intensity of the first and third peaks (375/396) in the emission spectrum of pyrene was indicative of the polarity of its microenvironment.[11] The concentration of pyrene was maintained at 0.2 μM while the concentration of **NTBH** was changed from 10 to 1500 μg/mL. The excitation wavelength was set at 339 nm, and the emission intensities were measured at 375, 382, and 396 nm (Figure S8). The relative emission intensities of 396/375 nm were varied as a function of polymer **NTBH** concentration (Figure 2a). The CAC was determined from the **NTBH** concentration value at which the relative fluorescence intensity ratio began to change. The observed CAC was 400 μg/mL.

After measuring the critical aggregation point, the **NTBH** in water solution was characterized by DLS, the noninvasive method of analyzing aggregates in solution. This **NTBH** self-assembled into vesicles with average diameter of approximately 96 nm in water. Figure 2b shows the average hydrodynamic sizes (D_h) of the vesicles. Light scattering measurements confirmed the presence of vesicles in solution with an average diameter of 96 nm, which was in excellent agreement with the AFM results (Figure 3a). To investigate the correlation between the assemblies in the solution and a dry sample on

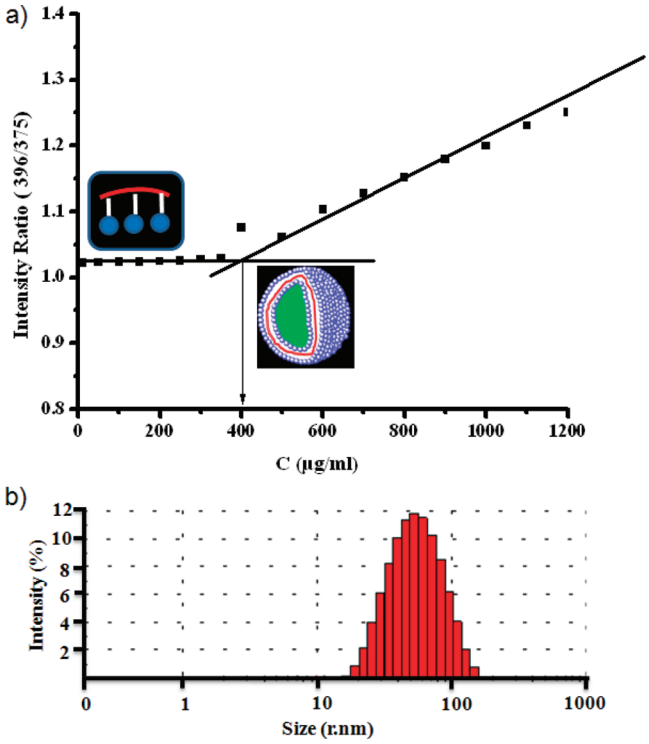


Figure 2. (a) Plot of concentration of **NTBH** in water vs the intensity ratio of emissions at 375 and 396 nm from pyrene (SI). The observed CAC was 400 μg/mL; (b) DLS measurement of **NTBH** vesicles in aqueous solution. The size of the micelles was about 96 nm with 0.189 PDI.

the substrate, the vesicular morphologies of the amphiphilic homopolymers were studied by AFM, SEM, and TEM. A histogram of 150 structures, collected from several AFM samples, revealed an average diameter of 105 nm for the vesicles (Figure S16). SEM images (Figure 3b) also revealed that the structures had diameters of around 100 nm. To collect the evidence for the hollow spherical vesicle, AFM was done with special parameters set for the AFM tip with increasing speed and force.[13] Observed images, as shown in Figure 3c, indicated that the size and morphology of the vesicles did not change except that the vesicles were collapsed. A cross-sectional analysis of the collapsed vesicle suggested the hollowness in their middle part (Figure 3c, inset). A series of TEM images were collected and a representative image, as shown in Figure 3d (stained with RuO_4), confirmed that the structures previously observed by AFM and SEM were vesicles. The sizes and diameters of these spherical vesicles observed by TEM were similar to those visualized by AFM and SEM. The observed open-mouth vesicles were due to the exposure to the high vacuum of TEM (Figure 3d). The CryoTEM images captured on **NTBH** vesicles stained with iodine further confirmed the presence of spherical assemblies with a dark outer ring expected from the 2D projection of vesicular structures, typically observed by TEM (Figure 3e). The inset in Figure 3e shows an enlarged picture of the vesicle core. A tomographic 3D view of closed (Figure 3f) and open vesicles (Figure 3g) were visualized using the FEI Xplore 3D software (a tiltseries was recorded from −65° up to +65° with 2° increments at a defocus of −10 μm).

Hydrophilic doxorubicin (DOXY) in its salt form and hydrophobic Nile red were selected as model candidates to test

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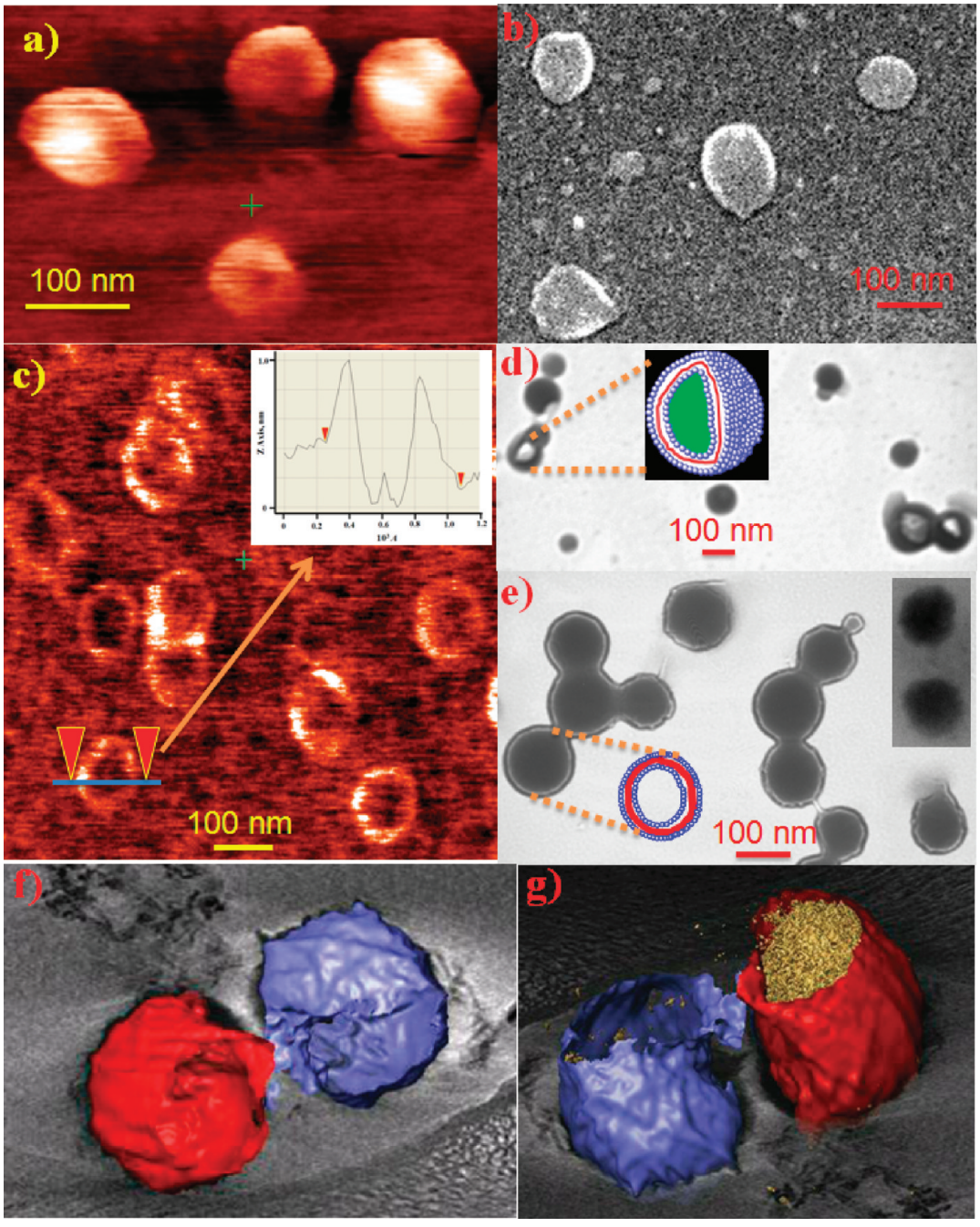

Figure 3. (a) Representative AFM image of **NTBH** vesicles spin-coated on a silicon surface; (b) SEM image of the sample used for AFM studies; (c) Tapping mode images from AFM show that the vesicles have collapsed after setting the special parameters for the AFM tip with increasing speed and force.[13] Inset shows the hollow middle part of a collapsed vesicle from cross sectional analysis; (d) TEM images of **NTBH** vesicles dip-casted on carbon-coated copper grids show the open-mouth vesicles. The inset shows the proposed self-assembled structure of **NTBH** in aqueous environment; (e) iodine-stained Cryo TEM images of NTBH vesicles reveal the bilayer wall as shown in the cartoon. Inset is an enlarged picture of the core of the vesicles; (f,g) Tomographic 3D view of closed (f) and open vesicles (g). Tiltseries is recorded from −65° up to +65° with 2° increments at a defocus of −10 μm using FEI Xplore 3D suit software.

whether these novel **NTBH** vesicles were capable of being a nanoreservoir for both hydrophobic and hydrophilic molecules. Nile red was insoluble in water, as evidenced by the lack of absorption corresponding to the dye molecule. However, when the dye was dissolved in the hydrophobic solvent and sonicated with **NTBH** in water, it could be encapsulated into the vesicles. The encapsulation was confirmed by the decrease of the Nile red absorbance in the hydrophobic solvent layer (Figure S9). However, the dye encapsulated **NTBH** vesicles remained colorless in the aqueous layer as the dye molecules were encapsulated in the interior of the vesicle due to the amphiphilic nature of **NTBH,** as shown in Figure 4a (colorless bottom layer of the UV-cuvette). This result indicated that the microenvironment provided by the amphiphilic **NTBH** assembly was hydrophobic in nature and was capable of sequestering lipophilic guest molecules. To test the dye release from the **NTBH** vesicles under the lipophilic environment, the most accepted octanol−water system was chosen.[14] Nile red

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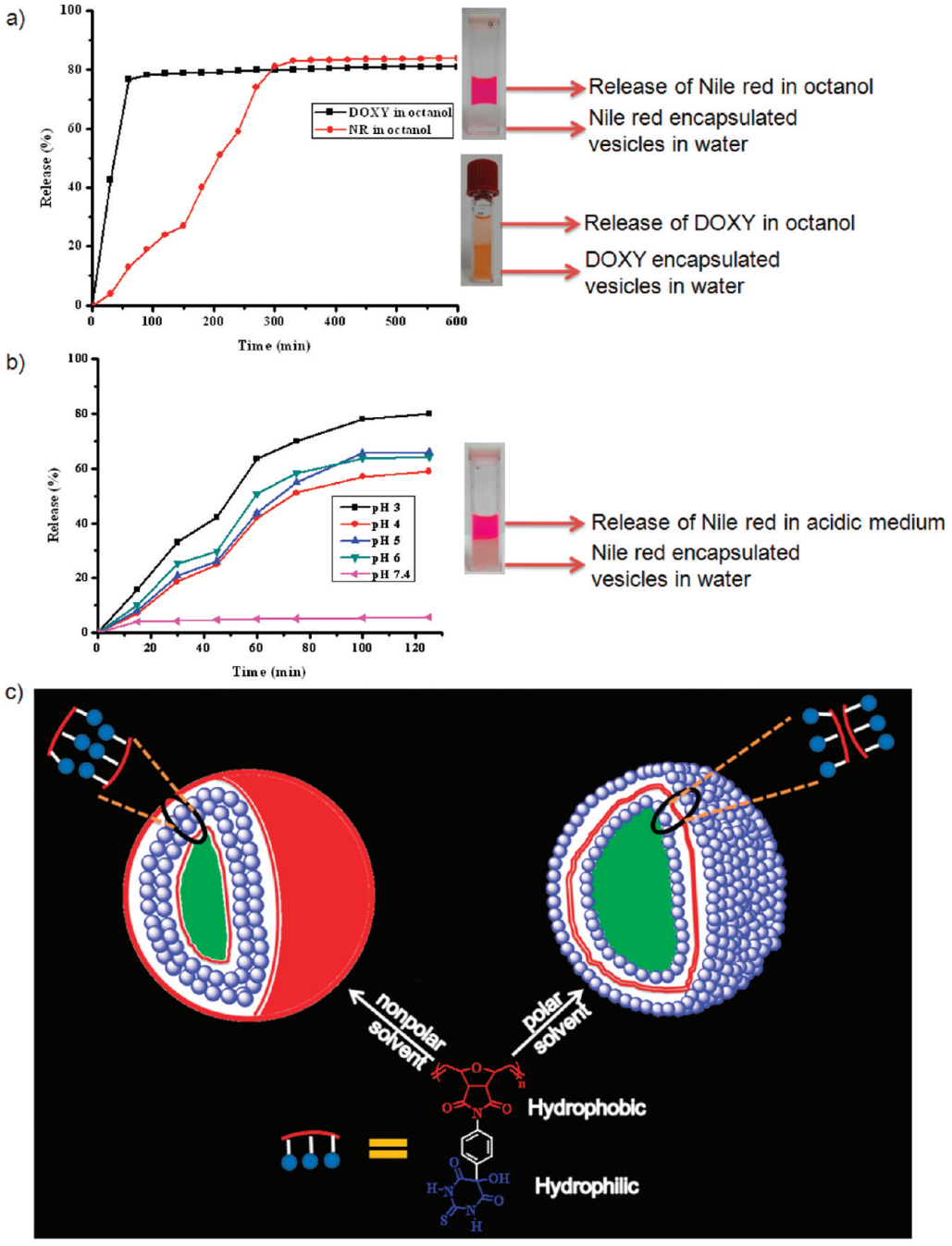

Figure 4. (a) Hydrophobic Nile red and hydrophilic DOXY (salt form) encapsulation (bottom layer) and release studies (upper layer) from **NTBH** vesicles in a lipid environment (octanol) is shown in a quartz cuvette. For Nile red release, absorbance maximum at 540 nm is measured with varying time intervals while for the DOXY release emission maximum at 580 nm is measured with varying time intervals (SI); (b) Nile red release from **NTBH** vesicles with varying pH is shown. The absorbance maximum at 520 nm is measured with varying time intervals; (c) cartoon representation of the newly designed amphiphilic homopolymer and its self-assembled vesicle structures in both hydrophobic and hydrophilic environment.

encapsulated **NTBH** vesicles were taken in a vial along with 1 mL of octanol. An aliquot of the sample was removed from the octanol layer and the absorbance at 540 nm was measured as an indication of the release of Nile Red (Figure 4a; violet upper layer of the UV-cuvette). After taking measurements in the UV range, the sample was added back to the vial to constantly maintain the volume of the solution. This procedure was repeated every 30 min and the results were observed for 10 h. Observations recorded after 9 h showed no significant increase in the intensity of absorbance (Figures 4a and S10). These results strongly suggested the release of dye in lipophilic environments.

Next, to verify the encapsulation of the hydrophilic molecule, 1 mg of **NTBH** and 1 mg of DOXY[11] in its salt form were dissolved in 1 mL of water separately. Both of the solutions were then mixed in a vial and stirred for 30 min. Then, the mixture was loaded in a dialysis tube (3500; Dalton cutoff) and dialyzed against 100 mL of water five times. An aliquot of the sample was removed each time and its fluorescence emission was measured at 552 and 587 nm as an indication of the release of nonencapsulated DOXY in the peripheral of the **NTBH**

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vesicles. A complete disappearance of DOXY emission at 552 and 587 nm was observed when the mixture was dialyzed for the fourth time. After confirming the complete disappearance of the DOXY emission by dialyzing for the fifth time, the solution loaded in the dialysis tube was taken in a vial and equal amount of octanol was added (2 mL). An aliquot of the sample was removed from the octanol layer every 15 min and its fluorescence was measured. Emissions from the free DOXY, released from the **NTBH** vesicles, were observed at 552 and 587 nm by exciting the solution at 510 nm. This procedure was repeated every 15 min, for 1 h, by constantly maintaining the volume of the solution. Observations recorded after 1 h showed no significant increase in the intensity of fluorescence (Figures 4a and S11). Thus, we hypothesize that the hydrophilic DOXY (salt form) could be encapsulated only if the hydrophilic head of the **NTBH** was inside (Figure 4c). Our hypothesis was supported by the following observations: (i) both polymer and drug were dissolved ONLY in water for the encapsulation studies while previous literatures often used a binary solvent system; (ii) spectroscopic measurements of the samples, collected from the beaker containing the dialysis tube, confirmed the peripheral attachment of the drug. This was possible only if the polar heads were outside the vesicle, as described in the cartoon (Figure 4c); (iii) because the **NTBH** polymer was not soluble but the DOXY was soluble in octanol, after dialysis, the solution inside the dialysis tube released the drug molecules when it was extracted with octanol. Again this was only possible if the hydrophilic heads were inside the bilayer structure of the vesicles as shown in the cartoon (Figure 4c). The above encapsulation studies of hydrophilic DOXY and hydrophobic Nile red (Figure 4), strongly suggested that the **NTBH** vesicles were capable of being reservoir for both hydrophilic and hydrophobic drug molecules. These nanoreservoirs were stable in the solvent in which they were assembled in, even in the presence of another immiscible, but favorable, solvent.

For the pH triggered dye release,[6a] the Nile Red encapsulated **NTBH** vesicles in water were taken in a vial along with ethyl acetate. Then the pH of the organic layer was adjusted to between 3 to 7.4 and was stirred gently. An aliquot from ethyl acetate layer was removed and the fluorescence emission was measured to indicate the release of the Nile Red. The sample was then added back to the vial to maintain the volume of the solution. This procedure was repeated every 15 min, for 90 min (Figures S12−S15). Observations recorded after 90 min, showed no significant increase in the intensity of fluorescence (Figure 4b). Apparently, there was no significant release of the Nile red from the vesicles at pH 7.4. Thus, we hypothesize that the mechanism of releasing sequestered dye molecules in the acidic pH was due to the breakage of hydrogen bonding[6,8] between the barbiturate functionality. A detailed mechanism of the nature of hydrogen bonding that occurs among the barbiturate functionality will be reported in future. In summary, the novel norbornene-derived thiobarbiturate homopolymers are shown to self-assemble into vesicles in aqueous solution. The synthesis of the newly designed monomer (**NTB**) and polymer (**NTBH**) are confirmed by NMR, IR, and mass spectroscopy. DLS and CAC studies reveal the aggregate formation. AFM, SEM, and TEM are used to characterize the morphology of these assemblies with the average diameter of around 100 nm. To our knowledge, this report represents the simplest route for producing amphiphilic homopolymers containing a narrow polydispersity. Dye encapsulation studies confirm the potential of these vesicles as nanoreservoirs. This property indicates that these polymers have the potential to act as controlled drug release vehicles. This is the first demonstration of vesicle self-assembly from norbornene-derived thiobarbiturate homopolymers. In the future, we aim to concentrate on demonstrating and improving the targeted delivery at the cellular level. We will also explore the thiobarbituate functionality in the **NTBH** vesicles for its therapeutic nature and as a delivery vehicle.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and additional analytical results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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