VIP Polymer Vesicles

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Polymer Vesicles Containing Small Vesicles within Interior Aqueous Compartments and pH-Responsive Transmembrane Channels**

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Intermolecular packing of amphiphilic block copolymers into vesicles is of particular interest, owing to the fundamental importance of such systems as a new class of polymer assemblies with well-controlled structures and potential biomedical applications.[1–4] Similar to conventional liposomes, polymer vesicles usually form a continuous bilayer structure primarily consisting of the hydrophobic blocks of copolymers, but they exhibit markedly enhanced stability and feasibility of incorporating functional groups in response to external stimuli.[5] However, the major limitation of polymer vesicles as biofunctional containers arises from the lack of permeation pathway for hydrophilic cargoes owing to the requirement to maintain the architectural integrity.[6,7] The vesicles obtained from block co-polypeptides are imparted responsive channels upon the pH-induced conformational change of a polypeptide block.[7] Redox control of the permeability of multilayer microcapsules containing poly(ferrocenylsiliane) was reported.[8] Incorporating channel-forming proteins into the vesicle membranes while fully retaining the protein functions represents an important paradigm of equipping polymer vesicles with transmembrane channels.[9,10] Thus, the transport mechanism, being either size-selective or substrate-specific, can be tailored by the pore proteins selected. It is also desirable to have versatile vesicular assemblies that contain small vesicles within the interior aqueous compartments in a manner similar to discrete organelles within eukaryotic cells, which perform diverse functions and are one of the feature differences from prokaryotic counterparts. Unfortunately, such assembly structural control has not yet been achieved. Herein, we show the first example of polymeric multivesicle assemblies similar to the architectural arrangement of eukaryotic cells, in which both the vesicle membranes are equipped with pH-responsive channels permeable for hydrophilic solutes (Scheme 1).

Copolymers comprising acrylic acid (AAc) and acrylate of 1,2-distearoyl-*rac*-glycerol (distearin acrylate, DSA) were obtained from partial transesterification of poly(*N*-acryloxysuccinimide) (poly(NAS)) with distearin and then thorough hydrolysis of the unreacted NAS to AAc units. Polymer vesicles were prepared by a double emulsion technique in a water/oil/water ($w_1/o/w_2$) system, in which the copolymer was dissolved in the organic phase prior to emulsification. The experimental methods are described in detail in the Supporting Information. THF/CH_3Cl solutions of varying ratios, depending on the target vesicle size, were employed as the organic phase. Either water or buffers in the pH range of 4.0–5.5 were used as both the inner (w_1) and outer (w_2) aqueous phases. The vesicles formed upon the evaporation of organic solvents in $w_1/o/w_2$ emulsions. However, the copolymers assembled into micelles above pH 5.5 and large precipitates below pH 4.0. The vesicles were obtained mainly from copolymer with an average molecular weight of $2.97 \times 10^5\ g\,mol^{-1}$ and a composition of 9.1 mol% DSA, unless stated otherwise. Figure 1a confirms that the resultant assemblies are unilamellar vesicles. The laser scanning confocal microscopy (LSCM) image of polymer vesicles in aqueous suspensions was revealed by the fluorescence of Nile red associated with the vesicle membranes. The lyophilized vesicles can be observed by scanning electron microscopy (see the Supporting Information). The fact that such polymer colloids maintain their structural integrity when subjected to transition from the aqueous to dried state reflects their robust stability. Transmission electron microscopy (TEM) examination of the sectioned specimens (ca. 60–90-nm thickness) of polymer vesicles indicates that the wall thickness was approximately 25 nm (Figure 1b). The vesicle size can be controlled by adjusting either the THF/CH_3Cl ratio used during emulsification or the DSA content of copolymers to give vesicles with diameters ranging from 1 to 15 μm. For example, changing the DSA content of copolymers from 9.1 to 13.1 mol% increases the vesicle diameter by 3–4 μm on the average. In contrast, increasing the THF content of the THF/CH_3Cl solution from 2 to 20% (v/v) reduces the vesicle size significantly (Figure 2) because of the increased miscibility with water and the resulting decreased interfacial tension of the polymer-containing oil droplets in the aqueous phase.

When the ionization of AAc residues increases to some extent with increasing pH value, the vesicles become equipped with transmembrane channels that are permeable for hydrophilic solutes. Figure 3 shows that, while transport of calcein (a water-soluble fluorescence probe) across the membrane was prohibited at pH 5.0, the probe molecules freely diffused into the vesicular aqueous compartment when the external pH value was increased to 8.0. Calcein was then confined within the compartment simply by adjusting the

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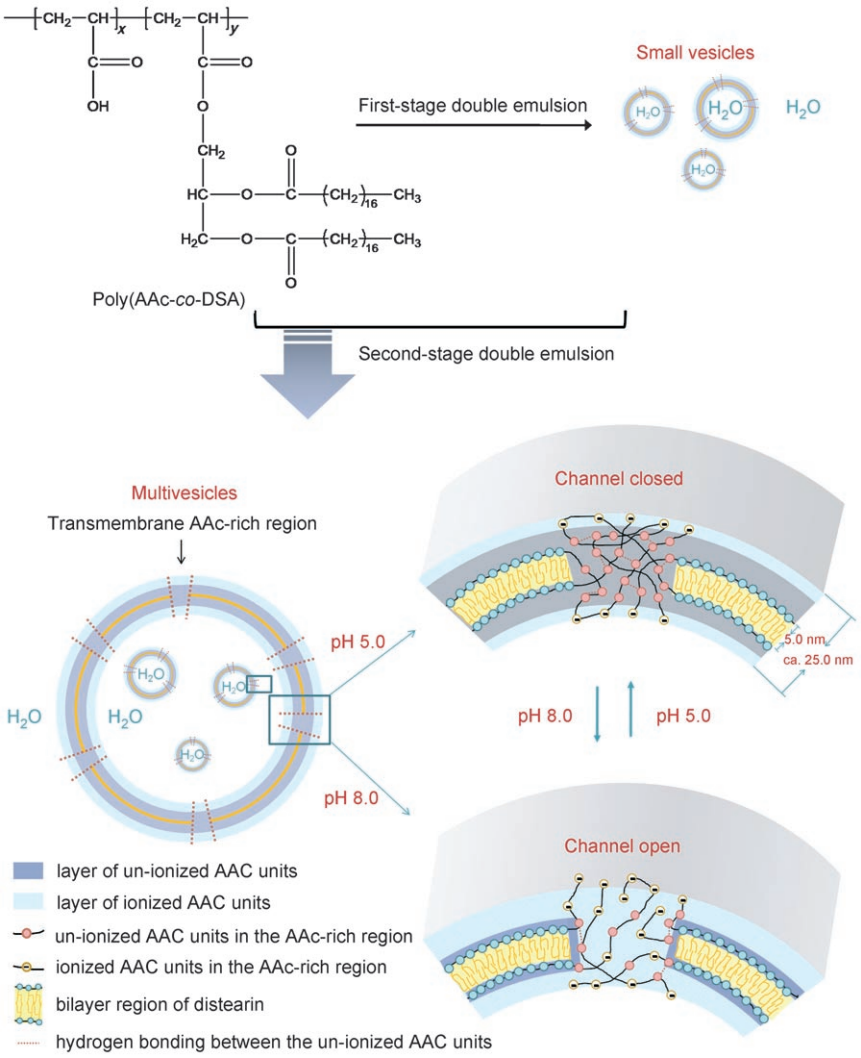

Scheme 1. Illustration of multivesicle assemblies equipped with pH-responsive transmembrane channels from two-stage double emulsion of poly(AAc-*co*-DSA). The AAc-rich regions and the bilayer islets within the vesicle membrane are not drawn to scale.

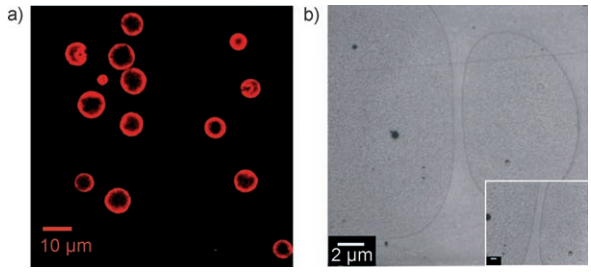

Figure 1. Images of a) polymer vesicles by LSCM and b) sectioned specimens of vesicles by TEM. The scale bar in the inset of the TEM image represents 500 nm.

pH value back to 5.0. This pH-responsive on/off process is reversible, and it relies on change in pH value in a narrow range between 6.4 and 6.5. These channels are also accessible to large cargos such as hemoglobin (see the Supporting Information). Polymer vesicles enclosing myoglobin or hemoglobin as oxygen carriers were reported.[6,11] The pH-responsive vesicles presented herein offer an alternative pathway to encapsulate chemicals (e.g. globular proteins) that are incompatible with organic solvents used in vesicle preparation.

A molecular packing model is illustrated in Scheme 1. The distearin grafts of copolymers are packed into discrete bilayer regions by hydrophobic association. The discrete bilayer islets act not only as nucleation loci for vesicle-wall formation but also as anchors for effectively stabilizing the membrane structure. These bilayer islets are surrounded by un-ionized AAc-rich regions. The separation of the two distinct regions is ascribed to the hydrophobicity difference. With the thickness of the DSA bilayer islets estimated to be at most 5 nm and that of the vesicle membranes approximately 25 nm, the AAc-rich layers residing above and below the bilayer islets are approximately 10 nm in thickness on the average. The un-ionized AAc-rich regions contribute markedly to the stabilization of vesicles by polymer-chain entanglements and covalent connections of polymer backbones to DSA residues in the bilayer islets in addition to the extensive hydrogen bonding and hydrophobic association. The outermost charged AAc surface layers prevent vesicles from aggregating. The un-ionized AAc-rich regions on the sides of the bilayer islets (parallel to the aligned lipid chains) are responsible for forming pH-responsive channels. It is recognized that the AAc (co)polymers embedded within sufficiently hydrophobic and compact domains are more

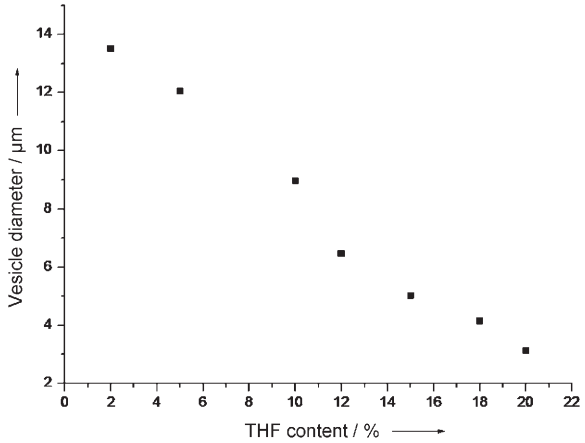

Figure 2. Dependence of the number-average vesicle size on the THF content of organic THF/CH_3Cl solutions in preparing polymer vesicles.

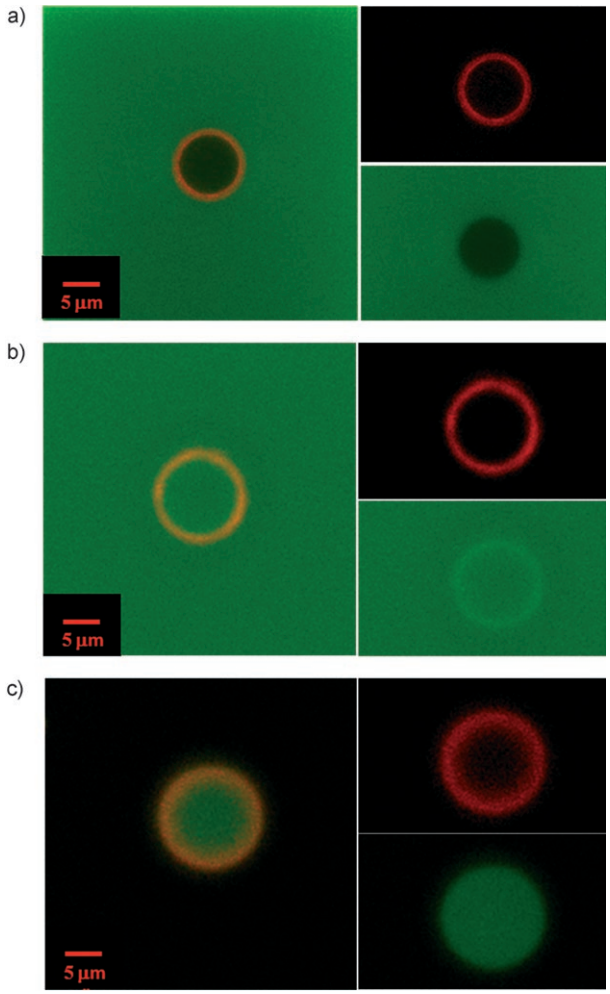

Figure 3. LSCM images of Nile-red-stained vesicle suspensions a) with the addition of calcein at pH 5.0 (calcein could not enter the vesicle), b) after pH adjustment to 8.0 (calcein diffused into the vesicle), and c) after replacement with fresh buffer of pH 5.0 (calcein was confined within the vesicle). Each multistained image (left) was derived from combining the two images to its right, in which the upper image was obtained at $\lambda_{ex} = 543$ nm and $\lambda_{em} = 560$–700 nm (red color arising from Nile red) and the lower at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 505$–550 nm (green color arising from the combination of calcein and Nile red). The intense colors of the membranes (yellow on the left photographs or green on the lower right) were caused by the fluorescence of Nile red associated with the membranes (see the Supporting Information).

difficult to deprotonate than those in an extended state.[12] Hence, with increasing pH value of vesicle suspensions, AAc ionization occurs mainly in the AAc-rich regions (those that lack DSA), including the transmembrane channels. When the pH value is increased to 6.5, the channels become permeable upon abrupt disruption of hydrogen bonds and hydrophobic association of un-ionized AAc units by the presence of a critical amount of ionized AAc units within the channels. The tendency toward forming vesicles is largely influenced by the distribution of DSA grafts within the copolymers, the hydrophilic/hydrophobic balance, and the stiffness of copolymers,[2,13] which, in turn, is closely related to the conjugation approach and the extent of DSA and ionization degree of AAc units along the polymer backbones. As the copolymers were prepared by postmodification of polymeric precursor, it is reasonable to postulate that distribution of DSA within the copolymers occurred in a multiblock manner with pertinent main-chain spacing from the AAc units (for decoupling the motion of the polymer main chain and the lipid grafts),[14] thereby promoting the intermolecular association[15] and formation of lipid bilayers and AAc-rich channels within vesicle membranes. In contrast, randomly distributed DSA units along the copolymer backbone may result in quite different self-assembly behavior. The effects of the structure of polymeric amphiphiles, especially the hydrophilic–lipophilic balance values and geometric factors, on their assembly through intermediate micelle structure into varying types of lyotropic mesophases were reported.[16] Apart from the copolymer of 9.1 mol% DSA, vesicles can also be obtained from the copolymer of 13.1 mol% DSA in the same buffer pH-value range, in contrast to micelle-like assemblies from the copolymer of 4.3 mol% DSA, which does not form vesicles owing to the insufficient hydrophobic lipid grafts.

Owing to the vesicles' excellent stability and controllable size over a wide range, the multivesicle assemblies shown in Scheme 1 could be obtained by suspending small vesicles (prepared in the first stage) in the w_1 phase of the second-stage double emulsion process that was used to produce large vesicles. Figure 4a shows the LSCM image of multivesicle

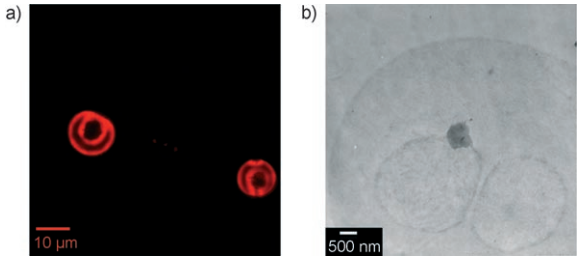

Figure 4. Images of a) multivesicle assemblies (LSCM) and b) a sectioned specimen (ca. 80-nm thickness) of the multivesicle assembly (TEM).

assemblies. The TEM photograph also confirms the architectural arrangement of multivesicle assemblies, as shown by enclosing two small vesicles within a larger one in Figure 4b. More LSCM images of multivesicles are illustrated in the Supporting Information. While the multivesicles can be readily obtained by the present approach, further control of the number of small vesicles within a large one is needed. Such assemblies represent a supramolecular organization similar to biological eukaryotic cells and their subcellular organelles. Furthermore, these synthetic vesicles, delicately implemented with pH-responsive channels, show great potential as biofunctional encapsulants by providing individual compartmentalization of varying chemicals within a vesicle and facile control of their encapsulation and release through the responses of the vesicle membrane to environmental stimuli (pH in this case).

Although many structural issues remain to be studied, such functional multivesicular architectures represent a sig-

nificant advance in mimicking eukaryotic cells and extend potential applications of polymer vesicles. Further studies concerning the effects of molecular parameters on performance properties of vesicle assemblies and precise control of the multivesicle morphology, such as the number of small vesicles in large ones and channel size, are in progress.

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