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Microreactors

Encapsulation of Myoglobin in PEGylated Polyion Complex Vesicles Made from a Pair of Oppositely Charged Block Ionomers: A Physiologically Available Oxygen Carrier**

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Hollow capsules or vesicles in the mesoscopic size range are of great interest because of their fundamental importance as new colloidal structures, as well as their potential utility in biomedicine as drug- and gene-delivery carriers, artificial cells, and bioreactors.[1–9] The most versatile method for preparing hollow capsules is the approach of molecular self-assembly.[1–5,7] Vesicles formed through this approach have attracted more attention for the lack of a template in their formation process and the feasibility of encapsulating a variety of guest molecules.[1–3,7,9] In particular, polymer vesicles self-assembled from amphiphilic block copolymers are characterized by a high structural stability compared to that of conventional lipid vesicles and an attractive chemical diversity to integrate smart functions, such as stimulus sensitivity.[1,5] Nevertheless, the major limitation of these amphiphilic polymer vesicles as biofunctional materials is the lack of permeability of hydrophilic solutes as a result of the hydrophobic nature of their membrane. The harsh preparation conditions involving organic solvents become problematic for the encapsulation of biologically relevant compounds, such as proteins.

Recently, a novel entity of polymer vesicles with a polyion complex membrane (PICsome) was developed by our group to overcome these issues that emerged in the conventional systems.[8] Without the use of any organic solvents, a PICsome forms in a single aqueous medium through self-assembly of a pair of oppositely charged block ionomers with biocompatible polyethylene glycol (PEG) segments.[8] Hence, as is typical with common liposomal systems, water-soluble macromolecular compounds may be readily compartmentalized in the inner aqueous core of the PICsome partitioned from the exterior by the semipermeable PIC membrane sandwiched between PEG layers. Apparently, this type of compartmentalization of biofunctional macromolecules, such as proteins and nucleic acids, into a segregated mesoscopic cavity to exert integrated functions is one of the focusing topics in the field of chemistry.[9] Herein, we wish to communicate for the first time the successful compartmentalization of biologically relevant proteins into the PICsome, thus demonstrating the unique function derived from the semipermeable nature of the PICsome membrane in the physiological environment as well as the increased tolerability against protease attack, which is often an issue in the application of fragile proteins in the biomedical field.

Myoglobin (Mb), which forms stable oxygen adducts in muscle, was selected in this study as a compartmentalized protein in the PICsome cavity because its biological function may be monitored quantitatively by UV/Vis spectroscopy.[10] Furthermore, Mb or hemoglobin loaded in the PICsome may have feasibility as an oxygen carrier in the future, because of the inherent blood compatibility of the PEG-shell layer and the appreciable stability of the inner PIC layer even at physiological salt concentrations.[8] Here, an oppositely charged pair of poly(amino acid)-based block ionomers, polyethylene glycol-*b*-poly(α,β-aspartic acid) (PEG-P(Asp)) as an aniomer and polyethylene glycol-*b*-poly((5-aminopentyl)-α,β-aspartamide) (PEG-P(Asp-AP)) as a catiomer, was synthesized as the component of the PICsome from the single-platform polymer PEG-*b*-poly(β-benzyl aspartate), as previously reported (Figure 1).[8] Mb-loaded PICsomes were obtained by the simple mixing of an aqueous solution of PEG-P(Asp) containing Mb with an aqueous solution of PEG-P(Asp-AP) at an equal residual ratio of $-COO^-$ and $-NH_3^+$ units in the block ionomers. Laser diffraction measurements indicated the formation of PICsomes with sizes ranging from 500 nm to 5 μm (data not shown). Furthermore, the encapsulation of Mb in the PICsome was directly observed by confocal laser scanning microscopy (CLSM) using Mb labeled with tetramethylrhodamine isothiocyanate (TRITC-Mb; Figure 2). A CLSM image of the PICsome showed a uniform red fluorescence in the inner cavity, which demonstrates the successful loading of TRITC-Mb in the PICsome by an equimolar mix of the oppositely charged ionomers.

Mb-loaded PICsomes were purified by the removal of unencapsulated Mb from the PICsome solution by centrifugation (3 min at 14000 *g*) and the subsequent exchange of supernatant with pure buffer, which was repeated five times. Absence of the free Mb in the final supernatant was

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

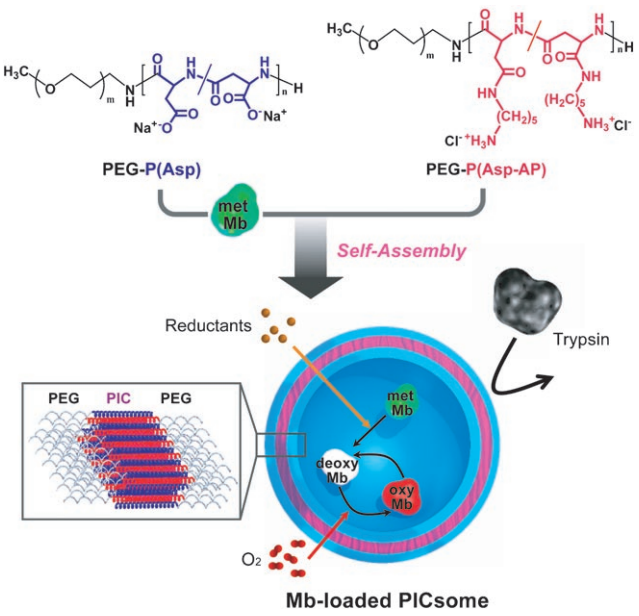

Figure 1. Reversible Mb oxygenation inside the PICsome self-assembled from a pair of oppositely charged block ionomers.

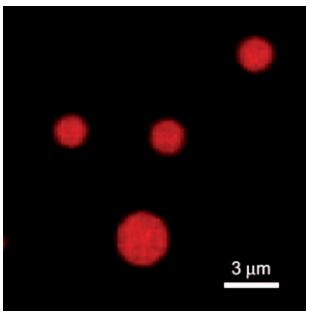

Figure 2. Cross-sectional image of TRITC-Mb loaded in PICsomes observed by CLSM.

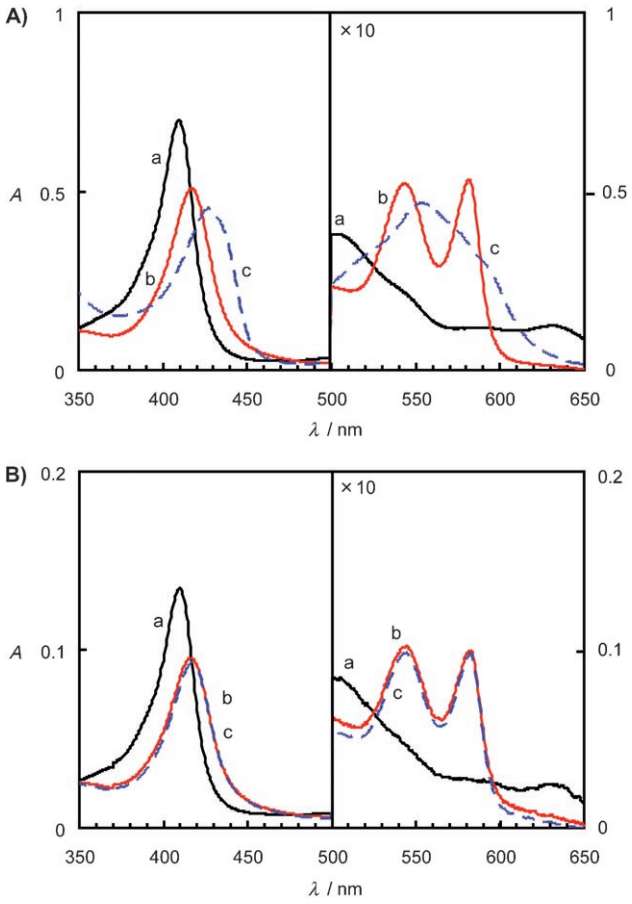

Figure 3. A) Electronic absorption spectra a) of free Mb solution (60 μg mL^{-1}; black solid line), b) after reduction and further introduction of O_2 gas in the absence of trypsin (red solid line), and c) after incubation with trypsin (37 °C, 4 h; blue dashed line). B) Electronic absorption spectra a) of the solution of Mb-loaded PICsomes (black solid line), b) after reduction and further introduction of O_2 gas in the absence of trypsin (red solid line), and c) after incubation with trypsin (37 °C, 4 h; blue dashed line).

confirmed by measuring the absorption at 409 nm, which corresponds to the Soret band of metmyoglobin (metMb; see the Supporting Information). The redispersed suspension of the PICsome obtained by vortex mixing revealed a clear Soret band of metMb at 409 nm, thus demonstrating the successful encapsulation of Mb in the PICsome (as shown in Figure 3 B, peak a).

To assess the oxygen-binding capability of Mb, metMb was reduced to deoxymyoglobin (deoxyMb) by the use of $Na_2S_2O_4$ (Supporting Information). Upon the addition of $Na_2S_2O_4$ to the PICsome solution, the Soret band of metMb at 409 nm shifted to 434 nm, which corresponded to that of deoxyMb, clearly showing $Na_2S_2O_4$ permeation through the PIC membrane (Supporting Information). Figure 4 shows the time course of the change in the absorbance at 434 nm after the addition of an aqueous solution of $Na_2S_2O_4$ to the PICsome solution, which demonstrates the effective reduction of metMb to deoxyMb loaded in the PICsome. After the steep change in the initial 12–18 s, the absorbance gradually reached a plateau within 20 s, which corresponds well to the

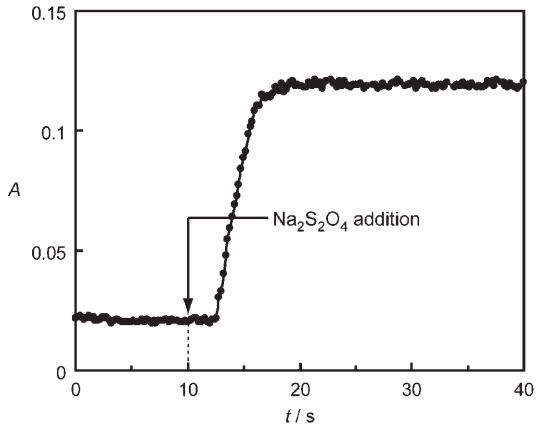

Figure 4. Time course of the absorbance at 434 nm after addition of $Na_2S_2O_4$ reductant to a metMb-loaded PICsome solution.

observed metMb reduction found in the PICsome solution. This result suggests the complete reduction of metMb even in the PICsome cavity (Supporting Information), and clearly

indicates the rapid penetration of the $S_2O_4^{2-}$ reductant across the semipermeable PICsome membrane.

Next, the introduction of O_2 gas to a solution of deoxyMb-loaded PICsomes clearly induced the blue shift of the Soret band to 413 nm as well as a change in the shape of the Q band around 550 nm, which demonstrates the generation of oxyMb inside the PICsome (Figure 3B, peak b). Notably, oxyMb completely returned to deoxyMb after bubbling of the solution with Ar gas for deoxygenation, as confirmed by the spectral change of the Soret and Q bands in a reversed manner (Supporting Information). This oxygenation/deoxygenation cycle of Mb in the PICsome with alternate bubbling of O_2/Ar was completely reversible (see Figure 5), which suggests the practical feasibility of this system as an oxygen carrier.

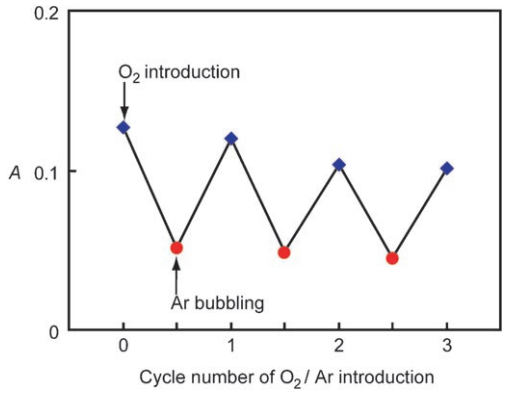

Figure 5. Change in the absorbance at 434 nm of the Mb-PICsome by the alternating introduction of O_2 (◆)/Ar (●) gas to the solution.

It is of further interest to examine the tolerability of compartmentalized protein in the PICsome under harsh operating conditions with potential protease attack in the medium. Therefore, the oxygenation/deoxygenation cycle of free and PICsome-encapsulated Mb was compared in a medium containing trypsin. As illustrated (Figure 3A, peak c), incubation of free Mb (60 μg mL^{-1}) in the trypsin solution (500 μg mL^{-1}) at 37 °C for 4 h[11] resulted in the complete disappearance of the O_2-binding activity, as indicated by the lack of change in spectral shift corresponding to oxyMb generation. However, Mb in the PICsome retained the initial reduction (Supporting Information) and O_2-binding activity even after incubation with trypsin at 37 °C for 4 h, as observed by the identical spectral changes in the Soret and Q bands upon O_2 bubbling between the conditions with medium containing trypsin (Figure 3B, peak c) and without trypsin (Figure 3B, peak b). Consequently, this result proved a protease resistance of PICsome-loaded Mb most likely caused by the steric barrier of the semipermeable PIC membrane surrounded by a PEG palisade.

In conclusion, the preparation and functionality of Mb-loaded PICsomes are demonstrated here as the first successful pathway to fabricate functional nano/microcontainers for a variety of proteins through the self-assembled vesicular formation of a pair of oppositely charged block ionomers. The Mb-loaded PICsome was indeed readily prepared in an aqueous medium by simple mixing of the block ionomer solutions containing Mb. Loaded metMb was smoothly reduced to deoxyMb by $S_2O_4^{2-}$ that had permeated through the PIC membrane, and reversible oxygenation/deoxygenation of the Mb in the PICsome was revealed even in the presence of trypsin in the outer medium. The biocompatible nature of this Mb-loaded PICsome, composed of poly(amino acid)s and a bioinert PEG shell, may also be feasible for further development of a new oxygen carrier for use in vivo. Furthermore, the approach of encapsulating biologically relevant macromolecules, including proteins, in the cavity of the semipermeable PICsome, as demonstrated here, may provide a general way to assemble novel carrier-system platforms useful in drug delivery as well as functional nano/microbioreactor systems available for the diagnostic and therapeutic fields.

Experimental Section

Materials: Equine heart Mb, sodium dithionite ($Na_2S_2O_4$), and bovine pancreas trypsin were obtained from Sigma (St. Louis, MO, USA). PEG-P(Asp) (PEG weight-average molecular weight, M_w = 2000 g mol^{-1}; unit number of P(Asp) = 75), PEG-P(Asp-AP) (PEG M_w = 2000 g mol^{-1}; unit number of P(Asp-AP) = 69), and TRITC-Mb were synthesized as previously reported.[8,12]

Instruments: Fluorescence observation of the PICsome was performed by using a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Germany) with a 63× objective (C-Apochromat, Carl Zeiss, Germany) at an excitation wavelength of 543 nm (He–Ne laser). Electronic absorption spectra were measured with a V-570 spectrophotometer (Jasco, Japan). Centrifugation was carried out with a micro high-speed centrifuge (MX-300, TOMY, Japan). Laser diffraction measurements were completed with a Shimadzu SALD-7100 instrument.

Preparation of Mb-loaded PICsomes: Solutions of PEG-P(Asp) (2 mg mL^{-1}) and PEG-P(Asp-AP) (1 mg mL^{-1}) were prepared separately in phosphate-buffered saline (PBS, 50 mM, pH 7.4) containing NaCl (150 mM). Equine heart Mb (pI = 7) was dissolved in the same buffer (10 mg mL^{-1}) and then mixed with the same volume of the PEG-P(Asp) solution to prepare a PEG-P(Asp) solution (1 mg mL^{-1}) containing Mb (5 mg mL^{-1}; PEG-P(Asp)/Mb solution). Subsequently, the PEG-P(Asp)/Mb solution was mixed with the PEG-P(Asp-AP) solution, in an equal unit ratio of $-COO^-$ and $-NH_3^+$ in the block ionomers, and vigorously stirred by vortex to prepare Mb-loaded PICsomes.

Removal of unencapsulated Mb: The mixed solution of PEG-P(Asp)/Mb and PEG-P(Asp-AP) (3.9 mL) was centrifuged at 14000 *g* for 3 min, and the supernatant (3 mL) was exchanged with pure buffer. The buffer exchange was repeated five times to completely remove the unencapsulated Mb from the solution, as confirmed by the absorbance at 409 nm. The final concentration of the encapsulated Mb in the solution was determined spectrophotometrically (0.88 μg mL^{-1}) based on the calibration curve shown in the Supporting Information.

Evaluation of Mb activity: Spectroscopic studies on Mb were carried out by using a quartz cell (optical length: 1 cm) equipped with an isolation vessel, which allows O_2 or Ar gas to be introduced under an inert atmosphere. Reduction of metMb was monitored every 0.2 s with stirring from the change in the absorbance at 434 nm immediately after the addition of a freshly prepared aqueous solution of $Na_2S_2O_4$ (5 equiv) to the solution. In a similar manner, the oxygenation/deoxygenation cycle of free Mb or PICsome-encapsulated Mb

was monitored from the spectrum change according to the alternating introduction of O_2 gas for 30 min and Ar gas for 2 h.

Tolerability against trypsin: Bovine pancreas trypsin was dissolved in PBS (50 mM, pH 7.4) containing NaCl (150 mM) to form a solution (500 $\mu g\,mL^{-1}$). Next, the trypsin solution was added to either the metMb solution or the metMb-loaded PICsome solution. The molar ratio of trypsin to metMb in the solution was adjusted to 1:20. The solution containing trypsin was kept at 37 °C with stirring for 4 h, and subsequently transferred to a quartz cell for spectroscopic studies.

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