Rational design of polyion complex vesicles (PICsomes) as versatile platforms for nanocarriers and nanoreactors

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Synthetic nanovesicles, possessing an ability to encapsulate versatile compounds with different properties such as surface charge, solubility and size, have been exploited in various biomedical applications, including cancer therapy. However, precise control of their physical and biological properties remains challenging. Recently, unilamellar polyion complex vesicles (PICsomes), formed by simple vortex mixing of polyethylene glycol (PEG)-based block aniomers and homocatiomers in aqueous media, have been reported to possess semi permeability, size tunability and sensitivity to physiological stimuli (ionic strength, temperature, and so on). In addition, their facile encapsulation of proteins and contrast agents, combined with long blood circulation time and selective tumor accumulation after crosslinking suggest their potential utilities as nanocarriers and nanoreactors. Nevertheless, development of PICsome is still immature considering that few combinations of oppositely charged polymers has been reported for PICsome formation. Also, to maintain the stability against environmental stimuli, PICsomes need crosslinking, which complicates their preparation, and possibly brings about long-term toxicity and deactivation of biologically active guests. Moreover, more examples of PICsomes with biological activity are needed to extend their use in pharmaceutical arena. Toward these goals, this study is devoted to establish factors directing to PICsome formation via studying on length and composition of polymers to generalize PICsome concept. Following, instead of crosslinking, utilizing intermolecular interaction to create physiologically stable PICsomes with potential biodegradable is investigated. Finally, to broaden biological usages of PICsomes, crosslinking-free enzyme-loaded PICsomes and PICsomes containing small interfering RNA (siRNA)-intercalated membranes were innovated.

To clarify critical factors to direct PICsome formation, PEG-based block aniomers with different length of charged segments, PEG-PAspx [$M_n$ of PEG = 2,000, and degree of polymerization (DP) of anionic chain (PAsp) is shown as x], and homocatiomer Homo-P(Asp-Cn), (number of carbon atoms in homocatiomer side chain is shown as n, and DP of P(Asp-Cn) is shown as y) were synthesized and used for PIC formation. Firstly, to see the effect of PAsp length in PEG-PAsp, PEG-PAspx (x = 30, 52, 60, 75 and 100) were complexed with Homo-P(Asp-C5)y that was used to obtain unilamellar PICsomes with the diameter of 100 nm in the previous reports, at charge ratio of unity. Note that PEG weight fraction ($f_{PEG}$) increase as x decrease. From dynamic light scattering (DLS) and transmission electron microscope (TEM) analyses, at $f_{PEG} \leq 9.6\%$, PICsomes with the diameter of 100 nm were observed, while spherical micelles with the size of 50 nm formed at $f_{PEG} \geq 10.9\%$. All formulations show narrow size distribution (polydispersity index (PDI) < 0.1). It is convincible that higher steric repulsion of surface PEG (higher $f_{PEG}$) inhibits lamellar growth and gives micelles, contrariwise, balance in repulsion of PEG and lamellar growth might provide PICsomes. Second, the effect of n and x are examined through mixing of
Homo-P(Asp-Cn), \( n = 2–8 \) with PEG-PAsp, at charge ratio of unity. At \( f_{\text{PEG}} < 10\% \), PICsomes with size of 100 nm or 70 nm were selectively formed for \( n = 5–6 \) and 8, respectively. Noteworthy, smaller size of PICsomes with \( n = 8 \) might be explained by higher hydrophobic interaction driving rapid vesicular enclosure. Also, formation of PICsomes with small lamellar fragments can be observed even at relatively high \( f_{\text{PEG}} \) (~9.8%) when \( n = 8 \). Alternatively, for combinations having \( n = 2–4 \), mixture of cylindrical micelles and spherical micelles (mixture of micelles) was observed. At \( f_{\text{PEG}} > 10.4\% \), 50 nm spherical micelles solely form, regardless of \( n \). Exceptionally, for formulations with \( n = 4 \), mixture of micelles formed in the range of \( 8.2 \leq f_{\text{PEG}} < 11.4\% \), whilst PICsomes formed at \( f_{\text{PEG}} \geq 6.3\% \), suggesting that the vesicle regime is slightly shifted to the lower \( f_{\text{PEG}} \) region. Third, to study the effect of nature of block copolymers, PEG-P(Asp-C5)_{70}, in which a PEG chain is attached to the oppositely charged chain compared to those used in previously reported PICsomes, was complexed with Homo-PAsp_{70} for PIC formation at stoichiometric point. 100-nm-scaled PICsomes with PDI < 0.1 were observed, indicating that PICsomes can be formed regardless of block copolymer combination. From these results, longer aliphatic spacer is preferable for PICsome formation possibly due to increased hydrophobicity and flexibility facilitating lamellar formation. Lower hydrophobicity or less volume of PICs having shorter aliphatic length might be unfavorable for lamellar packing, giving micelles. Taking together, \( f_{\text{PEG}} \) of less than 10% and long aliphatic spacer satisfy the requirement of PICsome formation.

Assuming that longer aliphatic spacer stabilizes lamella by providing tight side chain packing due to increasing intermolecular interaction, stability of PICsomes with \( n = 8 \) (C8-PICsomes) in the presence of 150 mM NaCl at 37 °C, is examined compared with PICsomes having shorter side chains (\( n = 5; \) C5-PICsomes), without crosslinking. Note that, all experiments were performed at pH 7.4. As revealed by DLS and TEM, 100-nm C5-PICsomes were suddenly transformed into μm-sized PICs upon addition of 150 mM NaCl at 37 °C. On the contrary, 70-nm C8-PICsomes preserved their vesicular morphology. In addition, vesicular structure and monodispersity of C8-PICsomes can be maintained even at 70 °C (0 mM NaCl). Higher saline and thermal stability of C8-PICsomes might be accounted for by stronger hydrophobic interaction.

Aiming to utilize the C8-PICsomes as nanoreactors, nano-sized vessels carrying catalysts (such as enzymes, etc.), β-galactosidase (β-gal), a potent prodrug-activating enzyme used in cancer therapy, was encapsulated inside C8-PICsomes and C5-PICsomes (control), through simple vortex mixing. Resulting non-crosslinked β-gal-loaded C8- and C5-PICsomes were challenged with 150 mM NaCl and trypsin (protease) at 37 °C for 24 h, followed by evaluation of enzymatic activity after addition of tokyogreen-β-gal (TG-β-gal; non fluorescent substrates which is converted by β-gal to fluorescent product). From DLS and fluorospectrophotometry, β-gal-loaded C8-PICsomes showed preservation of physiological stability and catalytic activity, while β-gal-loaded C5-PICsomes lost their vesicular structure and catalytic activity. Presumably, higher hydrophobicity helps C8-PICsomes to keep vesicular structure retaining β-gal inside, while the semipermeable membranes inhibit transportation of large trypsin but allow penetration of small TG-β-gal, providing appreciable enzymatic activity.

In addition to the hollow spaces of PICsomes, the PIC membrane can be also utilized for loading bioactive molecules, especially, highly charged molecules. In this regard, siRNA, an oligonucleotide possessing potent and specific gene silencing ability, was adapted as anionic source for PICsome formation instead of PAsp to create vesicular oligonucleotide carriers and further generalize concept of PICsome formation. PEG-P(Asp-C5)_{70} is used to complex with siRNA, considering that PICsomes can be
successfully formed after complexing this polymer with Homo-PAsp\textsubscript{78} as described above. From DLS and TEM, no significant multimolecular PICs was observed at stoichiometric ratio, however, increasing N/P (molar ratio of amino to phosphate groups) lead to formation of PICosomes having siRNA-embedded membranes (C5-siRNAsomes) and negative surface charge at N/P of 1.5, and those with positive surface charge at N/P = 2.2–2.4. In fact, after ultracentrifugation treatment, some amount of PEG-P(Asp-C5)\textsubscript{70} which were not incorporated into the C5-siRNAsomes was detected, and the actual N/P of C5-siRNAsome formed at N/P = 1.5 and 2.3 was calculated to be 0.9 and 1.2, respectively. Also, the elemental PIC structures are postulated to consist of both neutral PIC segments, driving C5-siRNAsome formation, and ionized segments that have free phosphate groups (for PICs formed at actual N/P = 0.9) or free amino groups (for PICs formed at actual N/P = 1.2), determining charge sign of C5-siRNAsomes.

As representatives, C5-siRNAsomes formed at actual N/P = 0.9 and 1.2 were crosslinked with gluteraldehyde (GA) to maintain their vesicular structure, size, and PDI in the presence of 150 mM NaCl and 10% serum at 37 °C. Following, gene knockdown ability of crosslinked C5-siRNAsomes was assessed using Luciferase gene as a model. As a result, C5-siRNAsomes formed at actual N/P = 1.2 and crosslinked at molar ratio of GA to primary amino groups of polymers ([GA]/[NH\textsubscript{2}]) = 0.4 show the highest gene knockdown ability, which is possibly attributed by their highest cellular uptake and siRNA release as confirmed by confocal laser scanning microscope and gel retardation assay. This formulation can also demonstrate significant and selective silencing ability against VEGF gene, overexpressed in cancer cells, as revealed by real time-PCR and CCK-8 assay, respectively. Combining with ability to load water-soluble macromolecules (TRITC-Dex 70k), C5-siRNAsomes are promising as multifunctional oligonucleotide carriers. Finally, taking an advantage of octyl side chain \((n = 8)\), enhanced saline resistance was confirmed upon using PEG-P(Asp-C8) as a cationic building block.

Overall, this study shed new light on design, stabilization and application of PICosomes towards biomedical applications. PICosomes can be formed by various kinds of materials opening a window of opportunity for tuning physical and biological properties for desired applications. Especially, without crosslinking, using homocatiomers having long aliphatic spacer improves physiological stability of PICosomes by means of intermolecular interactions, mainly hydrophobic and electrostatic interaction. This method is fascinating due to the simplicity and potential biodegradability, suggesting their use in practical applications. Besides, since PICosomes with longer aliphatic spacer is quite stable even at elevated temperature, after loading thermoresistant enzymes they might be used in chemical synthesis performed at high temperature. Moreover, PICosomes having siRNA-loaded membrane, illustrating gene silencing ability and encapsulation ability are appealing as versatile platforms for co-delivery of siRNAs and other materials, including enzymes, contrast agents, antigens, and so forth for diverse applications.