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Design of Polydiacetylene-Phospholipid Supramolecules for Enhanced Stability and Sensitivity

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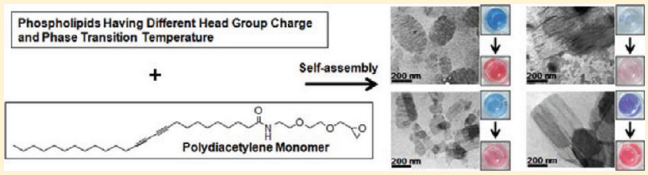
Supporting Information

ABSTRACT: We present polydiacetylene (PDA) liposome assemblies with various phospholipids that have different headgroup charges and phase transition temperatures (T_m). 10,12-Pentacosadiynoic acid (PCDA)-epoxy was used as a base PDA monomer and the insertion of highly charged phospholipids resulted in notable changes in the size of liposome and reduction of the aggregation of PDA liposome. Among the various phospholipids, the phospholipid with a moderate T_m demonstrated enhanced stability and sensitivity, as measured by the size and zeta potential over storage time, thermochoromic response, and transmission electron microscopy images. By combining these results, we were able to detect immunologically an antibody of bovine viral diarrhea virus over a wide dynamic range of 0.001 to 100 μg/mL.



1. INTRODUCTION

A conjugated polymer, polydiacetylene (PDA) has a special optical property, which changes its color, for example, from blue to red and emits weak red fluorescent light in response to various external stimuli such as heat,[1,2] light,[3] ions,[4−6] chemicals,[7−10] biomolecules,[11−15] and bacteria.[16−19] The color change is originated from distortion of π-conjugated backbone of PDA by external stimuli, which makes PDA an efficient component for self-signaling (label-free) and signal amplifying biosensor.[20−22]

The commonly used method for PDA biosensor is mediated by a liposome assembly. The monomers of PDA are generally amphiphilic in water; this lipidic property leads to self-assembly of the monomers that is analogous to liposome. After assembly and ordering of lipidic monomers by cooling (5 °C), UV (254 nm) polymerization makes sensory PDA liposome. The lipidic property of PDA monomer may also accommodate various natural or synthetic lipids, which has been extensively utilized for colorimetric detection of biointerfacial interactions such as receptor−ligand interaction,[10,23] membrane permeabilization by bacterial toxin[16] or antimicrobial peptides,[24] and enzymes−lipids interactions.[25]

In general, the insertion of lipids into PDA liposome has a merit to modify the physical properties of PDA liposome such as size,[26] surface charge, and packing of lipids,[19] which, in turn, affect the sensitivity and stability of PDA liposome biosensor.[7,12,14,15,19,27,28] It has been revealed that a PDA liposome of small size appears to improve sensitivity by compartmentalization of receptor sites. Namely, a smaller liposome can generate an equivalent signal to a larger liposome in spite of less binding of target molecule on the liposome surface.[15,29] An appropriate level of surface charge of liposome can also introduce electrostatic repulsion providing resistivity against aggregation and fusion.[27] In parallel, weak packing of lipids (or high membrane fluidity) in PDA liposome enhances sensitivity by making easy distortion of π-conjugated chain of PDA.[7,12,14,19] However, ironically, the weak packing interrupts polymerization into a stable conjugated chain[2] and deteriorates the degree of fusion.[28]

Recently, there have been some trials to increase the sensitivity of PDA liposome biosensor by controlling the degree of packing between lipids.[12,14,18,19] Despite these efforts, there is lack of comprehensive studies incorporating various effects of the inserted lipids. Herein, we choose 10,12-pentacosadiynoic acid (PCDA)-epoxy as a base PDA monomer because (i) epoxy group has good reactivity with amine group, which is abundant in biological molecules like DNA, protein, and antibody, and (ii) it is stable over long-term storage for biosensor applications.[6] Various PDA-phospholipid liposomes were formed by assembling PCDA-epoxy with three types of phospholipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA), and 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP), which have different

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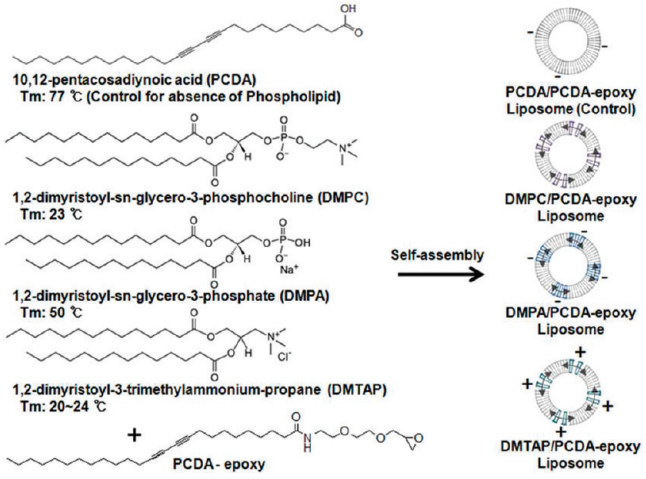
headgroup charges and phase transition temperatures (T_m indicating the degree of intermolecular attraction and packing between lipids) as schematically drawn in Scheme 1. As a general

Scheme 1. Schematic Illustration for the Design of Different Kinds of PDA-Phospholipid Liposomes



PDA monomer, PCDA was also used as a negative control without phospholipids. Finally, we investigated the effects of the inserted phospholipids on the PDA liposome biosensor applications.

2. MATERIALS AND METHODS

Materials. DMPC, DMPA, and DMTAP were purchased from the Avanti Polar Lipids. PCDA was purchased from the Sigma-Aldrich Chemicals and PCDA-Epoxy was synthesized according to the reference.[6] The other materials such as buffer and blocking agents were also purchased from the Sigma-Aldrich Chemicals. Monoclonal antibodies for bovine viral diarrhea virus (BVDV) B20.24 antigen and classical swine fever virus (CSFV) LOM1 antigen, and BVDV B20.24 antigen were kindly supplied from the JenoBiotech Corp. (Chuncheon, South Korea).

PDA Liposome Assembly. PDA-phospholipid liposomes were assembled with PCDA-epoxy and phospholipids or PCDA at 4:1 molar ratio which had been already optimized in our preliminary experiment (Figure S1 of the Supporting Information). For higher concentrations (1:1 or 1:3 molar ratio) of non-polymerizable phospholipid, the responses were lowered owing to the small amount of conjugated chain and unstable violet polymerization.[10] The 4:1 molar ratio was also reported to be optimal for sensory applications,[6] being in the range of widely used molar fraction of phospholipids, 0.2−0.4.[14,18,19] PCDA-Epoxy and Phospholipids/PCDA were dissolved in 0.2 mL tetrahydrofuran with 4:1 molar ratio with the final concentration of 0.5 mM. The solution was injected into 20 mL 5 mM HEPES buffer of pH 8 and probe-sonicated with 120 W for 5 min. The liposome solution was filtrated through 0.8 μm cellulose acetate syringe filter twice and stored for 24 h at 5 °C refrigerator before use.

Measurement of Size and Zeta Potential of PDA-Phospholipids Liposomes. The size and zeta potential of PDA-phospholipid were measured by Otsuka Electronics ELS-8000 Electrophoretic Light Scattering Spectrophotometer at day 1 and 7 after assembly. For the study period, liposomes were stored at 5 °C.

UV Polymerization of PDA-Phospholipids Liposomes. A small amount (200 μL) of 0.5 mM PDA-phospholipid liposome solution polymerized under 254 nm 1 mW/cm^2 UV irradiation for different time periods was located in an oven at 70 °C for optimization of polymerization time at which shows the highest colorimetric response (CR). UV−vis absorption spectra taken from PerkinElmer Lambda 45 UV−vis spectrometer were used to measure CRs about thermal stimuli.

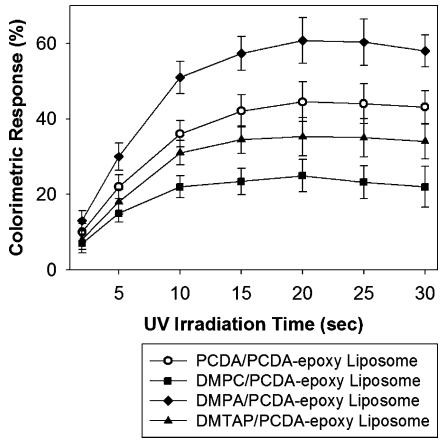


Figure 1. CRs of PDA-phospholipid liposomes after 254 nm UV polymerization for a series of time periods and heating at 70 °C (n = 3).

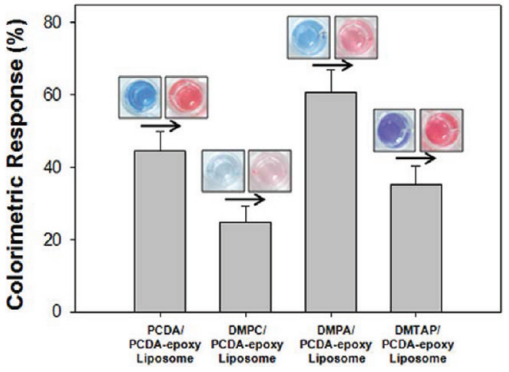


Figure 2. CRs of PDA-phospholipid liposomes (n = 3) and their optical images after 24hrs cooling following 254 nm UV polymerization for 20 s (left image) and heating at 70 °C for 3 min (right image).

Table 1. Sizes and Zeta Potentials of Various PDA-Phospholipid Liposomes with Exposure Time (1 and 7 Days)

	PCDA/PCDA-epoxy liposome	DMPC/PCDA-epoxy liposome	DMPA/PCDA-epoxy liposome	DMTAP/PCDA-epoxy liposome
size (nm) at 1 day	280 (6.5)[a]	>5000	187 (3.7)	289 (2.7)
size (nm) at 7 day	327 (6.1)		208 (4.3)	308 (3.1)
increasing ratio (%)	16.2		11.3	6.5
zeta potential (ZP) (mV)	−20.2 (0.5)	−3.2 (0.5)	−30.1 (0.5)	27.1 (0.6)

[a]Standard deviation (n = 3).

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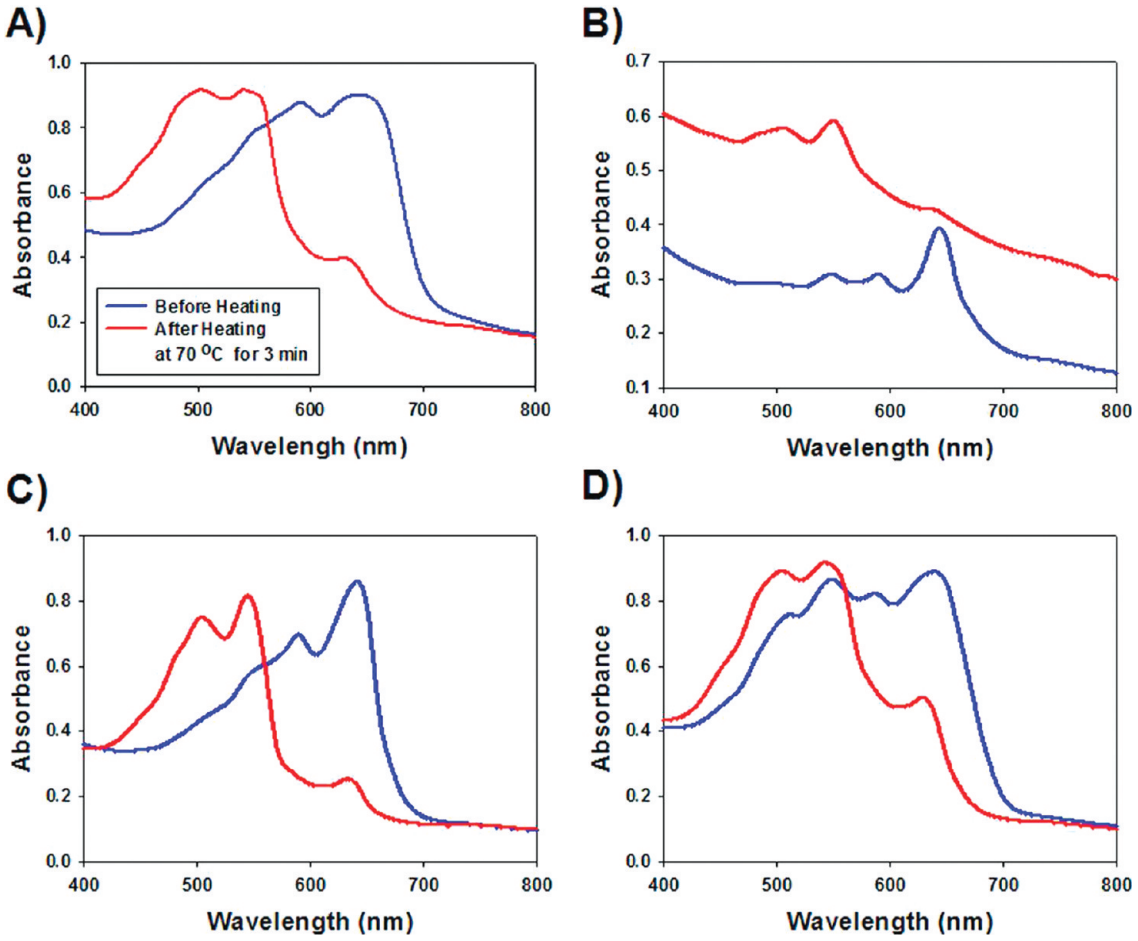

Figure 3. UV−vis spectra of PDA-phospholipid liposomes before and after heating at 70 °C for 3 min: A) PCDA/PCDA-epoxy liposome, B) DMPC/PCDA-epoxy liposome, C) DMPA/PCDA-epoxy liposome, D) DMTAP/PCDA-epoxy liposome.

The CRs of PDA-phospholipids liposomes were calculated by the well-known equation.[2] Here, the blue percentage (PB) is defined as PB = $A_{blue}/(A_{blue} + A_{red}) \times 100\%$ where A_{blue} is the absorbance at the peak around 640 nm and A_{red} is the absorbance at the peak around 540 nm. Then, the CR is defined as CR = (initial PB − final PB)/initial PB × 100%.

Comparison of Thermochromic Responses of PDA-Phospholipid Liposomes. PDA-phospholipid liposome solution polymerized under an optimal UV irradiation for 20 s was located in an oven at 70 °C for 3 min and the CRs were measured with UV/vis absorption spectra. Thermochromic responses at several intermediate temperatures between 25 and 70 °C were measured (Figure S2 of the Supporting Information).

Transmission Electron Microscopy (TEM) Image. The PDA-phospholipids liposomes were deposited on a carbon-coated copper grid. JEOL JEM-1010 electron microscope was used to take TEM images. The width and length of liposomes on TEM images were measured by the image processing software, ImageJ (National Institutes of Health).

Antibody Detection with PDA-Phospholipid Liposome Solution. A mixture of 0.5 mM PDA-phospholipid liposome solution (900 μL) and BVDV B20.24 antigen (100 μL) were incubated at 5 °C overnight. Unbounded antigens were removed twice by centrifugation at 13 000 rpm for 10 min. Remaining epoxy groups were blocked by reacting with amine group of 5 wt % BSA in 5 mM HEPES buffer of pH 8 for 1 h at room temperature. 0.05 mM liposome solution was polymerized by UV irradiation for 20 s and reacted with antibodies in PBS solution overnight. UV−vis absorption spectra taken from PerkinElmer Lambda 45 UV−vis spectrometer were used to measure CRs. In detail, to optimize the probe concentration, the PCDA/PCDA-epoxy liposomes incubated by varying final concentrations of BVDV B20.24 antigen at 5.0, 7.5, 10.0, and 12.5 μg/mL were reacted with 100 μg/mL BVDV antibodies and the CRs were measured (Figure S3 of the Supporting Information). Subsequently, the PDA liposomes incubated with an optimal, 10 μg/mL BVDV B20.24 antigen were reacted with 0.001, 0.01, 0.1, 1, 10, and 100 μg/mL BVDV antibodies, and 100 μg/mL CSFV antibodies in PBS solution overnight. To confirm that the degree of protein binding on each PDA-phospholipids liposome is similar, FITC conjugated BSA (FITC-BSA) was used. After binding of the probe (BVDV B20.24 antigen) on each liposome, remaining epoxy groups were reacted with 100 μg/mL FITC-BSA for 1 h at room temperature. The solutions of unbounded FITC-BSA on each liposome were separated by centrifugation. Then, the degrees of absorbance of unbounded FITC-BSA solutions were taken at 492 nm and compared to the calibration curve (Figure S4 of the Supporting Information).

3. RESULTS AND DISCUSSION

Size and Stability of PDA-Phospholipid Liposomes. The size and zeta potential (ZP, degree of repulsion between

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liposomes) of the designed PDA-phospholipid liposomes were measured by electophoretic light scattering on day 1 and 7 after forming the assemblies. As shown in Table 1, the liposomes inserted by charged lipids such as DMPA (negative), DMTAP (positive), and PCDA (negative) show good assemblies, as measured by their higher values of ZP. In the case of DMPC/PCDA-epoxy liposome, however, the aggregation was observed with naked eye after 4 h cooling in a refrigerator and thus its size could not be measured by the scattering method (initial size > ~5 μm). This is because DMPC/PCDA-epoxy liposome has a low ZP (−3.2 mV) due to neutral charge of DMPC, a zwitterion, resulting in rapid aggregation as compared to DMTAP/PCDA-epoxy liposomes having lipids of similar T_m but positive charge (inducing relatively high ZP, 24.9 mV). The assembly with DMPC and PCDA instead of PCDA-epoxy (molar ratio DMPC:PCDA = 1:4) was also successful because of the negative charge of PCDA (data not shown). These results indicate that the insertion of charged lipid can reduce aggregation of PDA liposome even in the presence of high flexibility. Table 1 shows that DMPA/PCDA-epoxy liposome has the smallest size because its highest charge makes the liposome easily broken down to small vesicles upon sonication.[30] Such a small size together with good membrane flexibility allows for the high sensitivity of DMPA/PCDA-epoxy liposome, which is described in the following section.

On day 7 (stored at 5 °C), the size of each liposome was measured again and compared with the day 1 result to confirm the degree of aggregation. As shown in Table 1, the DMPA/PCDA-epoxy (increasing ratio: 11.3% ZP: −30.1 mV) and DMTAP/PCDA-epoxy liposomes (6.5%, 27.1 mV) show smaller increase of size than that of the PCDA/PCDA epoxy liposome (16.2%) having a relatively low ZP (−20.2 mV). This suggests that the insertion of highly charged phospholipid can reduce the aggregation of liposome while still keeping its high flexibility.

Thermochromic Responses of PDA-Phospholipid Liposomes. After 24 h cooling for packing, the liposomes were polymerized by irradiating 254 nm UV light for different time periods to determine an optimum polymerization time, in which the CRs of PDA-phospholipids liposomes were measured by UV−vis spectrometer. As shown in Figure 1, the optimized polymerization time for the maximum thermochromic responses turned out to be around 20 s. The increase of UV irradiation time up to 20 s enhanced the CRs, while the changes were saturated after 20s UV irradiation.[2,9]

The optical images of PDA-phospholipids liposome polymerized by 20s UV irradiation are presented in Figure 2. Interestingly, the DMTAP/PCDA-epoxy liposome shows violet color presumably due to unstable π-conjugation by weak packing. The DMPA/PCDA-epoxy liposome shows blue color, which is lighter than the color of the PCDA/PCDA-epoxy liposome. It can be explained by a small amount of π-conjugated polymerized chains by weak packing and reduced amount of PDA monomers. In contrast, the DMPC/PCDA-epoxy liposome shows very weak blue or violet color for its instability. The π-conjugated polymerization was also confirmed by UV−vis spectra as shown in Figure 3. Here, the width and intensity of absorbance peak (especially around 650 nm) in the UV−vis spectra qualitatively shows the respective amount of stable π-conjugated PDA chains that is blue in color.[2,9] As seen from the figure, the DMPA/PCDA-epoxy liposome (before heating) has a narrower band around 650 nm than PCDA/PCDA-epoxy liposome, which means a smaller amount of π-conjugated polymerized chains showing the lighter blue color. The DMTAP/PCDA-epoxy and DMPC/PCDA-epoxy liposomes show absorption peaks around 550 and 650 nm respectively indicating that a relatively unstable π-conjugated polymerization has occurred.

To evaluate the sensor performance, we measured the CR (Figure 2, detailed spectra are shown in Figure 3) of liposomes by applying heat at 70 °C for 3 min. Then, the color changes from blue to red as shown in Figure 2. The DMPA/PCDA-epoxy liposome has the highest CR with the help of relatively low T_m and small size despite its light-blue color. To characterize the structure and morphology of the liposomes, TEM images were taken as shown in Figure 4, which apparently matched the size and degree of aggregation based on the electophoretic light

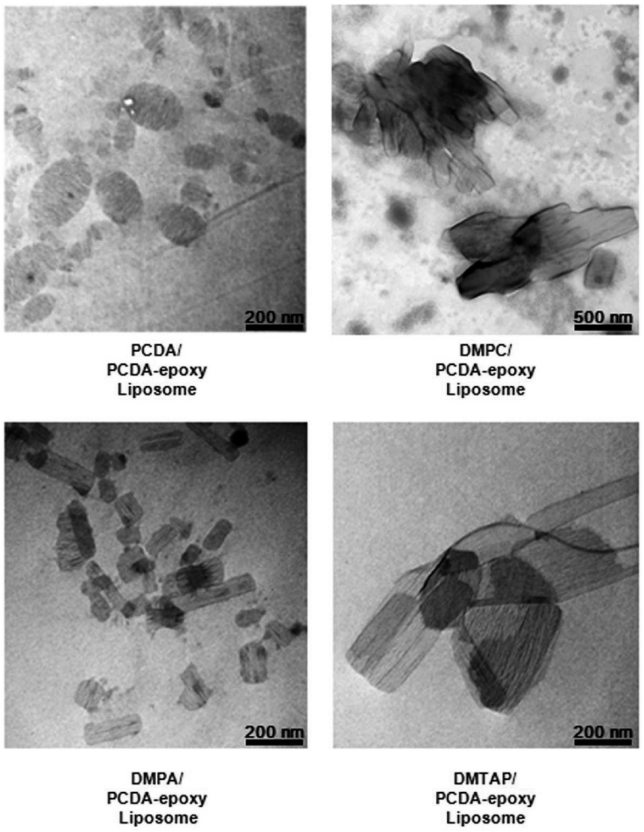

Figure 4. TEM images of the different kinds of PDA-phospholipid liposomes.

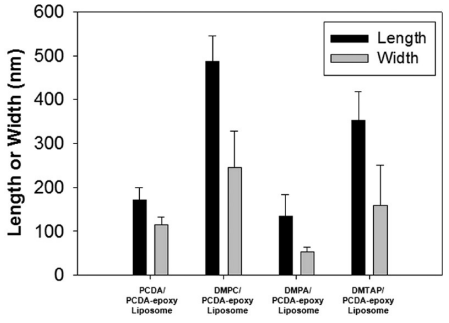

Figure 5. Quantitative analysis of the width and length of PDA-phospholipids liposomes (n = 30) with TEM images. The size of nonaggregated liposome was measured for the DMPC/PCDA-epoxy liposome.

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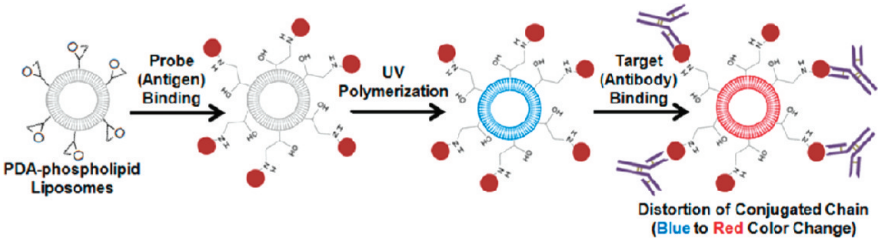
Scheme 2. Schematic Illustration of Biosensor Applications Using PDA-Phospholipid Liposomes



scattering measurement. In general, the insertion of phospholipid transforms the shape of liposome from round to rectangular sheet, according to previous findings.[14] Also, the DMPC/PCDA-epoxy liposome shows a great aggregation (scale bar, 500 nm), which also agrees with the scattering experiment summarized in Table 1. Furthermore, as the inserted phospholipid has a lower T_m, the sheet shape became wider and longer as shown in Figure 5. These observations reveal changes in the packing and ordering of lipids (monomers) and thus enhanced membrane flexibility. For the inserted DMPA (T_m, 50 °C), the change in the packing of lipids resulted in enhanced sensitivity, whereas, for the low T_m lipids like DMPC (T_m, 23 °C) and DMTAP (T_m, 20–24 °C), the sensitivity was reduced presumably due to unstable π-conjugation with weak packing of monomers.

Enhanced Sensitivity to Immunological Detection of Biomolecules. To utilize the PDA-phospholipid liposomes for liposome immunoassay,[31,32] we detected an antibody of bovine viral diarrhea virus (BVDV) which is known to induce reproductive failure in breeding stock. BVDV is a pandemic disease which requires early diagnosis for safe and economical livestock industry. We designed an indirect detection scheme using the BVDV antigen as a probe and detected the BVDV IgG antibody as shown in Scheme 2. The rationale for this design is that the IgG antibody (~150 kDa) is bigger than the antigen, glycoprotein of virus (~53 kDa), and thus the binding of the target molecule can generate a greater force to PDA backbone.[8] In the experiment, the BVDV antigen was attached to epoxy groups on the surface of liposomes before UV polymerization at the solution state. After overnight incubation in the BLDV antibody solution of 0.001, 0.01, 0.1, 1, 10, and 100 μg/mL, the CRs were measured as shown in Figure 6 (detailed spectra are shown in Figure S4 of the Supporting Information). It should be noted that the CR of DMPC/PCDA-epoxy liposome could not be measured due to its rapid aggregation. Similar to the CR results in response to heat, the sensitivity was increased in the order of DMTAP/PCDA-epoxy, PCDA/PCDA-epoxy, and DMPA/PCDA-epoxy liposomes where the DMPA/PCDA-epoxy liposome shows the highest sensitivity. This is because its smaller size and weaker packing of lipids causes easy distortion of conjugated backbone of PDA. A linear characteristic was obtained over a wide dynamic range, 0.001–100 μg/mL, and the absence of nonspecific binding was also confirmed by incubation with classical swine fever virus (CSFV) IgG antibody 100 μg/mL. Results showed that the CR change in this case was less than 1.6% for all liposomes tested (Figure S5 of the Supporting Information).

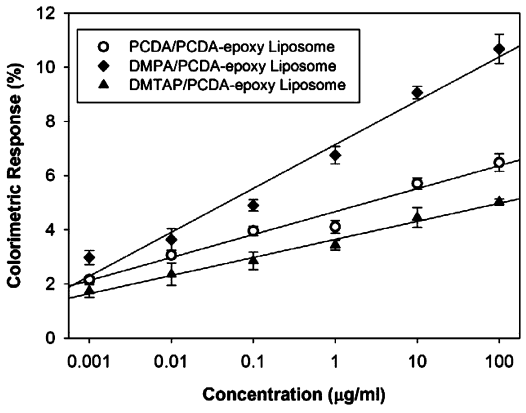


Figure 6. Correlation curve between the CR and concentration of BVDV antibody after 24 h incubation in the antibody solution (n = 3).

4. CONCLUSIONS

We have demonstrated that the insertion of phospholipids into PDA liposome reduced aggregation of PDA liposome and generated smaller size and higher flexibility. These features appeared to increase sensitivity in biosensor applications in a synergistic manner. By assembling with highly negative charged DMPA lipids of moderate T_m, the PDA liposome biosensor demonstrated enhanced stability and sensitivity. The present design of PDA-phospholipid liposomes is potentially useful for various PDA liposome-based sensor systems.

■ ASSOCIATED CONTENT

Supporting Information

Colorimetric responses of PDA-phospholipid liposomes for the different ratios of nonpolymerizable phospholipids after heating at 70 °C for 3 min. Colorimetric responses of the PDA-phospholipid liposomes after heating at several intermediates temperatures between 25 and 70 °C for 3 min. Colorimetric responses of PCDA/PCDA-epoxy liposome bonded as a function of the probe (BVDV B20.24 antigen) concentration against 100 μg/mL BVDV antibody. Measurement of the degree of protein binding on PCDA/PCDA-epoxy, DMPA/PCDA-epoxy, and DMTAP/PCDA-epoxy liposomes. UV–vis spectra of PDA-phospholipid liposomes bonded by BVDV antigen as a probe and reacted with 0.001, 0.01, 0.1, 1, 10, and 100 μg/mL BVDV antibodies, and 100 μg/mL CSFV antibodies overnight to confirm nonspecific binding. 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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