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# Alkyl Chain-Grafted Poly(L-lysine) Vesicles with Tunable Molecular Assembly and Membrane Permeability

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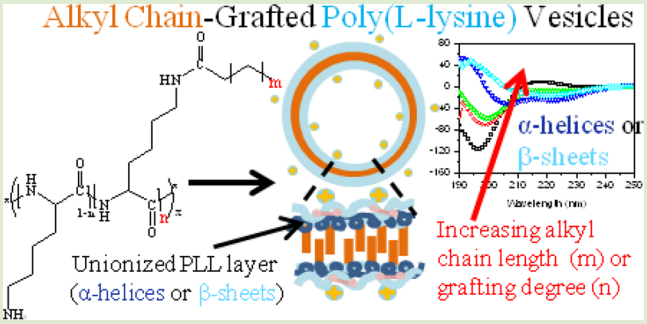
Supporting Information

**ABSTRACT:** The preparation of alkyl chain-grafted poly(L-lysine) (PLL) vesicles with tunable molecular assembly in aqueous solution and the evaluation of their membrane permeability by drug release experiments have been investigated. Upon grafting long alkyl chains, polypeptides confined in the assembled nanostructures adopted ordered conformations such as $\alpha$-helices or $\beta$-sheets/turns, leading to the dense packing of membranes and, consequently, the decreases in vesicular size and membrane permeability. The vesicles can also be cross-linked by genipin to form stable structures with tunable membrane permeability. Additionally, these vesicles exhibited noticeable pH-sensitive behavior, depending on the grafted alkyl chain and cross-linking.




Polymer vesicles that self-assembled from amphiphilic copolymers have attracted great interest because of their analogous structure with cell membranes and potential biomedical applications as bionanoreactors or delivering vehicles.[1−7] Comparing with conventional liposomes, polymer vesicles exhibited improved stability, superior membrane properties, and versatile chemistry for functionalization.[1−3] Recently, polypeptide-based block or graft copolymers that can self-assemble to form various structures such as micelles or vesicles have been extensively studied due to their advantages, including biocompatibility, stimuli-responsiveness, and possessing the essential structures and functions of proteins.[6−10] Polypeptides that adopted an ordered conformation such as $\alpha$-helices or $\beta$-sheets would influence the amphiphilic nature and morphology of the assemblies.[10−12] For polymer vesicles, the molecular assembly and amphiphilic nature of the polymer chains in the membranes would dictate their membrane permeability, which is essential for applications such as drug delivery and protein encapsulation.[2−5]

Herein, we report the synthesis of alkyl chain-grafted poly(L-lysine) (PLL) that can self-assemble to form vesicles with different chain conformations and sizes, which were readily controlled by the degree of substitution (DS) and alkyl chain length. The interplay between the hydrophobic interaction and the conformational changes upon alkyl chain substitution determined their molecular assembly and amphiphilicity and, subsequently, their membrane permeability. Also, they can be conjugated with functional ligands and stabilized by genipin-cross-linking, making them potentially useful for applications in drug delivery and biomimetic encapsulants.

Poly(L-lysine) (PLL), which was synthesized by ring-opening polymerization (ROP) of Z-Lys-NCA and followed by the removal of Z groups using HBr, was partially substituted with various alkyl chains including hexanoyl, decanoyl, and tetradecanoyl groups, denoted as PLH, PLD, and PLT, respectively. The detailed synthesis procedure was shown in the Supporting Information (Scheme S1). The number-averaged molecular weight of PZLL derived from GPC was 79400 with a narrow molecular weight distribution ($M_w/M_n \sim 1.23$), as shown in Figure S1. The degree of polymerization was calculated to be about 300. The feed molar ratio of the anhydride to lysine was set to be 20 and 40%. $^1$H NMR spectra of these resultant polymers demonstrated the substitution of alkyl chains, evidenced by the presence of the characteristic signals corresponding to the protons of alkyl chains (Figure S2). The DS value was determined by the ratio of the $\varepsilon$ protons with or without alkyl chain substitution on PLL (Figure S1). The substitution efficiencies were calculated to range between 80 and 90% (Table S1). The substitution efficiency for the PLT prepared at the feed molar ratio of the anhydride to lysine of 40% was found to be lower than 70%.

The polymer assemblies were prepared in phosphate buffer saline (pH 7.4, $I$ = 0.01 N) by dialysis method. Their average hydrodynamic diameters (or sizes), $D_h$, were determined by DLS using cumulant program. These resultant assemblies have monomodal distributions (Figure S3). It was found that the size and critical aggregation concentration (cac) of the assemblies decreased upon grafting longer alkyl chain and increasing DS (Table 1, Figures S3 and S4), which led to the stronger hydrophobic interaction and, subsequently, the formation of more compact assemblies. The zeta potential revealed that

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Table 1. Degree of Substitution (DS), Size ($D_h$), Polydispersity (PD), Critical Aggregation Concentration (cac), and Zeta Potential (with standard deviation, $\sigma$) of Alkyl Chain-Grafted PLL Assemblies ($n$ = 3)

| sample code | obtained DS (%) | $D_h$ (nm) | PD (nm) | cac × $10^{-2}$(mg/mL) | zeta potential (mV) | $\sigma$ (mV) |
|---|---|---|---|---|---|---|
| PLH4 | 35.0 | 230 ± 3 | 127 ± 7 | 9.5 | 16.0 ± 0.4 | 10.2 ± 1.1 |
| PLD2 | 16.9 | 181 ± 5 | 120 ± 11 | 5.2 | 14.1 ± 1.7 | 8.4 ± 0.5 |
| PLD4 | 34.3 | 73 ± 2 | 27 ± 2 | 3.1 | 12.5 ± 0.4 | 7.3 ± 0.3 |
| PLT2 | 17.0 | 88 ± 8 | 34 ± 9 | 1.4 | 9.1 ± 0.4 | 4.5 ± 0 5 |

these assemblies carried positive charge due to the presence of amino groups. These assemblies were stained by $RuO_4$ for TEM characterization. PLH4, PLD2, and PLD4 self-assembled to form vesicles, evidenced by the observed ring structures (Figures 1a and S5). Rather TEM image of PLT2 assemblies

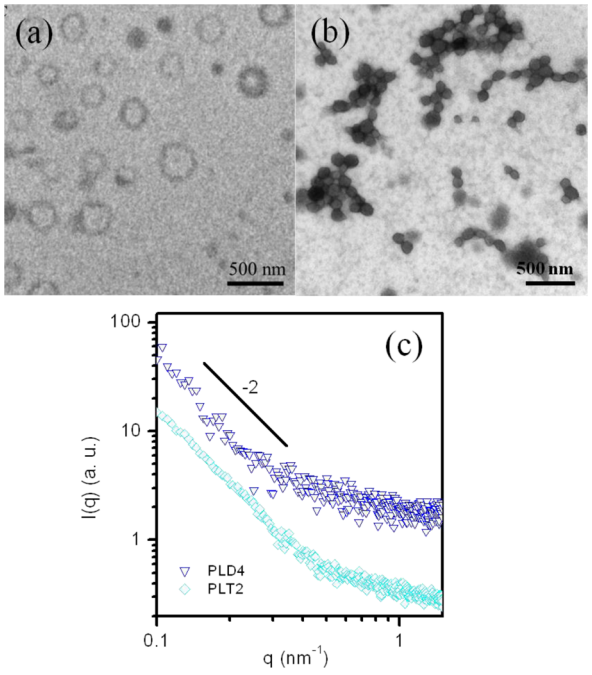
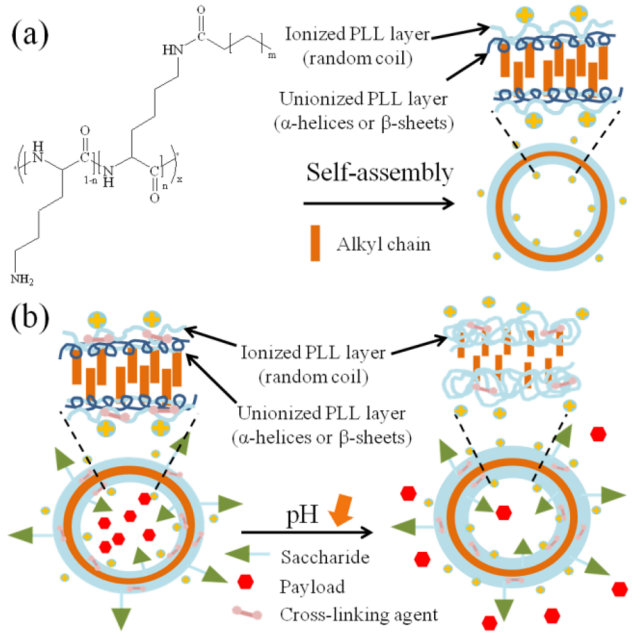



**Figure 1.** TEM images of (a) PLH4 and (b) PLD4 assemblies with $RuO_4$ staining and (c) SAXS patterns of PLD4 and PLT2 assemblies.

revealed the observed dense cores, which could be due to staining (Figure S5b). To gain more insight into the molecular packing architecture of these assemblies, static light scattering (SLS) and small-angle X-ray (SAXS) analyses were performed on PLD4 and PLT2 assemblies. Their hydrodynamic radius ($R_h$) and radius of gyration ($R_g$) were evaluated by SLS and the $R_g/R_h$, which is sensitive to assembly morphology,[13] was then determined. The respective $R_g/R_h$ values for PLD4 and PLT2 assemblies were calculated to be, respectively, 0.99 and 1.14 (Figure S6), suggesting the formation of vesicles. SLS measurements of PLH4 and PLD2 assemblies were not reliable due to their broad size distributions. SAXS measurements of PLD4 and PLT2 assemblies showed the scattering intensity ($I$) with characteristic $I(q) \propto q^{-2}$ at low scattering vectors ($q$) (Figure 1c), indicating the formation of vesicular structures,[14] consistent with SLS analysis. The results revealed that the alkyl chain-grafted PLL self-assembled to form vesicles (Scheme 1a).

The chain conformation adopted by the polypeptide in the vesicles at neutral condition was characterized by circular dichroism (CD) and computed using a software CD-fit 4.[15] Figure 2a showed that PLH4 and PLD2 adopted mainly coil conformation (>70%), evidenced by the well-known doubly

**Scheme 1. Schematic Presentation of (a) Self-Assembly of Alkyl Chain-Grafted PLL and (b) the Drug Release from the Saccharide-Conjugated, Cross-Linked Vesicles upon pH Change**




inflected curves. Rather, the CD spectrum for PLD4 exhibited the double minima, suggesting that the polypeptide chains adopted mainly $\alpha$-helical conformation (60%). Additionally, PLT2 mainly adopted $\beta$-sheet/turn conformation (71%) at neutral condition. The results suggested that the secondary conformation adopted by the polypeptide chains can be tuned by varying the alkyl chain length or DS. The increase in hydrophobic interaction due to increasing the alkyl chain length or DS resulted that the polypeptide chains were embedded in much confined space,[9] which consequently facilitated the polypeptides adopted more un-ionized, ordered conformation. The titration curves showed that the degree of deprotonation ($\alpha$) for PLD4 was higher than that for PLD2 at given pH and the drastic increase of $\alpha$ value from 0.17 to 0.40 for PLD4 as the pH value changed from 7.2 to 7.7 (Figure S7), supporting that PLD4 adopted more un-ionized, ordered conformation than PLD2 at neutral condition (see the discussion in Supporting Information). It is reasonable to surmise that the PLL segments with un-ionized, ordered conformation would probably reside above and below the hydrophobic regime, leading to the increase in the hydrophobicity of the vesicular membrane and consequently the formation of compact assemblies. CD analysis revealed that a significant conformational transition from $\alpha$-helix/$\beta$-sheet to random coil was observed for PLD4 and PLT2 vesicles as the pH was decreased from 7.4 to 4.7 (Figure 2b). Upon decreasing the pH to 4.7, the zeta-potential values of these vesicles increased to respective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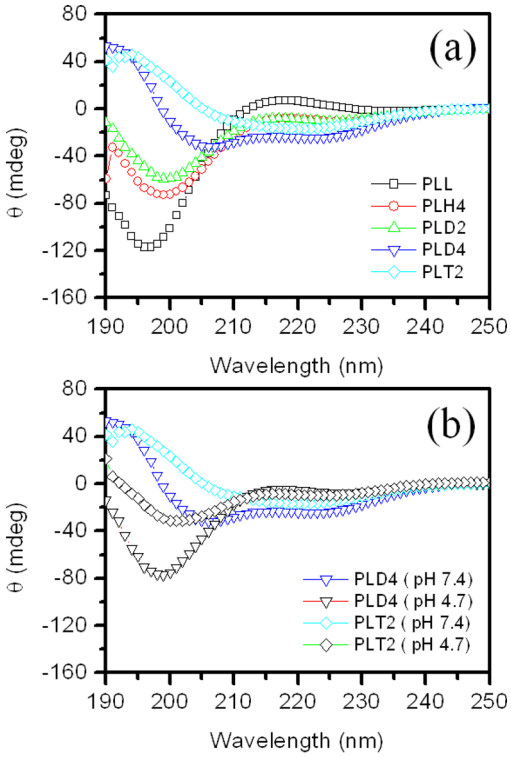


**Figure 2.** (a) CD spectra of alkyl chain-grafted PLL (0.5 mg/mL) at pH 7.4 and (b) CD spectra of PLD4 and PLT2 (0.5 mg/mL) at pH 7.4 and 4.7.

38.3 ± 2.1 and 27.1 ± 1.2, suggesting the protonation of unionized PLL-rich layers. The results suggested that the conformational changes can act as a solubility switch to tune membrane permeability.

Conjugation of cell-targeted ligand onto the polypeptide assemblies can lead the as-prepared polypeptide vesicles to be served as cell-targeted, pH-responsive drug carriers. To demonstrate that, lactobionolactone (Lac), which is a model targeted ligand to HepG2 liver cells with a galactose unit,[16] was selected for conjugation. The detailed synthesis procedure was shown in the Supporting Information (Scheme S1). The conjugation degree of Lac was calculated to be 15.5, 12.1, and 6.6% with the feed ratio of 20% for PLH4, PLD4, and PLT2 copolypeptides, respectively (Table S2 and Figure S8). The low substitution efficiency for PLT2 copolypeptide can be attributed to the steric hindrance and aggregation in methanol due to the grafted long alkyl chain. The particle sizes of Lac-PLH4, Lac-PLD4, and Lac-PLT2 vesicles were measured to be, respectively, 237 ± 3.0, 84 ± 1.0, and 89 ± 0.6 nm, which were comparable with those of the corresponding vesicles. The zeta potential revealed that these assemblies still carried positive charge. The carbohydrate–lectin binding measurements were first performed to evaluate the bioactivity of the saccharide-conjugated vesicles (Figure S9). *Ricinus communis* Agglutinin ($RCA_{120}$) is a known specific lectin for the selective binding of galactosyl residues.[17] Concanavalin A (Con A) lectin, which is selective for glucosyl and mannosyl residues but not for galactosyl residues,[17] was used as the control. The changes in absorbance of Lac-PLD4 vesicle/$RCA_{120}$, PLD4 vesicle/$RCA_{120}$, Lac-PLD4 vesicle/Con A, and PLD4 vesicle/Con A solutions at 450 nm were recorded. The Lac-PLD4 vesicle/Con A and PLD4 vesicle/Con A solutions showed no change in absorbance at 450 nm, suggesting no change in turbidity. The absorbance for Lac-PLD4/$RCA_{120}$ was much higher than that for PLD4 vesicle/$RCA_{120}$, indicating that the significant change in turbidity for Lac-PLD4 vesicle/$RCA_{120}$ solution. The results revealed that the galactosyl residues on the surface of the Lac-PLD4 vesicles can mediate the interaction with the target biomolecules and no nonspecific binding of $RCA_{120}$ onto PLD4. The results signified the highly specific and noncovalent carbohydrate–lectin interactions.

To evaluate the membrane permeability of these vesicles and their use as pH-responsive drug carriers, doxorubicin (DOX) was chosen as the model drug for encapsulation. A high DOX loading level was achieved with the aid of sonication as a pH gradient applied between the outside and inside of the vesicular membrane.[18,19] The loading content (LC) of DOX for Lac-PLH4, Lac-PLD4, and Lac-PLT2 vesicles ranged between 28 and 36% (Table 2). The DOX release of these drug-loaded vesicles was studied at neutral and acidic conditions (Figure 3a). DOX was released rapidly from the Lac-PLH4 vesicles,

**Table 2. Degree of Saccharide Substitution, Loading Content, Size, and PD of the Drug-Loaded Vesicles ($n$ = 3)**

| sample cede | DS of saccharide (%) | LC (%) | $D_h$ (nm) | PD (nm) |
|---|---|---|---|---|
| DOX-loaded Lac-PLII4 | 15.5 | 35.4 ± 2.0 | 245 ± 5 | 131 ± 13 |
| DOX-loaded Lac-PLD4 | 12.1 | 28.0 ± 3.0 | 127 ± 7 | 74 ± 2 |
| DOX-loaded Lac-PLT2 | 6.6 | 30.6 ± 2.2 | 102 ± 7 | 55 ± 3 |

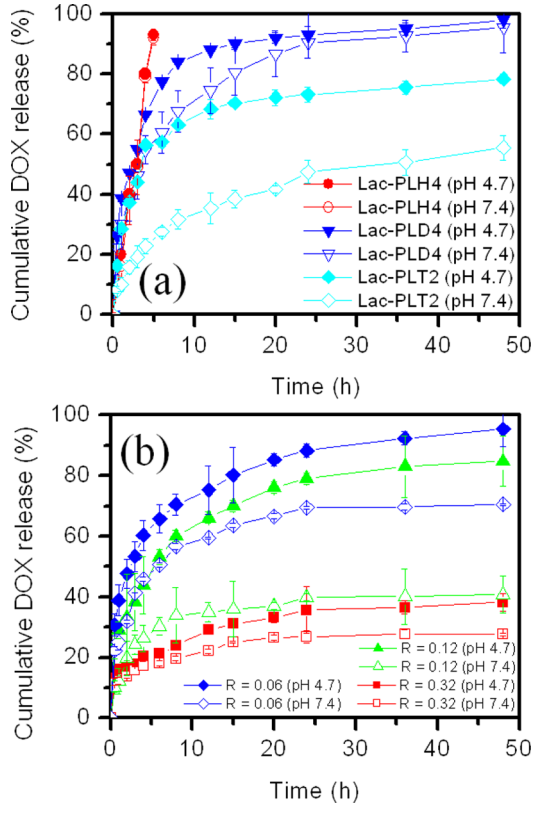


**Figure 3.** Cumulative drug release profiles of (a) the Lac-PLH4, Lac-PLD4, and Lac-PLT4 vesicles and (b) the cross-linked Lac-PLD4 vesicles with different $R$ at (close) pH 4.7 and (open) 7.4 ($n$ = 3).


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indicating that the vesicular membrane was highly permeable. Rather, the drug release from the Lac-PLD4 and Lac-PLT2 vesicles was slower than that from the Lac-PLH4 vesicles. The results revealed that the vesicles comprising more ordered chain conformation, leading to the increase of hydrophobicity and, consequently, the lower membrane permeability, exhibited slower drug release rate. Upon decreasing the solution pH, the drug release rate was accelerated due to the increase in membrane permeability, resulting from the protonation of un-ionized PLL-rich layers accompanying with conformational changes and subsequently the increase in the hydrophilicity of the vesicular membrane. In addition, the water solubility of DOX molecules increased at acidic condition. The results suggested that the polypeptide chain conformation in the assemblies can be tuned by varying grafted alkyl chain length and DS, which can be a means to control membrane permeability.

The effect of genipin-cross-linking on the membrane permeability of the Lac-PLD4 vesicles was then investigated. Cross-linking of vesicular membrane has been shown to be an effective means to tune membrane permeability.[2,19] Upon cross-linking at different genipin to lysine feed ratios (R), DLS and TEM analyses revealed that the size and morphology of the drug-loaded Lac-PLD4 vesicles were not significantly perturbed (Figures S10 and S11). As shown in Figures 3b and S12a, the payload release from the cross-linked Lac-PLD4 vesicles slowed down upon increasing the *R* value from 0.06 to 0.32, suggesting that the increase of *R* value resulted in the more densely packed membrane, thereby retarding the outflow of drug payloads from vesicles. The results revealed that the membrane permeability can be readily tuned by varying *R*. The cross-linked vesicles displayed an accelerated drug release at acidic condition. This is primarily ascribed to that, in addition to the increase in the water solubility of DOX at pH 4.7, the increase in the permeability of membrane due to the protonation of un-ionized PLL layer, thus, facilitating cargo efflux (Scheme 1b). The highest relative ratio of the cumulative drug release at pH 4.7 to that at pH 7.4 for 24 h was found to be at $R = 0.12$ (Figure S12b), suggesting that this *R* value (0.12) was optimal in this case.

In conclusion, we demonstrated that the molecular assembly and membrane permeability of alkyl chain-grafted vesicles were influenced by alkyl chain length and degree of substitution. The amphiphilic copolypeptides could assemble to form spherical vesicles with ordered chain assemblies. At neutral condition, the increase of the DS or length of alkyl chain resulted in the increase of the relative content of ordered conformation due to confinement and subsequently the formation of more compact assemblies with lower permeability. The membrane permeability can also be tuned by genipin-cross-linking without perturbing their size and morphology. Due to the stimuli-responsiveness and readily functionalization of these assemblies, the application of these micelles as targeted drug delivery and biomimetic encapsulants is under way in our laboratory.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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