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Tunable Vesicles

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CO_2-Responsive Polymeric Vesicles that Breathe**

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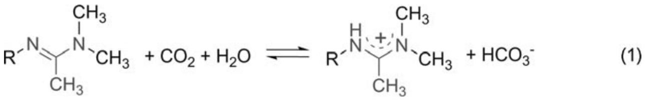
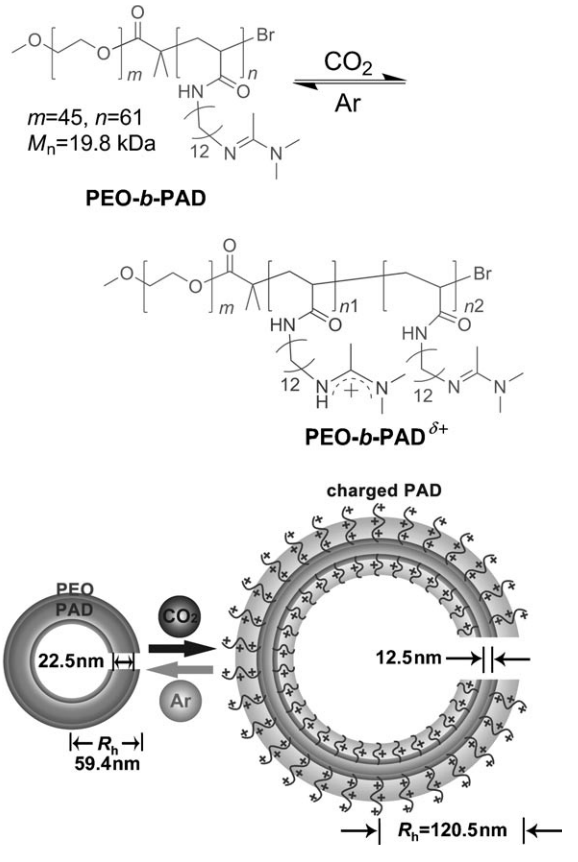
Stimuli-responsive polymeric vesicles have attracted great attention in recent years owing to their prospective biological applications,[1] such as in drug-delivery and gene-transport systems to enhance drug efficacy and mitigate side effects.[2] These "smart" polymer assemblies can undergo reversible changes (phase inversion, shape variation, and systemic assembly and disassembly) in response to external stimuli such as pH value,[3] temperature,[4] light,[5] reducing or oxidizing agents,[6] and even voltage.[7] In order to utilize this technique in a living body and inhibit biological incompatibility, it is crucial to exploit new trigger modes as close to physiological conditions as possible. Carbon dioxide (CO_2), as a key metabolite in cells, possesses good biocompatibility and membrane permeability. More importantly, it can stabilize the intracellular pH value by a series of equilibrium reactions ($CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^-$). Additionally, CO_2 metabolic disorder is related to certain metabolic diseases.[8] Considering the nontoxic nature of CO_2 and the fact that the influence of gas on polymer assemblies has rarely been investigated, construction of a CO_2-resonsive polymer system and exploration of how a gas can affect the self-assembly mechanism is quite challenging.

Herein, we have developed the new idea of utilizing a specific amidine-containing block copolymer to fabricate CO_2-responsive vesicles with a biomimetic "breathing" feature. Amidine is a type of gas-switchable molecule that was pioneered by Jessop et al. [Eq. (1)]. It can transform into a charged amidinium species upon reaction with CO_2, and this reaction is reversible upon exposure to argon.[9] Inspired by this switchable group, we wondered if we could obtain a gas-responsive polymer by introducing amidine species to the polymer chain. However, our attempt to synthesize polyamidine failed because the amidine unit displays low polymerization activity (Supporting Information, Figure S3).



$$\mathrm{R{-}N{=}C(CH_3){-}N(CH_3)_2 + CO_2 + H_2O \rightleftharpoons R{-}NH{-}C(CH_3){=}N^{+}(CH_3)_2 + HCO_3^-} \qquad (1)$$

To overcome this problem, we designed and synthesized the difunctional monomer (*N*-amidino)dodecyl acrylamide (AD). AD contains a highly reactive acrylic head and a pendant amidine group. Using a poly(ethylene oxide)-based macroinitiator, the target diblock copolymer PEO-*b*-PAD (Scheme 1) was prepared at room temperature using an atom-transfer radical polymerization protocol (see the Supporting Information),[10] which provided the product with well-defined molecular weight ($M_n = 19.8$ kDa) and monodispersion ($M_w/M_n = 1.14$).



Scheme 1. Gas-switchable structural change of amidine-containing diblock copolymer PEO-*b*-PAD (top) and schematic representation of its self-assembly into vesicles and their reversible gas-responsive "breathing" in aqueous media (bottom).

The gas-responsiveness of this copolymer in aqueous media was confirmed by conductivity experiments.[9a] When

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CO_2 was passed through the polymer solution for 20 min, the conductivity rose dramatically from 3.4 to 26.9 $\mu S\,cm^{-1}$ accompanied by a decrease of the pH value from approximately 6.94 to 5.68, implying that a number of protonated species formed in the copolymer chains. The original low conductivity was restored upon treatment with Ar owing to an opposite deprotonation effect. This procedure is reversible over three cycles (Figure 1). Considering the fact that reaction with CO_2 can convert the PAD chain into a cationic polyelectrolyte, we desired to understand the degree of protonation of the PAD chain. In a reference experiment, a pH–conductivity correlation indicates that the pK_a of PAD homopolymer is approximately 5.4. Furthermore, according to the final pH value of the PEO-*b*-PAD solution upon CO_2 treatment (pH ≈ 5.68), a partial charged model of $PAD^{\delta+}$ chain state (δ is the degree of protonation) can be deduced, that is, in a given PAD chain, 37 % of amidine side groups are protonated and 63 % remain in the uncharged state (Supporting Information, Figure S4–S5). This result means that when CO_2 is added continuously into the PEO-*b*-PAD solution until saturation, δ can gradually increase to an upper critical value of 0.37. This change could significantly impact the behavior of the PAD block in solution.

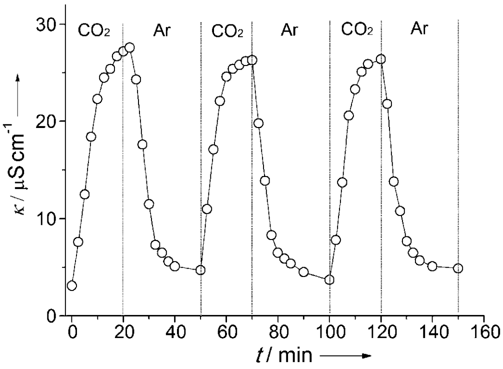


Figure 1. The conductivity of the PEO-*b*-PAD in aqueous solution plotted versus time upon alternating CO_2 and Ar stimuli (○). Polymer concentration $c = 1.2\ mg\,mL^{-1}$.

Next, we aimed to explore the self-assembly of PEO-*b*-PAD in aqueous media. When dissolved in water, PEO-*b*-PAD can self-assemble owing to its amphiphilic nature, in which the PEO portion is hydrophilic and the PAD portion is hydrophobic. The critical aggregation concentration (CAC) is approximately 0.16 $mg\,mL^{-1}$, as monitored by the fluorescent probe method (Supporting Information, Figure S6). These aggregates were studied by transmission electron microscopy (TEM). Figure 2a displays how they self-assemble into a spherelike morphology. The clear contrast between the dark periphery and the hollow center indicates that these spheres are vesicular. TEM images show that the average size of the vesicles is (110 ± 5.5) nm, which is consistent with the hydrodynamic radius R_h of (59.4 ± 2.1) nm determined by dynamic laser scattering (DLS, Figure 2b). In view of the good agreement, this image is used to evaluate the wall thickness, which statistically yields a thickness of (22.5 ± 2.0) nm. In the absence of stimuli, these aggregates are stable in water, and there was no obvious change in either morphology or size over three months.

It is worth noting that these vesicles can expand as CO_2 is passed through the PEO-*b*-PAD solution. As shown in Figure 2c, much larger intact vesicles with a diameter of (205 ± 25) nm are found after CO_2 treatment for 20 min. For these aggregates, $R_h = (120.5 \pm 9.2)$ nm, as determined by DLS (Figure 2d), corresponding to the TEM results. Compared with its counterpart without gas stimulus, the volume of these new vesicles after gas stimulus increases strikingly by 835 % and the wall thickness is halved ((12.5 ± 2.0) nm). The evolution of the average vesicular radius is plotted versus the duration of gas treatment in Figure 3. It is clear that the curve is discontinuous at a time of 20 min: one segment extends from start to 20 min, while the other covers the range from 20 min to the end of the experiment. In the first part, the radius increases linearly by a total of 95 % (from 59.4 to 120.5 nm). In the second part, it maintains a plateau (from

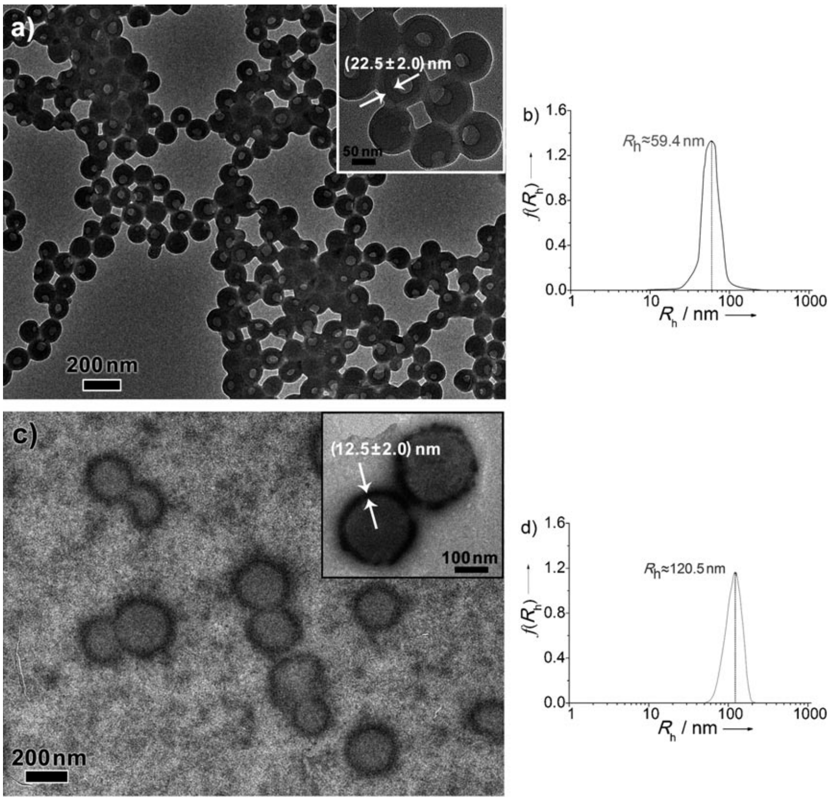


Figure 2. a) TEM image and b) DLS data for self-assembled PEO-*b*-PAD aggregates in the absence of CO_2; c) TEM image and d) DLS data for self-assembled PEO-*b*-PAD aggregates in the presence of CO_2. Insets are magnified images.

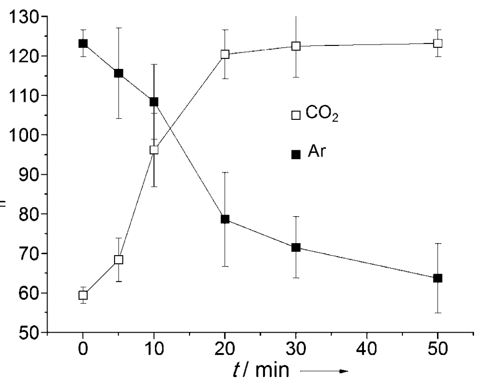


Figure 3. Reversible change of the average vesicular radius as a function of the time of CO_2 or Ar exposure.

120.5 to 123.2 nm). These findings suggest that the vesicular radius follows a trend analogous to the variation of δ. Moreover, it is interesting to stress that these vesicles can shrink back to their initial size in the presence of Ar. A possible explanation for the observation of volume swelling or collapse could be the fusion or fission of these polymer vesicles. Static light scattering experiments, however, show that the aggregate number $\langle N_{agg} \rangle$ of these vesicles before and after treatment with CO_2 varies only slightly from 1.82×10^2 to 1.68×10^2, thus eliminating the above possibility (Supporting Information, Table S1). Thus, in view of the reversible expansion and contraction in each polymersome in response to gas, the shape evolution of the vesicles is in many ways reminiscent of "breathing" features.[11] The two extreme sizes can be identified as the beginning and end of the breathing mode.

In general, a double hydrophilic block copolymer is destined to dissolve in water, while an amphiphilic one will self-assemble into structures such as vesicles. In the case of PEO-*b*-PAD, although the PAD block has been transformed into a polyelectrolyte upon CO_2 treatment, the vesicles only swell and do not disassemble. A key factor is the incomplete protonation of PAD in CO_2 environment. To further validate this viewpoint, a zeta potentiometer was employed to detect the change of surface charge in the vesicles.[12] Before CO_2 was added, the solution has a weak signal ($\zeta_{CO_2} = +0.5$ mV), thus indicating that the initial vesicles carry few surface charges. The value of ζ_{CO_2} progressively climbs to $+28.6$ mV, accompanied by a reduction in solution pH value from 6.94 to 5.68 upon CO_2 treatment for 20 min. However, even when the duration of CO_2 addition is prolonged, ζ_{CO_2} and the pH value both remain constant (Figure 4a, gray dot in 3D and gray square in 2D). In a controlled pH titration experiment, we recorded the continuous variation of ζ_{pH} and pH of PEO-*b*-PAD solution by means of a slow, homogeneous decrease of the pH value.[13] In contrast to CO_2 treatment, ζ_{pH} is analogous to ζ_{CO_2} in the same pH region of 7.0–5.68, whereas ζ_{pH} abruptly increases from $+24.3$ to $+69.8$ mV when the solution pH value is in the region of 5.68–3.40 (Figure 4a, black dot in 3D and black square in 2D). According to the pK_a of PAD, we can calculate that full protonation of the PAD chain ($\delta \approx 1$) takes place at pH 3.40, which allows us to estimate the value of δ ranging from 0 to a maximum of 0.41 ($\delta = \zeta_{CO_2}/\zeta_{pH,max}$) upon CO_2 treatment. This finding is consistent with the result of conductivity experiments (0 to 0.37).

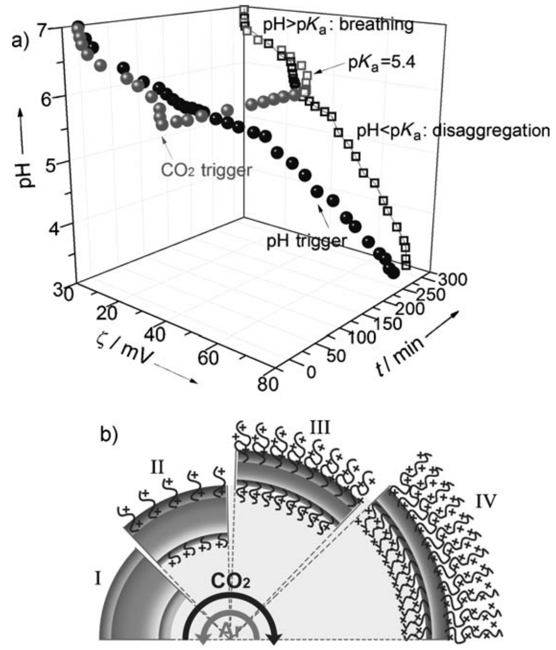
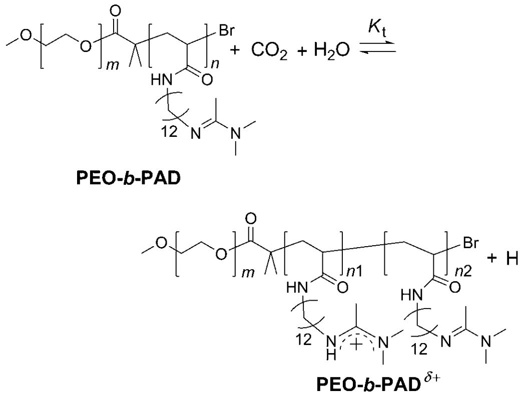


Figure 4. a) The change of zeta potential and pH value of the PEO-*b*-PAD solution as a function of time upon CO_2 treatment and upon continuous reduction of the pH value in a controlled titration experiment. b) Schematic illustrations of the changes of the vesicular radius and internal structures in response to CO_2 and Ar.

From these results, a δ-driven vesicular breathing mechanism has been proposed: 1) In the absence of CO_2, the vesicles present a common one-layer wall structure with corona on each side (Figure 4b, state I).[14] 2) In the presence of CO_2, the charged PAD species are extruded to solution, forming a new corona, whereas uncharged PAD species still serve as the inner layer.[15] To compensate the electrostatic repulsive force in the corona, these vesicles are obliged to expand to achieve lower interaction free energies (states II and III). 3) Limited by the protonation equilibrium of PEO-*b*-PAD [$K_t = 0.1$, Eq. (2)] and the final acidity pH > pK_a upon CO_2 treatment, the degree of protonation is insufficient ($\delta <$ 0.41) to transform PAD into an entirely hydrophilic chain. Hence the vesicles can only swell to a given degree (state IV). DLS experiments further support this conclusion. When the



(2)

pH value is higher than the PAD pK_a of 5.4, the vesicles can "breathe"; once the pH value is lower than the pK_a, the vesicles disaggregate into fragments, as each PAD chain carries a sufficient number of positive charges to become water-soluble (Supporting Information, Figure S7). 4) The alternating changes of the vesicular interfacial tension between 32 and 55 mN m^{-1} upon CO_2 and Ar treatment confirms their reversible swelling and collapse (Supporting Information, Figure S8).[16]

We developed this kind of CO_2-responsive vesicle to perform drug-release experiments. Fluorescent rhodamine B (RB) was used as a model, and the RB-loaded vesicles were dialyzed against water until the water outside the dialysis tube exhibited negligible fluorescence. To understand the quantity of RB release upon CO_2 or Ar treatment, the excitation outside the tube was recorded by fluorescence spectrum.[7] The plot of release percentage versus time for different stimulus cases is shown in Figure 5. Clearly, these nanocapsules display a low-level free release (less than 35 % over 10 h) without stimuli. When CO_2 is added, the release quantity has a sudden increase to 25 % within 1 h and then remains roughly constant for the rest of the experiment. In this case, the greatly expanded surface area of the vesicles (from 0.044 to 0.18 μm^2) increases the membrane permeability and causes an accelerated RB leak. When we apply alternating gas stimuli with CO_2 and Ar, more interestingly, the release rate presents periodic acceleration–plateau features. We infer that this unprecedented result can be ascribed to the extension–retraction motion of the vesicles.

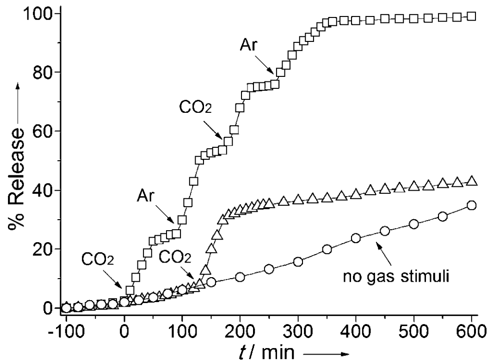


Figure 5. Controlled release of RB from the vesicles without stimuli (○), with only CO_2 stimulus (△), and with alternating CO_2/Ar stimuli (□).

In conclusion, we have designed and developed a new class of amidine-containing block copolymers. They can spontaneously form vesicles in aqueous media on the basis of their amphiphilicity. Specific functionalities in the polymer endow these vesicles with unique gas-responsivity. CO_2 can tune the size of these vesicles over a wide range by controlling the degree of protonation of amidine species. Alternating treatment with CO_2 and Ar realizes a smart expansion and contraction cycle of these vesicles, which can be considered as "breathing" nanocapsules. In contrast to other reported polymeric vesicles with changeable sizes,[11,17] these gas-sensitive ones have the advantages of their unique trigger mode, template-free synthesis, extensive size-tuning range, and excellent reversibility. As drug-delivery vehicles, the vesicles can control release in a tunable time and speed. Further studies inside cells are prospective. We envisage that this kind of CO_2-responsive nanocapsule will open up a new pneumatotherapeutic pathway.

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