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Proton Diffusion across Membranes of Vesicles of Poly(styrene-*b*-acrylic Acid) Diblock Copolymers

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Abstract: A study of proton diffusion across membranes of block copolymer vesicles in dilute solution is described. The vesicles were formed by the self-assembly of a diblock copolymer of poly(styrene-*b*-acrylic acid) (PS_{310}-*b*-PAA_{36}, where the numbers represent the degree of polymerization for individual blocks). A pH gradient was created across the vesicle membrane with the interior pH (pH_{in}) of ca. 2.9 and the exterior pH (pH_{out}) of ca. 8.5. The permeability of the polystyrene (PS) membrane was tuned by the addition of different amounts of dioxane (0−40 wt %) to the external aqueous solution. Proton concentrations in the solution outside of the vesicles were followed by monitoring the spectrum of a pH-sensitive fluorescent dye, namely 8-hydroxypyrene-1,3,6-trisulfonate. After the start of the experiment, the proton concentrations increase linearly with the square root of time, while the slopes of the lines increase with dioxane content. To calculate the diffusion coefficients of the protons across the vesicular membrane, the concentration data were fitted using a model, which describes the diffusion of species across the membrane of a reservoir. The apparent diffusion coefficient (D^*, which equals the true diffusion coefficient multiplied by the partition coefficient of protons between PS and water) increases from 1.1×10^{-18} cm^2/s at 7 wt % dioxane in the external solution to 1.2×10^{-14} cm^2/s at 40 wt %. The increase of D^* with dioxane content is related to its plasticization of the PS membrane, which can be used as a gating mechanism.

Introduction

Block copolymer vesicles have attracted considerable attention since Zhang and Eisenberg showed that this morphology is part of a morphological continuum for amphiphilic diblock copolymers in selective solvents.[1] Vesicles belong to a morphological family of polymeric aggregates, which also include spheres, rods, and many others.[2] All these aggregates can be formed by the self-assembly of block copolymers in solution. Block copolymer vesicles are hollow spheres with a hydrophobic membrane or wall and a hydrophilic corona on the inner and outer interfaces.[3]

Due to the robustness and stability of their wall, block copolymer vesicles are being explored in a variety of applications such as catalysis, drug delivery, and others where protection of contents against a hostile environment is useful. Before some of these applications can be exploited fully, especially those in which the vesicle structure is preserved, it is necessary to understand the transport of various species through the walls. While transport in and out of liposomes has been explored extensively, no comparable literature exists for block copolymer vesicles.

Recently, Choucair et al. loaded a drug, doxorubicin, into a vesicle system formed by an amphiphilic block copolymer of poly(styrene-*b*-acrylic acid) (PS-*b*-PAA) and explored the diffusional release of the drug from the vesicles.[4] Since the block copolymer vesicles have a thicker membrane (ca. 30 nm) than that of liposomes (3−5 nm) and also since PS has a much higher glass transition temperature (T_g) than that of lipids, the rate of drug diffusion through block copolymer vesicle membrane is slow. Therefore, Choucair et al. tuned the permeability of PS membrane by adding different amounts of dioxane to the external solutions of the vesicles. Dioxane partitions between the PS wall and aqueous phase so that the glassy vesicle membranes become plasticized. In a study of the partitioning of dioxane between homopolystyrene and water, Yu et al. showed that the dioxane content in the polystyrene-rich phase decreases from ca. 65 to ca. 40 vol % as the dioxane content in the aqueous solution decreases from 91 to 83 wt %.[5] In an equilibrated vesicle solution in a dioxane/water mixture, the

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dioxane partitions between the water and PS wall; the higher the dioxane content in the external solution, the higher the dioxane content in the PS wall, and the greater the permeability. While the dioxane contents cited above are considerably higher than those used for plasticization of vesicle walls, extrapolations suggest that even at the dioxane contents in water of 7−40 wt % used here, the dioxane contents of the PS wall should be significant. In a completely different approach to vesicle wall permeability, Ahmed and Discher showed that the permeability of the membranes of vesicles formed from poly(ethylene glycol-*b*-butadiene) (PEG-PBD) could be modified by blending a degradable copolymer, such as poly(ethylene glycol-*b*-lactic acid) (PEG-PLA), into the membranes of these PEG-PBD vesicles.[6]

Choucair et al. compared two methods for incorporating doxorubicin into the polymeric vesicles: passive loading and active loading. The difference between the two methods is that during the active loading process, there is a trans-membrane pH gradient ($pH_{in} = 2.5$; $pH_{out} = 6.3$), while no such gradient exists for passive loading ($pH_{in} = pH_{out} = 2.5$). In both cases, doxorubicin was added to the preformed vesicle solutions. For active loading, the amount of drug incorporated was related to the interior and exterior pH values. It was suggested that only the neutral form of the drug can diffuse through the PS membrane and that the concentrations of the neutral form, once diffusion equilibrium is reached, are equal on both sides of the membrane. The drug molecules become protonated once they are inside the vesicles, and the charged species do not diffuse out readily. Because of the lower pH inside the vesicles, the drug accumulates inside the vesicles, in contrast to the situation during passive loading. Successful drug accumulation inside the vesicles depends on the maintenance of a pH gradient during loading. In the active loading study by Choucair et al., it was found that, when the dioxane content was lower than 50 wt %, the loading efficiency increased with the dioxane content. However, a dioxane content higher than 50% led to a decrease in the loading efficiency, which was attributed to the progressive disappearance of the pH gradient due to the leakage of protons.

Although the presence of a pH gradient plays an essential role for a high loading efficiency during active loading, studies on how a pH gradient across vesicular membrane evolves are rarely seen in the literature even though proton diffusion across polymeric membranes such as Nafion has been studied extensively in the past.[7] One report is available on the lifetime of the pH gradient in liposomes.[8] A second report is available on the measurement of the initial change in absorbance at a certain wavelength of trapped pH indicator dyes (bromcresol green and bromthymol blue) in liposomes upon external imposition of a pH difference.[9] However, no reports could be found on the rate of proton diffusion either through the membrane of liposomes or of block copolymer vesicles. Approaches to characterize the pH gradient across liposome membranes include characterizing the distribution of radioactive or fluorescent amines with appropriate pK_a values[10] or using pH-sensitive fluorescent dyes such as 8-hydroxypyrene-1,3,6-trisulfonate (HPTS).

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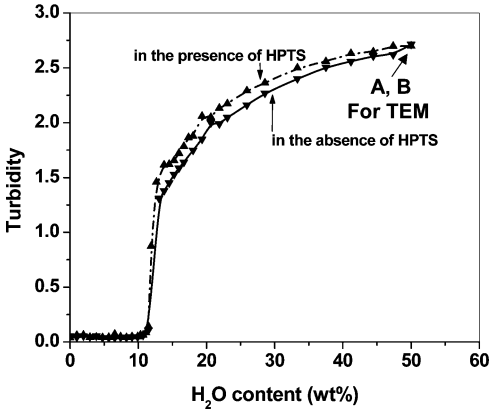

Figure 1. Turbidity profiles of a solution of PS_{310}-*b*-PS_{36} in dioxane upon adding water (lower curve) or HPTS solution (2.6 μM) (upper curve) to a final water content of 50 wt %.

In the amine distribution approach, a radioactive or fluorescent amine was introduced to a liposome solution. The pH gradient was deduced from the ratio of the amine concentrations inside and outside the vesicles. This approach allows one to measure the pH gradient statically. Another approach to characterize the pH gradient across vesicle membrane involves using pH-sensitive dyes. For example, Clement and Gould[8] studied the internal pH response of phospholipid vesicles to an external pH perturbation. HPTS was incorporated only into the interiors of liposomes. It was found that the relaxation time of the internal pH following the perturbation of pH in the exterior of vesicles was of the order of a few minutes.

In the present report, we describe experiments that enable us to quantify the proton diffusion behavior across vesicle membranes. These experiments also provide insight to the permeability of a PS membrane and its sensitivity to dioxane content in the exterior solution. A pH gradient was created across the vesicle membrane with pH_{in} of ca. 2.9 and pH_{out} of ca. 8.5. The proton concentration outside of the vesicles was followed as a function of time by monitoring the spectrum of a pH-sensitive fluorescent dye, HPTS. Apparent diffusion coefficients of protons across the vesicle membrane were calculated. The question was also addressed whether proton diffusion out of the vesicles or the compensating sodium ion diffusion into the vesicles is rate-determining. To our knowledge, this is the first study of proton diffusion across block copolymer vesicle membranes.

Experimental Section

For experimental details, refer to the Supporting Information.

Results and Discussion

HPTS as a pH-Sensitive Fluorescent Dye. Refer to the Supporting Information for how HPTS was applied in the current study.

Effect of HPTS on the Self-Assembly of PS-*b*-PAA. To explore whether HPTS has an effect on the self-assembly of PS_{310}-*b*-PAA_{36}, we studied its influence on the turbidity of a solution during water addition and on the final morphology of the resulting aggregates. Figure 1 shows the solution turbidities during vesicle formation both in the presence and in the absence of HPTS. One sees that the presence of HPTS does not affect the position of the critical water content (cwc; 12 wt % in this case), at which the polymers start to aggregate. Beyond the cwc,

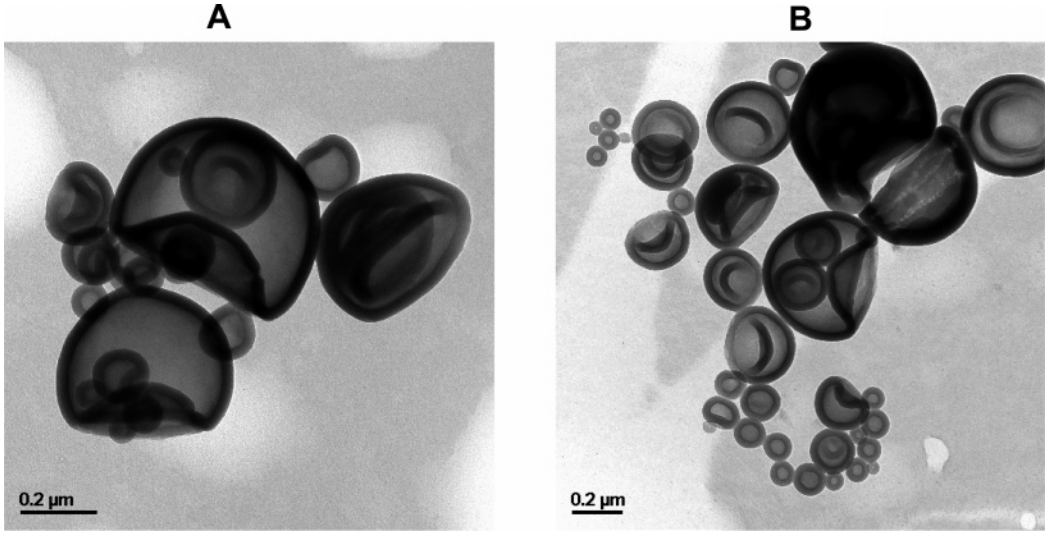

Figure 2. TEM images of vesicles formed from the self-assembly of PS_{310}-*b*-PAA_{36} in dioxane induced by adding pure water (A) (vesicle diameter = 150 ± 75 nm; membrane thickness = 32 ± 5 nm) or HPTS solution (2.6 μM) (B) (vesicle diameter = 175 ± 90 nm; membrane thickness = 33 ± 6 nm), A, B in Figure 2 correspond to points with the same letter in Figure 1 at which the samples were taken for TEM imaging.

the turbidity of the solution in the presence of HPTS is only slightly higher than that in its absence.

Figure 2A shows the TEM images of typical vesicles prepared in the absence of HPTS. The vesicles have an average size of ca. 150 ± 75 nm and a membrane thickness of ca. 32 ± 5 nm. Vesicles formed in the presence of HPTS have a somewhat larger average size (175 ± 90 nm, Figure 2B). The thickness of PS membrane (33 ± 6 nm) remains essentially the same.

From the results presented in Figures 1 and 2, we conclude that the presence of HPTS does not have a significant effect on the course of the self-assembly of PS_{310}-*b*-PAA_{36} or on the final morphology of the aggregates under the current experimental conditions. In the experiments involving pH determination of the external solution, the HPTS was added to the vesicle solutions after the vesicles had been formed, in which case the dye molecules are not, in principle, in equilibrium across the vesicle wall. However, since the presence of HPTS does not alter significantly the shape and size of vesicles thermodynamically (Figure 2B), we conclude that the presence of HPTS would not influence the morphology of the vesicles during our exterior pH determination experiments. In addition, we note that the HPTS concentration (2.6 μM) used for the experiments, the results of which are shown in Figures 1 and 2, was 26 times that (ca. 0.1 μM) of solutions used for the external pH determination.

Validity of Our Approach for the Study of Proton Diffusion. It is important to demonstrate the applicability of the approach used in the present study to measure proton diffusion. The method is based on measuring the pH of the solution outside of the vesicles. A uniform distribution of HPTS in the exterior solution and its absence or presence in, at most, negligible amounts in the interior of the vesicles are essential for this measurement. Because of its polyanionic character, HPTS does not bind significantly to the PS-*b*-PAA vesicle surface, which has a net negative charge under experimental conditions. Thus, a uniform HPTS distribution in the external solution is believed to exist.

In view of the size and ionic character of the HPTS, it is likely that the diffusion through the PS wall of the vesicle is very slow. Furthermore, the negative charge of the corona is likely to repel HPTS molecules approaching the vesicle. It should also be noted that there is no conceivable preference for the localization of HPTS in the interior of the vesicles, as there might be, for example, for a weakly basic dye. If, despite these aspects, HPTS molecules do diffuse into the interior of the vesicles, the maximum possible amount of HPTS in the interior relative to that in the exterior is likely to be the same as the volume ratio of the interior and the exterior. In the present system (average diameter = 150 nm; block copolymer composition = 92.5 wt % PS; average membrane thickness = 32 nm; polymer concentration = 0.05 wt %), the ratio is 0.0001 (or 0.01 vol %). One can thus conclude that the experimental spectra reflect the pH of the exterior of the vesicles.

The PAA in the external corona of the vesicles is not likely to complicate the proton diffusion process because, under the prevailing external pH at the beginning and end of the experiment (8.5 and 6.4), the PAA would be completely ionized, taking into account the pK_a of PAA, which is between 4 and 5.

Next, we turn our attention to the estimation of the internal pH of the vesicles. This pH has to be low enough so that a detectable signal is created when protons diffuse from the interior to the exterior of the vesicles. For this purpose, a model experiment was carried out by measuring the pH of an acetic acid solution with a concentration similar to that of AA units (2.5 M) in the interior of the vesicles. The concentration of AA units inside the vesicles was calculated to be ca. 2.5 M (0.02 mg PAA/mL solution) by assuming that the amounts of AA units on the internal and external surfaces are equal, a reasonable assumption for the vesicles found here (D_{ave} = 150 nm). Since the pH of the acetic acid in water (2.5 M) is ca. 2.9, we assume that the pH of the interiors of vesicles is similar to this value. Attempts to measure the internal pH independently were not successful.

Proton Diffusion across the Membranes of Block Copolymer Vesicles. Before the external pH determinations, specific amounts of dioxane were added to the individual vesicle solutions. The external pH was then adjusted to 8.5 ± 0.2 before adding the HPTS in the last step. Figure 3 shows the changes

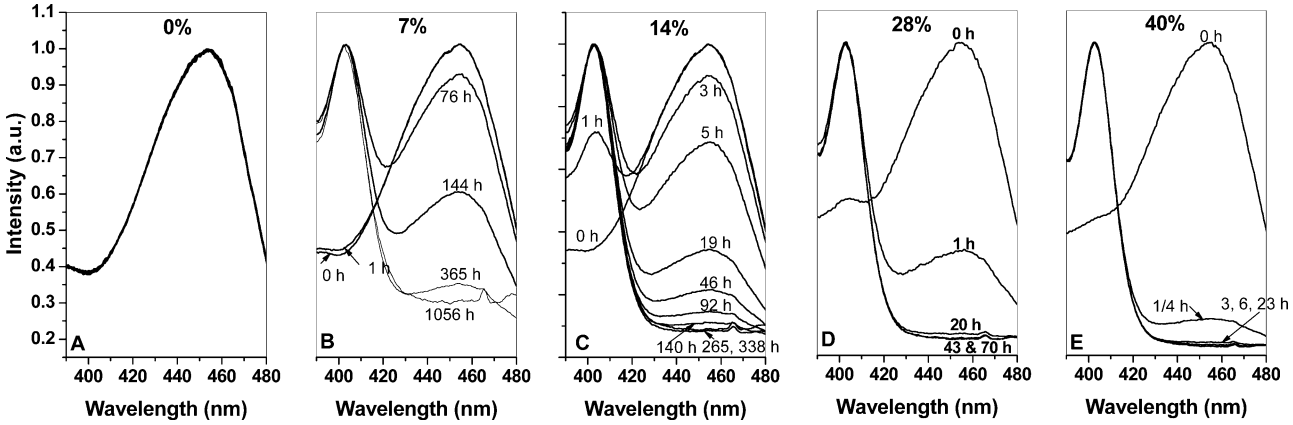

Figure 3. Time evolution of fluorescence excitation spectra from vesicle solutions with added HPTS at different dioxane contents. (A) 0 wt %. (B) 7 wt %. (C) 14 wt %. (D) 28 wt %. (E) 40 wt %. All spectra obtained at different times for the 14 wt % dioxane sample are shown in C. In B, D, and E, most were omitted for the sake of clarity. In A, all spectra overlap.

in the HPTS spectrum as a function of time for solutions containing different amounts of dioxane. At 0% dioxane, the HPTS spectrum remains constant for up to 300 h within experimental error. Figure 3A shows a superposition of ca. 20 spectra obtained at different times. We attribute the constancy of the spectrum to the high glass transition temperature (T_g) of PS (ca. 100 °C), which prevents protons from diffusing across the vesicle membranes at room temperature.

Figure 3B−E shows the evolution of the spectra at dioxane contents of 7, 14, 28, and 40 wt %, respectively. In all of these cases, the initial exterior pH was ca. 8.5. After 1 h at 7 wt % dioxane, the pH decreased to 8.48 (almost no change), at 14 wt % to 7.60, at 28 wt % to 6.87, and at 40 wt % to 6.12. Clearly, the higher the dioxane content, the more rapidly the pH drops. In the presence of 40% dioxane, the time required to reach steady state is ca. 2 h. The approximate equilibration times for 28, 14, and 7% dioxane are ca. 12, 35, and 450 h, respectively. Approximate equilibration times were estimated from the time point beyond which the spectrum remains identical. A further experiment, which was performed to ascertain that pH equilibrium had indeed been reached, was to heat the vesicle solution to 80 °C for half an hour and then to cool it to room temperature. The spectrum remained constant. Since the experiments described here involve applying dioxane (up to 40 wt %) into the exteriors of vesicle solutions, it is important to check the maintenance of the vesicular morphology in the presence of this amount of dioxane. In one experiment, we quenched the vesicle solution containing 40 wt % dioxane after the diffusion study. TEM images showed that the vesicular morphology was maintained with the diameter and the membrane thickness of the vesicles similar to those before adding dioxane and the diffusion study.

Proton concentrations, obtained from corresponding pH values, were plotted against the square root of time; the results are shown in Figure 4. At 0 wt % dioxane, the line is flat, indicating no change of proton concentration in the exterior solution. At 14, 28, and 40 wt % dioxane contents, the proton concentrations increase linearly with the square root of time. The slope of the line increases with dioxane content. Interestingly, two linear segments with an intersection at ca. 16 h were observed for the sample with 7 wt % dioxane (see inset in Figure 4). This suggests that there is an induction period for proton diffusion in this system. We are not clear about its origin. We

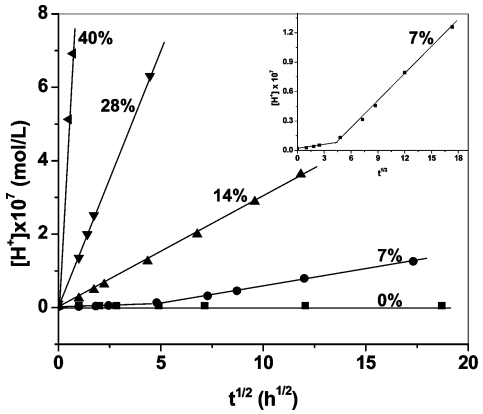

Figure 4. Plots of proton concentration [H^+] against the square root of diffusion time *t*.

speculate that this effect might be due to the relatively long time that it may take for the dioxane to plasticize the glassy PS wall at 7 wt % of dioxane in the water. As for all cases of diffusion out of containers of limited size, the diffusion rate decreases significantly when the internal protons are close to equilibrium, a phenomenon that manifests itself by lower slopes for times beyond those shown in Figure 4 (the longer time data are not shown).

We fit the data in Figure 4 with the equation below (see the Supporting Information for the derivation of this equation):

$$\frac{dC_{out}}{dt^{1/2}} \approx C_{in}\sqrt{\frac{2NV_{in}ADK_{H^+}}{V_{out}L}}$$

where C_{in} and C_{out} are proton concentrations in the interior and exterior of the vesicle, respectively, t is the time of diffusion, A is the average outer surface area of the vesicle (7.1×10^{-10} cm^2), D is the proton diffusion coefficient, K_{H^+} is the proton partition coefficient between polystyrene wall and water, N is the number of vesicles per unit volume (3.1×10^{11} per mL), V_{in} and V_{out} are internal and external volumes, respectively, and L is the thickness of the vesicle membrane. By replacing DK_{H^+} with an apparent diffusion coefficient (D^*) since K_{H^+} is not known, D^* can be calculated from the slopes of the curves in Figure 4, using the equation above. The D^* values are 1.1×10^{-18}, 1.2×10^{-17}, 2.5×10^{-16}, and 1.2×10^{-14} cm^2/s for the cases of 7, 14, 28, and 40 wt % dioxane content, respectively.

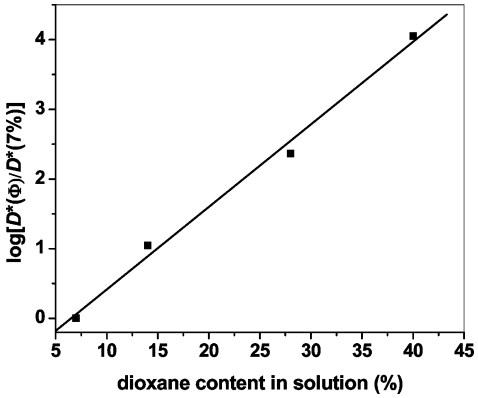

Figure 5. Plot of log[$D^*(\Phi)/D^*(7\%)$] against dioxane content.

One sees that as the dioxane content increases from 7 to 40 wt %, D^* increases by approximately 4 orders of magnitude. For the release of doxorubicin from PS-*b*-PAA vesicles, the diffusion coefficient was less sensitive to dioxane content, ranging from 7.8×10^{-17} cm^2/s when the dioxane content was 0 wt % to 6.0×10^{-16} cm^2/s when the dioxane content was 50 wt %.[4] If $K_H{}^+$ is independent of dioxane content, D^* and D are directly related. If, on the other hand, $K_H{}^+$ is not independent of dioxane content in the PS wall, then there will be a systematic divergence between the two.

Figure 5 shows a linear plot of log[$D^*(\Phi)/D^*(7\%)$] (where D^* at 7 wt % dioxane serves as a reference point) versus the dioxane content Φ. Linearity in semilog plots is frequently observed for the rate of polymer diffusion in the presence of different amounts of plasticizer at temperatures higher than T_g.[11] The presence of plasticizer lowers the T_g (by increasing the free volume) of the system so that a higher diffusion coefficient results. Although the T_g of the PS membrane in the presence of dioxane is not known and the diffusing species is a proton instead of a polymer chain or the plasticizer molecule itself, it is not unexpected that the increase of the rate of proton diffusion at higher dioxane contents is related to its plasticizing effect.

Finally, it should be recalled that during the proton diffusion process, electroneutrality must be maintained. When protons diffuse out of the cavities of the polymer vesicles, sodium ions must diffuse into the vesicles to compensate for the loss of positive charge. Studies of ionic mobility in aqueous solution have shown that proton diffusion is much faster than that of sodium ions [ionic mobility (relative to K^+): H^+: 4.8, Na^+: 0.7[12]] because the Grotthus mechanism is operative. It is therefore worth inquiring whether, under our experimental conditions, the diffusion of sodium ions is the rate-determining step rather than that of the protons. To determine which of the two cations is rate-determining, an experiment was performed in which the sodium ion concentration in the external solution was changed by a factor of 3 by the addition of NaCl to increase the sodium ion concentration by 20 mM. In the original solution, the sodium ion concentration from the NaOH used to maintain the pH was estimated to be 6 mM. We are thus comparing two solutions with extravesicular sodium ion concentration of 20 and 6 mM. In both cases, the external dioxane contents were the same (ca. 25%). We found that the rate of proton diffusion was independent of the exterior sodium ion concentration.

It is worth considering whether the Grotthus mechanism might be contributing to the proton diffusion process. In this connection, the following consideration should be borne in mind: as was pointed out before, the dioxane concentration in PS decreases from 65 to ca. 40 vol % as the water content of the external solution increases from 9 to 17 wt %.[5] In the present study, the water content in the external solution ranged from 60 to 100 wt %; therefore, the dioxane concentration inside the PS will be expected to be considerably lower than 40 vol %, possibly in the range of 10−20 vol %. The water content of this PS-rich mixture would be expected to be very low, not conducive to the presence of a continuous water path in the sample. The absence of a continuous path makes the possibility of a Grotthus mechanism of proton transport highly unlikely.

Summary

Proton diffusion across the membrane of block copolymer vesicles was investigated. A pH gradient was created across the vesicle membrane with a pH inside (pH_{in}) of ca. 2.9 and a pH outside (pH_{out}) of ca. 8.5. The permeability of the PS wall was tuned by adding different amounts of dioxane (0−40 wt %) to the external solution. The external proton concentrations increased linearly with the square root of time, and the slopes of the lines increased with dioxane content. The apparent diffusion coefficients D^* of protons at different dioxane contents in the external solution increased from 1.1×10^{-18} cm^2/s when the dioxane content was 7 wt % to 1.2×10^{-14} cm^2/s when the dioxane content was 40 wt %. The increase in the diffusion coefficient was attributed to plasticization of the PS membrane by the dioxane, which represents a convenient control mechanism for the diffusion process.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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