Novel Method to Load High Amount of Drugs and Macromolecules into Polyion Complex Vesicles (PICsomes)

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ABSTRACT SUMMARY
By utilizing unique properties of polyion complex vesicles (PICsomes), such as a crosslinkable PIC layer, fragmentation into single paired PICs consisting of a single molecule of PEG-PAsp and Homo-P(Asp-AP), tunable permeability, and so on, we succeeded in loading of high amount of cargoes into partially crosslinked PICsomes. More interestingly, stepwise loading of two kinds of cargoes with different molecular weight was performed based on this new method.

INTRODUCTION
Recently, there has been an increase in the number of studies devoted to morphological control of self-assembled nano-/micron-sized structures in aqueous media, as they have great potentials for biomedical applications. Very recently, we have developed novel polyion complex (PIC) nano-architectures prepared by the simple mixing oppositely charged block copolymers consisting of poly(ethylene glycol) (PEG) and poly(amino acid)s in an aqueous medium1,2. For example, we found the spontaneous formation of monodispersed PIC vesicles, nano-PICsomes in the aqueous media. Nano-PICsomes are characterized by facile tuning of vesicle size ranging from 100–400 nm, and their useful properties, such as semipermeability, facile loading of water-dispersed materials, long blood circulation after crosslinking, excellent tumor accumulation based on the enhanced permeability and retention (EPR) effect, and so on1,2. So far, we have demonstrated delivery of iron oxide nanoparticles to the tumor site after encapsulation into PICsomes, and succeeded in early-stage tumor detection in mice using MRI. However, loading efficiency of materials into PICsomes is limited, because PICsomes do not have an ability to concentrate materials in their inner aqueous phase. Also, mono-dispersed and size-controlled PICsomes cannot be obtained in the presence of high concentration of salt or macromolecules, that is, high viscosity condition. Herein, we report a new method to improve loading amount and efficiency of PICsomes, mainly using partially crosslinked PICsomes. Partially crosslinked PICsomes are expected to show enough stability, to load extremely high concentration of macromolecules inside in a moderate loading manner. Furthermore, macromolecules with different molecular weights can be sequentially loaded, using step-wise crosslinking of PICsomes (Scheme). This method will increase the value of PICsomes for nano-DDS.

EXPERIMENTAL METHODS
Preparation of partially crosslinked PICsomes and subsequent loading of cargoes. Solutions of PEG-b-poly(α,β-aspartic acid) (PEG-PAsp; $M_a$ of PEG = 2,000, degree of polymerization (DP) of PAsp = 75) and poly([5-aminopentyl]-α,β-aspartamide) (Homo-P(Asp-AP); DP of P(Asp-AP) = 82) were prepared separately in 10 mM phosphate buffer (PB; pH 7.4, 0 mM NaCl; typically, polymer concentration = 1 mg/mL). Subsequently, solutions of PEG-PAsp and Homo-P(Asp-AP) were mixed together in an equal unit ratio of –COO⁻ and –NH₃⁺ in the charged polymers, and vigorously stirred with a vortex mixer for 2 min, to give PICsomes. Obtained products were subjected to crosslinking with 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydro chloride (EDC). Typically, 0.5 equivalent of EDC to the number of –COO⁻ contained in the solution was used for partial crosslinking. Resulted partially crosslinked PICsomes were subjected to vortex mixing in the presence of specific amount of cargo macromolecules (Scheme a). In this study, we used 5–50 mg/mL of FITC-labeled dextrans (FITC-Dex; M.W. = 40,000) for loading. Finally, Dex-loaded PICsomes were crosslinked again for suppression of release of cargoes. For another example, we tried to load aluminum phthalocyanine disulfonate (AlPcS2a), which is a drug of photodynamic therapy, as an example of small molecular weight material. Obtained PICs were evaluated by dynamic light scattering (DLS), transmission electron microscopy (TEM), and fluorescence correlation spectroscopy (FCS).
**Double-stage loading of multiple cargoes into partially crosslinked PICsomes (Scheme b).** As a first cargo, we selected cytochrome c (cyt c; M.W. = 12 327). Cyt c was dissolved in PB, and then added to the uncrosslinked PICsome solution, followed by vigorous vortex mixing for 2 min. Obtained products were subjected to partial crosslinking with EDC using the procedure shown above. As a second cargo, we chose FITC-Dex (M.W. = 4 000). Cyt-c-loaded PICsomes were exposed to various concentration of the FITC-Dex (M.W. = 4 000) solution, followed by vortex mixing. Characterization was carried out by DLS, TEM, FCS, and HPLC.

**Scheme.** Schematic drawings of loading cargoes into partially crosslinked PICsomes: (a) Loading cargoes with high concentration, and (b) double-stage loading.

**RESULTS AND DISCUSSION**

Partial crosslinking was carried out by addition of 0.5 equivalent of EDC to PICsomes with a diameter of ~100 nm. After exposure to the FITC-Dex solution, size distribution and morphology of PICsomes was maintained irrespective of the FITC-Dex concentrations (Scheme a, Fig. 1a). In contrast, PICsomes prepared in the presence of FITC-Dex with concentrations more than 10 mg/mL gave much larger PICsomes with broader size distribution on the basis of the previously reported method (Fig. 1b). FCS measurements show that number of loaded FITC-Dex into a single PICsome is ~ 20, when the FITC-Dex concentration is more than 20 mg/mL. This value is ~6 times higher than the value obtained upon using the previous method at the upper limit concentration of FITC-Dex. This behavior can be accounted for the potential fragmentation property of PICsomes into single-paired PICs, or unit PICs.3 After crosslinking, some parts of PIC layer is considered to be open for loading. Also, loading efficiency can be improved by increasing the concentration of partially crosslinked PICsomes in the solution.

Next, we tried loading of relatively hydrophobic drugs, AlPcS2a, into partially crosslinked PICsomes by sonication. Interestingly, use of suspension of AlPcS2a resulted in much higher loading than its solubility limit. This method provides an effective way for loading slightly water-soluble drugs.

![Figure 1. TEM images of (a) crosslinked PICsomes after exposure to the 50 mg/mL FITC-Dex solution, and (b) PICsomes prepared in the solution containing 50 mg/mL FITC-Dex.](image)

For double-stage loading, first cargoes, cyt c, were loaded into PICsomes, followed by partial crosslinking. Resulted PICsomes were exposed to the solution of FITC-Dex (M.W. = 4 000), second cargoes (Scheme b). Finally, we confirmed that negligible release of the first cargo and co-existence of the second cargo in PICsomes. Thus, release of larger cargoes are suppressed by cross-linking. This result suggest that two kinds of cargoes with different molecular weight or size can be loaded in a stepwise manner with well-controlled concentrations.

**CONCLUSION**

The present study clearly demonstrates that limited loading amount and efficiency of PICsomes can be improved by using partially crosslinked PICsomes.

**REFERENCES**

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