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Multiscale and Bioinspired Design of Polydiacetylene-based Biosensor Systems
Multiscale and Bioinspired Design of Polydiacetylene-based Biosensor Systems

멀티스케일 및 자연모사 설계를 통한 폴리디아이아세틸렌 기반 바이오센서 시스템의 제작

지도교수 전 누 리

이 논문은 공학박사 학위논문으로 제출함

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Abstract

Multiscale and bioinspired methods for designing polydiacetylene-based biosensor systems are presented. Mechanism of the presented biosensor is mechanochromism of polydiacetylene (PDA) which can change its color from blue to red and emits red fluorescence by mechanical stresses from binding of bioanalytes.

For the fabrication of polydiacetylene-based biosensors, we exploited the liposome form of the polydiacetylene (PDA) materials, self-assembled from amphiphilic PDA monomers. Being inspired by the structural similarity between liposome and cell membrane, we coined the PDA liposomes containing phospholipid, the main component of natural cell membrane, with realizing the two achievements. First, we enhanced the sensitivity and stability of PDA liposomes by insertion of a phospholipid (1,2-dimyristoyl-sn-glycero-3-phosphate, DMPA) having high head group charge and moderate phase transition temperature. In comparative experiments with other lipids having different head group charges and phase transition temperatures, DMPA lipids is the most proper lipid for suiting the mechanical properties of PDA liposomes, such as size, shape, membrane flexibility, and zeta potential (surface charge) for sensory applications. Second, we utilized specific phospholipids as a receptor for highly sensitive and selective detection to
aminoglycosidic antibiotics, and lead (II) metal ion. For the facile detection of aminoglycosidic antibiotics, we designed a PDA-phospholipids liposome containing phosphatidylinositol-4,5-bisphosphate (PIP$_2$) phospholipids (PDA-PIP$_2$ liposome). The detection mechanism is inspired by the cellular membrane interactions between neomycin and PIP$_2$. We also developed PDA-lipid liposomes having 1,2-dipalmitoyl-sn-glycero-3-galloyl (DPGG) lipids (PDA-DPGG liposome) for lead (II) ion detection.

In addition to the above-mentioned bioinspired molecular and liposome designs, we conducted the multiscale integration of PDA liposomes to sensory devices, microarray and microbeads. First, PDA-PIP$_2$ liposome microarray was developed and showed a low detection limit of 61 ppb for neomycin, a kind of aminoglycosidic antibiotics, and was very specific to aminoglycosidic antibiotics only. Second, Janus-compartmental alginate microbeads having two divided phases of sensory PDA liposomes and magnetic nanoparticles were fabricated for facile sensory applications. The Janus microbeads having sensory PDA liposomes were usefully manipulated by magnetic field in sensory process such as washing and solvent exchange, stirring, and detection. Furthermore, the Janus microbeads embedding PDA-DPGG liposome conveniently enabled the label-free lead (II) detection, with lead (II) removal function of alginate. Finally, as a future work, we suggested a facile method for preparing PDA liposome coatings inspired by the strong adhesion
property of mussel protein in nature. By conjugating PDA liposomes with dopamine, a small molecule mimicking the chemicals groups on mussel protein, we realized material-independent coating of PDA liposomes, even on hydrophobic polytetrafluoroethylene (PTFE) surface. We are planning to fabricate paper microfluidic PDA biosensor utilizing hydrophilic PDA-dopamine liposome coating on hydrophobic surface.

**Keywords:**  Biosensor, Multiscale, Bioinspiration, Mechanochromism

Polydiacetylene, Liposome, Self-assembly, Phospholipid,

Microarray, Microbeads, Micro/nano fabrication, Microfluidics

Antigen, Antibody, Aminoglycosidic antibiotics, Lead (II) ion,

Mussel protein, Dopamine

**Student Number:** 2009-20648
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Chapter 1 Introduction

1.1 Biosensor

Engineered efforts to protect our life healthy against diverse biological threats, ranging from disease, polluted food, and hazardous environment to aging, essentially start from measuring the information of biosamples in analyzable signal. Biosensor is such a kind of sensory device identifying, quantifying, and converting the concentration, structure, or function of bioanalytes, to optical, electrical, or other human-readable signals. Since the first biosensor for glucose detection was invented by Clark in 1962[1], numerous types of biosensor have intensely suggested, and practically applied to various areas such as biological research, medical diagnosis, inspection of pollutants, and home healthcare.

 Biosensors are generally designed by combinations of two components, receptor and transducer, as shown in Figure 1.1, Receptor interacts with only targeted bioanalytes selectively, and transducer measures the intensity of the interaction between the analytes and the receptors, Representative receptors are antibody,
enzyme, peptide, aptamer, chemical ligand, and complementary oligonucleotide. In case of transducers, there are two classified mechanisms, labeled and non-labeled detection. Labeled detection quantify the receptor conjugated with observable objects (enzyme, fluorophore, nanoparticles, etc.) after capturing the analyte while non-labeled detection directly quantify the dynamic variations by the interaction between the analyte and the non-labeled receptor. Though labeled methods such as ELISA (Enzyme-Linked ImmunoSorbent Assay) have been mostly widely used methods, due to the complex and skillful steps of labeled detection, non-labeled transduction system recently attracts remarkable attentions.

In common with other sensory devices, the performance of biosensor is mainly evaluated by such following factors: sensitivity, response time, linearity, selectivity, and reproducibility. i) High sensitivity and fast response time are important for rapid action to prevent the unpredicted deterioration of the health. ii) Linearity enables facile quantification of bioanalytes or integration to automated feedback control systems. iii) Selectivity to targeted bioanalytes is needed to reduce the wrong evaluation or diagnosis. iv) Robust reproducibility unaffected by user or environment is also essential.

Recent achievements in materials science and micro/nano fabrication technology have brought innovations in biosensor development with notable
enhancements of the performance.[2-6] Advanced materials such as fluorophores, nanoparticles, nanowires, nanotubes, quantum dots, stimuli-responsive polymers, and conjugated polymers have been developed with suggesting novel signal transduction or amplification mechanism having high sensitivity and selectivity. In addition, such materials which can change their optical or electrical properties to environmental variations have realized the non-labeled detection system with replacing conventional labeled detection. Micro/nano fabrication technology has also led following improvements of biosensors. i) The micro/nano patterning methods placing the sensory materials on defined location in the device realize solid-type and non-labeled biosensors achieving small-volume analysis and user-friendly operation. ii) Micro or nano arraying of sensory materials enables the rapid detection of multiple targets for high-throughput screening. iii) At miniaturized scale, sensory materials obtain high sensitivity due to the enhanced surface area for increased interaction between receptor and analyte and rapid diffusion of analyte in confined geometry.
Figure 1.1 Schematic illustrations for components of biosensor
1.2 Polydiacetylenes for Colorimetric Biosensor

Polydiacetylenes (PDAs) are a kind of conjugated polymer, which is polymerized from diacetylene monomers. In contrast with general polymers, the conjugated polymer has unique electrical and optical properties (e.g. electric conductivity, color, fluorescence) resulted from its chemical structure [3, 7-8]. Main chain of the conjugated polymer consists of alternating unsaturated bonds (e.g. double and triple bond) and p-orbitals of unsaturated bonds with delocalized π-electrons are overlapped along the main chain. The conjugated p-orbitals generate electronic band structure which is an origin of electric and optical properties of conjugated polymer.

Conjugated PDAs have a specific optical property called “mechanochromism” which means change of color and fluorescence induced by mechanical stress. In general, PDAs originally have blue color and no fluorescence resulted from their well-oriented conjugated backbones. Blue PDAs can, however, change their color to red and emit weak red fluorescence, which is believed from mechanical distortion of conjugated backbone of PDA by external stimuli, as shown in Figure 1.2 A).

Due to attractive advantages, simple naked-eye and non-labeled detection, The mechanochromism of PDA has been applied to colorimetric sensor systems to
various stimuli such as heat[9-10], mechanical stress[11], metal ions[12-20], chemicals[15, 21-24], biomolecules[25-26], and bacteria[27-29]. In case of PDA-based biosensor, the mechanical stresses induced by molecular interaction between bioreceptor and bioanalytes induce the mechanochromism as shown Figure 1.2 B). When PDA combined with bioreceptor, the mechanical stresses by molecular interaction can be transferred to and distort the conjugated backbone of PDA, and thus result in the blue-to-red color change and red fluorescence. Representative mechanical stresses by the molecular interaction are steric force, Van der Waals force, hydrophobic interaction, hydrogen bonding, and electrostatic forces.
Figure 1.2 Schematic illustrations for A) mechanochromism of polydiacetylene and B) mechanism of PDA-based biosensor.
1.3 Design of Polydiacetylene-based Biosensor

1.3.1 Bioinspired Design of Polydiacetylene-based Biosensor

A representative preparation method of PDA material for biosensory applications is liposome assembly utilizing amphiphilic PDA monomers [9]. Liposome is a vesicular type of supramolecular structure enclosed by lipid bilayer and is generated when amphiphilic molecules (lipids) having hydrophobic tail and hydrophilic head group are self-assembled in aqueous solution. The liposome structure is same as the structure of cell membrane mainly consisting of lipid bilayer and therefore has high biocompatibility and attractive potentials in various bioengineering applications.

The monomers of polydiacetylene are generally amphiphilic in water and the lipidic property leads to self-assembly of the monomers to liposome with size of 100 ~ 200 nm. Due to their bioinspired cell membrane-like structures, the liposome-type of PDA materials has following advantages and properties in biosensory applications.

i) Water-compatibility of liposome structure is proper to sensing aqueous biomolecules while most of conjugated polymer is not insoluble in water due to its hydrophobicity of unsaturated bond in its conjugated chain [3]. ii) The most important
function of cell membrane is the precise signal transduction from extracellular signals to cytoplasm. Therefore, cell membranes fundamentally have high sensitivity to desired extracellular signals and inherent resistance to undesired fluctuation from extracellular environment. Similarly, PDA liposomes have high affinity to targeted bioanalytes through well-oriented head groups of PDA liposomes, and have high selectivity to non-specific binding of undesired molecules [30]. iii) Cell membrane-like structure makes the polydiacetylene be good platform to observing various phenomena like cell-signaling occurred at cell membrane [30]. iv) Cell membrane often regulates their cellular functions through changing the mechanical properties of the cell membrane. Similarly, the sensory property of PDA liposomes, such as sensitivity is closely related to the mechanical property of liposomes such as size, membrane flexibility, and surface charge [25]. Owing to this reason, the sensitivity can be tuned by the changing of mechanical property of liposomes through modification of PDA monomer or insertion of non-PDA lipids.

Apart from bioinspired properties, liposome structure of PDA monomers is also important for preparation of polymerized PDA. The assembled PDA monomers should be polymerized and having well-oriented blue conjugated chain for sensory use. The amphiphilic PDA monomers in liposome can be well-ordered in such a cooling process and can be readily polymerized by several minutes of 254 nm UV
light irradiation resulting in conjugated blue PDA [9]. Such polymerization process is
called “topochemical polymerization” and it is quite simple and does not need any
additional chemicals such as initiator in the process.
Figure 1.3 Schematic illustrations for preparation of bioinspired PDA liposomes
1.3.2 Multiscale Design of Polydiacetylene-based Biosensor

Constructing polydiacetylene-based biosensor systems needs multiscale design process ranging from monomer or lipid molecules, and nano/micro liposome to integrated device. First, in molecular scale, a careful design process combining receptor and PDA liposomes is necessary for sensory PDA liposomes.[3, 31] For high sensitive signal generation, when the receptor conjugated with PDA interacts with bioanalytes, the interaction should effectively exert strong mechanical stress to conjugated PDA chain. Representative mechanical stresses which can generate sensory signal of PDA are electrostatic force, steric force, Van der Waals force, and hydrophobic interaction. The receptor can be conjugated with headgroup of PDA monomer before or after self-assembly, or be incorporated to the PDA liposomes as type of lipids.

Second, after the self-assembly process to nano or micro liposomes, the PDA liposomes should have proper mechanical property such as size, uniformity, surface charge, and phase transition temperatures.[25] In addition, liposome design should consider the robustness and stability of liposome because inherent disadvantage of liposome is the aggregation and fusion which prevents their long-term storage.[25]

Third, though the assembled PDA liposome can be self-inclusively used in a
solution-type sensor system, there are some limitations in solution-type PDA liposome sensor: i) long term storage of liposomes is difficult because of inherent aggregation,[25] ii) buffer exchange or washing steps (e.g. dialysis, centrifuge) for removing unbounded probe or target is skillful and can cause the damage of liposomes, and iii) sensitivity is lowered due to homogeneous dispersion of targets in solution. Therefore, the various micro/nano fabrication methods for integration of PDA liposomes into or on solid substrate have been suggested. The integrated devices such as PDA liposome- microarrays,[18, 32], thin films,[22, 24, 28, 33] microfibers,[17, 34-35] or microbeads.[36-38] have attracted attentions realizing convenient, user-friendly and versatile biosensor platforms.
Figure 1.4 Schematic illustrations for multiscale design of PDA-based biosensor device. It is noted that PDA-based biosensor devices are not limited to microarrays; other types of devices such as microbeads and microfluidic devices are also included.
1.4. Thesis Objective and Outline

In this thesis, I present the multiscale and bioinspired approaches for designing polydiacetylene-based biosensor systems such as molecular design for enhancing sensitivity and stability of PDA liposomes, cell membrane-like PDA liposomes for detection of desired target bioanalytes, and integration to biosensor devices, PDA liposome microarray and PDA liposome-embedded microbeads.

*In chapter 2*, we present polydiacetylene (PDA) liposome assemblies with various phospholipids that have different head group 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.

*In chapter 3*, we rationally design highly sensitive and selective polydiacetylene (PDA)-phospholipids liposomes for the facile detection of
aminoglycosidic antibiotics. The detecting mechanism mimics the cellular membrane interactions between neomycin and phosphatidylinositol-4,5-bisphosphate (PIP₂) phospholipids. The developed PDA-PIP₂ sensory system showed a detection limit of 61 ppb for neomycin and was very specific to aminoglycosidic antibodies only.

In chapter 4, we report Janus-compartmental alginate microbeads having two divided phases of sensory PDA liposomes and magnetic nanoparticles for facile sensory applications. The Janus microbeads having sensory PDA liposomes were usefully manipulated by magnetic field in bead concentration for washing and solvent exchange, stirring, and detection. In addition, Janus microbeads applied to the lead (II) detection with newly designed PDA liposomes having 1,2-dipalmitoyl-sn-glycero-3-galloyl (DPGG) lipid, which have a lead (II) sensitive galloyl head group. The Janus microbeads conveniently enabled the label-free lead (II) detection, with lead (II) removal function of alginate.

Finally, in chapter 5, we present the conclusion of our previous works and suggest a future work. The future work is developing a simple and efficient coating method of PDA liposomes for facile integration to multiscale biosensor systems. For the purpose, our on-going research is being inspired by the strong adhesion property of mussel protein in nature. By conjugating the PDA liposomes with dopamine, a mussel-inspired small molecule, we realized the material-independent coating of PDA
liposomes. Furthermore, we anticipate that catechol groups on PDA-dopamine liposomes can be used for universal tethering of the receptors. As an application, we are planning to fabricate paper microfluidic PDA biosensor utilizing hydrophilic PDA-dopamine liposome coating on hydrophobic surface.
Chapter 2 Molecular Design of Polydiacetylene-Phospholipid Liposome for Enhanced Stability and Sensitivity

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2.1 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[9, 39], light[40], ions[18-19, 41], chemicals[15, 23, 42-43], bio-molecules[27, 44-47], and bacteria[28-29, 48-49]. 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[3, 31, 50].

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 bio-interfacial interactions such as receptor-ligand interaction[23, 51], membrane permeabilization by bacterial toxin[48] or antimicrobial peptides[21], and enzymes-lipids interactions[52].

In general, the insertion of lipids into PDA liposome has a merit to modify the physical properties of PDA liposome such as size[53], surface charge, and packing of lipids[49], which, in turn, affect the sensitivity and stability of PDA liposome biosensor[15, 27, 46-47, 49, 54-55]. 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[47, 56]. An appropriate level of surface charge of liposome can also introduce electrostatic repulsion providing resistivity against aggregation and fusion[54]. 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[15, 27, 46, 49]. However, ironically, the weak packing interrupts polymerization into a stable conjugated chain[9] and deteriorates the degree of fusion[55].
Recently, there have been some trials to increase the sensitivity of PDA liposome biosensor by controlling the degree of packing between lipids[27, 29, 46, 49]. 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[18]. 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 head group charges and phase transition temperatures ($T_m$ indicating the degree of intermolecular attraction and packing between lipids) as schematically drawn in Figure 2.1. As a general 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.
Figure 2.1 Schematic illustration for the design of different kinds of PDA-phospholipid liposomes
2.2 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[18]. 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 (see Figure 2.2). 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.[23] The 4:1 molar ratio was also reported to be optimal for sensory applications,[18] being in the range of widely used molar fraction of phospholipids, 0.2 ~ 0.4.[29, 46, 49] 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 20ml 5mM 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 hr at 5°C refrigerator before use.
Figure 2.2 Colorimetric responses of PDA-phospholipid liposomes for the different ratios of non-polymerizable phospholipids after heating at 70°C for 3 min
Measurement of Size and Zeta Potential of PDA-Phospholipid 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-Phospholipid Liposomes

A small amount (200 µl) of 0.5 mM PDA-phospholipid liposome solution polymerized under 254 nm 1 mW/cm² 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. The CRs of PDA-phospholipids liposomes were calculated by the well known equation.[9] Here, the blue percentage (PB) is defined as \[ \text{PB} = \frac{A_{\text{blue}}}{A_{\text{blue}} + A_{\text{red}}} \times 100\% \] where \( A_{\text{blue}} \) is the absorbance at the peak around 640 nm and \( A_{\text{red}} \) is the absorbance at the peak around 540 nm. Then, the CR is defined as \[ \text{CR} = \frac{\text{initial PB} - \text{final PB}}{\text{initial PB}} \times 100\%. \]
Comparison of Thermochromic Responses of PDA-phospholipid liposomes

PDA-phospholipid liposome solution polymerized under an optimal UV irradiation for 20 sec 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 (see Figure 2.3).
Figure 2.3 Colorimetric responses of the PDA-phospholipid liposomes after heating at several intermediates temperatures between 25 and 70°C
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 5mM HEPES buffer of pH 8 for 1 hr 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, in order 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 (see Figure 2.4). 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 hr 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 (see Figure 2.5).
Figure 2.4 Colorimetric responses of the PCDA/PCDA-epoxy liposomes for the different concentrations of the probe (BVDV B20.24 antigen) against 100 µg/ml BVDV antibody
Figure 2.5 Measurement of the degree of protein binding on PCDA/PCDA-epoxy, DMPA/PCDA-epoxy, and DMTAP/PCDA-epoxy liposomes. 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 hr at room temperature. Then, the degrees of absorbance of unbounded FITC-BSA solutions were taken at 492 nm and compared to the calibration curve.
2.3 Results and Discussion

2.3.1 Size and Stability of PDA-Phospholipid Liposomes

The size and zeta potential (ZP, degree of repulsion between liposomes) of the designed PDA-phospholipid liposomes were measured by electrophoretic light scattering on day 1 and 7 after forming the assemblies. As shown in Table 2.1, 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 hr 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.[57] 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 $5^\circ C$), 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.
<table>
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<tr>
<th></th>
<th>PCDA/PCDA-epoxy liposome</th>
<th>DMPC/PCDA-epoxy liposome</th>
<th>DMPA/PCDA-epoxy liposome</th>
<th>DMTAP/PCDA-epoxy liposome</th>
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<tr>
<td><strong>Size (nm)</strong></td>
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<tr>
<td>at 1 day</td>
<td>280 (6.5)(^a)</td>
<td>&gt; 5000</td>
<td>187 (3.7)</td>
<td>289 (2.7)</td>
</tr>
<tr>
<td>at 7 day</td>
<td>327 (6.1)</td>
<td>-</td>
<td>208 (4.3)</td>
<td>308 (3.1)</td>
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<tr>
<td><strong>Increasing Ratio (%)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>16.2</td>
<td>-</td>
<td>11.3</td>
<td>6.5</td>
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<tr>
<td><strong>Zeta Potential (ZP) (mV)</strong></td>
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<td></td>
<td>-20.2 (0.5)</td>
<td>-3.2 (0.5)</td>
<td>-30.1 (0.5)</td>
<td>27.1 (0.6)</td>
</tr>
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</table>

Table 2.1 Sizes and zeta potentials of various PDA-phospholipid liposomes with exposure time (1 and 7 days).

\(^a\) Standard deviation (n=3)
2.3.2 Thermochromic Responses of PDA-Phospholipid Liposomes

After 24hr cooling for packing, the liposomes were polymerized by irradiating 254 nm UV light for different time periods in order to determine an optimum polymerization time, in which the CRs of PDA-phospholipids liposomes were measured by UV-Vis spectrometer. As shown in Figure 2.6, 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.[9, 43]

The optical images of PDA-phospholipids liposome polymerized by 20 s UV irradiation are presented in Figure 2.7. 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 2.8. 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.[9, 43] 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 nm and 650 nm, respectively, indicating that a relatively unstable π-conjugated polymerization has occurred.

In order to evaluate the sensor performance, we measured the CR (Figure 2.7 detailed spectra are shown in Figure 2.8) 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 2.9, which apparently matched the size and degree of aggregation based on the electrophoretic light scattering measurement. In general, the insertion of phospholipid transforms the shape of liposome from round to rectangular sheet, according to previous findings.[46] Also, the DMPC/PCDA-epoxy liposome shows a great aggregation (scale bar: 500 nm), which also agrees with the scattering experiment summarized in Table 2.1. Furthermore, as the inserted phospholipid has a lower T_m, the sheet shape
became wider and longer as shown in Figure 2.10. 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.
Figure 2.6 CRs of PDA-phospholipid liposomes after 254 nm UV polymerization for a series of time periods and heating at 70 °C (n=3).
Figure 2.7 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).
**Figure 2.8** 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.
Figure 2.9 TEM images of the different kinds of PDA-phospholipid liposomes.
Figure 2.10 Quantitative analysis of the width and length of PDA-Phospholipids liposomes (n=30) with TEM images. The size of non-aggregated liposome was measured for the DMPC/PCDA-epoxy liposome.
2.3.3 Enhanced Sensitivity to Immunological Detection of Biomolecules

To utilize the PDA-phospholipid liposomes for liposome immunoassay,[58-59] 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 Figure 2.11. 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[42]. 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 2.12 (detailed spectra are shown in Figure 2.13). 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 \(\mu\)g/ml, and the absence of nonspecific binding was also confirmed by incubation with classical swine fever virus (CSFV) IgG antibody 100 \(\mu\)g/ml. Results showed that the CR change in this case was less than 1.6 % for all liposomes tested (Supplemental Figure S5).
**Figure 2.11** Schematic illustration of biosensor applications using PDA-phospholipid liposomes.
Figure 2.12 Correlation curve between the CR and concentration of BVDV antibody after 24hr incubation in the antibody solution (n=3).
Figure 2.13 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 (for confirming nonspecific binding) overnight. Enlarged spectra for the wavelength of 620–660 nm are also presented to demonstrate reduced adsorption of red light with respect to the increased concentration of target, BVDV antibodies: A) PCDA/PCDA-epoxy liposome, B) DMPA/PCDA-epoxy liposome, C) DMTAP/PCDA-epoxy liposome
2.4 Summary

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.
Chapter 3 Biomimetic Polydiacetylene-Phospholipids Liposome for Aminoglycosidic Antibiotics Detection

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3.1 Introduction

Neomycin is a representative aminoglycosidic antibiotic prevalently used in hospitals and the livestock industry. The antibiotic mechanism of neomycin is based on the inhibition of protein synthesis by binding to a ribosomal RNA[60] and is very effective against most clinically harmful bacteria. However, neomycin is much more nephrotoxic compared to other aminoglycosides. Therefore, the abuse and misuse of neomycin can cause an allergic response,[61] organ damage (such as ear and kidney), and nerve system malfunction,[62-63] as well as the emerging super bacteria having a tolerance to antibiotics.[60] Consequently, many agriculture, food, and drug regulatory authorities such as World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) have set a tolerance limit of neomycin in meat and dairy products including milk and eggs. Accordingly, various
detection strategies have been developed to detect neomycin including high performance liquid chromatography (HPLC),[64] enzyme-linked immunosorbent assay (ELISA),[65-66] aggregation based sensors,[67] and competitive impedimetric assay.[68] However, these techniques are complex, expensive, and often require highly sophisticated heavy equipment and skillful operation.

Polydiacetylene (PDA), a unique conjugated polymer, has been applied to various label-free and colorimetric sensory systems since PDA changes its color from blue to red upon exposure to various external stimuli such as heat,[9, 33, 69] mechanical stress,[70] ions,[18-19] chemicals,[15] bio-molecules,[21, 52, 71] and bacteria.[27, 48-49, 51] In addition to this convenient colorimetric self-signaling property, the transformed red phase PDA also emits red fluorescence, enabling the convenient and sensitive dual detection capability. The most commonly used and convenient form of PDA is liposome because amphiphilic PDA monomers can be easily designed and self-assembled into a liposome shape. After the self-assembly, PDA monomers in the liposomes are photo-polymerized by 254 nm UV irradiation to become conjugated PDA having blue color.[32] Distortion of the conjugated yne-ene backbone of PDA by external stimuli is believed to cause the color change and the red fluorescence development.

PDA liposomes can accommodate various natural and/or synthetic lipids
such as phospholipids. Molecular interactions via such a lipid inserted into a PDA liposome, for example, enzymes-lipids interactions,[52] membrane permeabilization by antimicrobial peptides[21, 71] or bacterial toxin,[48] and receptor-ligand interaction,[51] have been investigated in various colorimetric biosensor development. We coined such a molecular interaction into a PDA sensory system design to selectively and sensitively detect possible residual neomycin in dairy product or meats. Neomycin is known to bind to phosphatidylinositol-4,5-bisphosphate (PIP_2) lipids in the cellular membrane.[72-74] Molecular and cellular biology research revealed that PIP_2 decomposes into diacylglycerol (DAG) and 1,4,5-triphosphate (IP_3) by phospholipase C (PLC) through stimulating various hormones and growth factors.[75] As shown in Figure 3.1 A), neomycin binds to PIP_2, inhibiting the PIP_2 degradation by PLC and thus inhibiting the IP_3-related signal cascade, which is a known side effect of neomycin.[72-74] In our convenient colorimetric PDA sensor design, as schematically described in Figure 3.1 B), PIP_2 was used as a selective receptor to detect neomycin and co-assembled into a PDA liposome (PDA-PIP_2). As designed and anticipated, the interaction between neomycin and PIP_2 exerted stress on the conjugated backbone of PDA-PIP_2 and consequently produced a sharp color change and fluorescence development as dual sensory signals.
Figure 3.1 A) Schematic illustration of the inhibition of PIP$_2$-PLC signal pathway by neomycin binding to PIP$_2$ lipid. B) Chemical structure of neomycin. C) Schematic illustration of neomycin detection mechanism by the designed PDA-phospholipids liposome including PIP$_2$ phospholipids as a specific receptor toward neomycin.
3.2 Materials and Methods

Materials

Phospholipids, L-α-phosphatidylinositol-4,5-bisphosphate (PIP$_2$) and 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) were purchased from Avanti Polar Lipids. Other chemicals such as a PDA monomer, 10,12-pentacosadiynoic acid (PCDA), neomycin, other antibiotics, solvents and buffers were purchased from Sigma-Aldrich Chemicals. Amine-coated glass slides for the microarray experiment were fabricated similarly to a reference.[15] Glass slides were cleaned with chloroform, acetone, and IPA for a 5 minute bath sonication each and activated in sulfuric acid with ammonium persulfate for a 1 hr bath sonication. After thorough washing with water and drying, the slides were dipped in toluene containing 1 wt% 3-aminopropyltriethoxysilane toluene for 1 hr. After washing with toluene and subsequent drying, the slides were baked at 110 °C for 15 minutes and the unreacted silane molecules were removed by bath sonication in toluene, toluene:methanol (1:1), and methanol for 3 minutes each.
Liposome Assembly

As schematically illustrated in Scheme 1B, PDA-PIP2 liposomes were assembled by the following thin film method. PCDA, DMPA, and PIP2 lipids were dissolved in chloroform (7:2:1 molar ratio) to have the final concentration of 0.5 mM. After removing chloroform by thorough N2 blowing, the lipids were suspended in 10 ml 0.1X PBS and 120W probe-sonicated at 80 °C for 10 minutes. The liposome solution was filtrated through a 0.8 μm cellulose acetate syringe filter and stored at 5 °C for 24 hr before use.

Aminoglycosidic Antibiotic Detection with PDA-PIP2 Liposome in Solution

0.5 mM PDA-PIP2 liposome solution was polymerized by 254nm UV irradiation for 1 minute. 50 μM aminoglycosidic antibiotic solution was introduced into the solution. UV/Vis adsorption spectra taken from PerkinElmer Lambda 45 UV/Vis spectrometer were used to quantify the colorimetric response by means of CR values.8a Fluorescence spectra were obtained on PTI QuantamasterTM spectrofluorometer.
Microarray Experiment with PDA-PIP$_2$ Liposome

0.5mM PDA-PIP$_2$ liposome solution was spotted on amine-coated glass slides by using a manual microarrayer (V&P scientific, INC) and incubated at above 70% humidity for 24 hrs to prevent droplet drying out too fast. The liposomes were covalently immobilized on amine coated glass slides through the carbodiimide chemistry between the carboxylic groups on the liposome surface and the amine groups on the substrate. (Or the liposomes were physically immobilized on amine coated glass slides with interaction between negative-charged liposome and positive-charged amine.) After washing with 0.1X PBS for 10 minutes followed by drying with N$_2$ blowing, the immobilized PDA-PIP$_2$ liposomes on the glass slides were subsequently polymerized by 254 nm UV irradiation for 1 minute. For detection tests, the glass slide was dipped into an antibiotics solution at 37 °C for 20 minutes. Fluorescence microscopic images were obtained on Olympus BX 71 microscope with mercury lamp and 540 nm excitation, 600 nm cut-off emission filters.
3.3 Results and Discussion

3.3.1 Neomycin Detection with PDA-PIP$_2$ Liposome

The PDA-PIP$_2$ liposome containing PIP$_2$ showed a sharp color change upon exposure to a neomycin solution as shown in Figure 3.2 A). The optimized molar ratio of three components of the PDA-PIP$_2$ liposome was 7:2:1 (PCDA:DMPA:PIP$_2$) as Figure 3.3 B) shows that the sensitivity at that composition is highest. The role of DMPA in our liposome design is to enhance the sensitivity by making the PDA backbone in the liposome more mobile.[49] PDA liposomes having no PIP$_2$ did not show any noticeable color change as presented in Figure 1B, indicating the recognition of neomycin originates from PIP2 in the PDA-PIP$_2$ liposome. The affinity between neomycin and PIP$_2$ stems from the charge-charge interaction between negative charges on the PIP2 head group and positive charges of neomycin (Scheme 1).[72-74][76] The neomycin-PIP$_2$ complex formation exerts stress on the conjugated backbone of PDA either due to the steric hindrance between surface-bound adjacent neomycin or the reconstruction of the liposome surface induced by charge alteration.

We further developed a more convenient solid-state sensory system based on the solution results.[32] A PDA-PIP$_2$ microarray was fabricated byspotting the PDA-
PIP₂ liposome on an amine-coated glass slide. Figure 3.3 A) shows fluorescence microscope images of the PDA-PIP₂ liposome microarrays before and after a 20-minute exposure to a 50 μM neomycin solution. The fluorescence emission intensity of the liposome spot became stronger as the concentration increased (Figure 3.3 B)). We could develop a good correlation between the fluorescence signal intensity and the neomycin concentration as plotted in Figure 3.3 C). The detection limit of the present system is 0.1 μM (61 ppb), which is good enough to detect the regulation limits of neomycin defined by WHO and FAO (500 ppb in meat and egg and 1500 ppb in milk).
Figure 3.2 A) UV-vis spectra and corresponding optical images of a PDA-PIP₂ liposome solution after 20-minute of incubation with 50 μM neomycin at 37 °C. B) Optimization of the mole ratio of PCDA:DMPA:PIP₂ in the PDA-PIP₂ liposome. Colorimetric Response was measured after 20-minute incubation with 50 μM neomycin at 37 °C.
Figure 3.3 A) Fluorescence microscope images of PDA-PIP<sub>2</sub> liposome arrays before and after 20-minute reaction with 50 μM neomycin at 37 °C. B) Enlarged fluorescence microscope images of PDA-phospholipid liposome spots after 20-minute incubation with various concentrations of neomycin from 0.1 to 50 μM at 37 °C. C) Correlation curve between the fluorescence intensity and the concentrations of neomycin.
3.3.2 Fluorescence Responses of PDA-PIP$_2$ Liposome about Other Aminoglycosidic Antibiotics

We extended our detection study to other aminoglycosidic antibiotics because PIP$_2$ lipids are known to bind to other aminoglycosidic antibiotics that have a similar chemical structure to neomycin.[73-74] As anticipated, the PDA-PIP$_2$ liposome also developed a certain level of fluorescence sensory signal to other aminoglycosidic antibiotics such as gentamicin, tobramycin, and streptomycin as shown in Figure 3.4 and Figure 3.5 (PL spectra). The signal intensity for other aminoglycosidic antibiotics, however, was much weaker and showed an interesting trend. As one can see in Figure 3.4 A), the signal intensity displays an exponential drop rather than a linear decline as the net charge[77-78] of the aminoglycosidic antibiotics decreases. The signal intensity is likely a product of the molecular size as well as the charge density of the aminoglycosidic antibiotics. Neomycin having the largest charge density among the aminoglycosidic antibiotics also has the largest molecular weight of 614.64 g/mol compared to gentamicin (477.60), Tobramycin (467.52), and streptomycin (581.57). Therefore, the larger charge density will cause stronger interaction with the PDA-PIP$_2$ liposome and the larger size will induce greater stress and a brighter sensory signal.
Figure 3.4  A) Fluorescence emission intensity and B) Fluorescence microscope images of the PDA-PIP$_2$ liposome microarrays after 20-minute incubation with 50 μM concentration of various aminoglycosidic antibiotics at 37 °C and a physiological pH. The net charge of each aminoglycosidic antibiotic is given in the parentheses.
Figure 3.5 Fluorescence Spectra of the PDA-PIP₂ Liposomes after 20-minute incubation with 50 μM concentration of various aminoglycosidic antibiotics at 37 °C and a physiological pH
3.3.3 Confirmation of Nonspecific Binding

We also tested non-specific binding of non-aminoglycosidic antibiotics such as penicillin G, oxytetracycline, and sulfamethazine to the PDA-PIP<sub>2</sub> microarray. As shown in Figure 4, these antibiotics, having a different antibiotic mechanism from the aminoglycosidic antibiotics, did not produce any sensory signals, which confirmed a good selectivity of the developed PDA-PIP<sub>2</sub> sensory system toward aminoglycosidic antibiotics. Furthermore, we found that even a cocktail solution of 50 μM penicillin G, oxytetracycline, and sulfamethazine do not hinder the specific interaction between neomycin and the PDA-PIP<sub>2</sub> liposome. While the cocktail without having neomycin did not produce any noticeable signal generation, the same cocktail having 1 μM neomycin generated fluorescence signal as demonstrated in Figure 3.6.
Figure 3.6 A) Fluorescence intensities of the PDA-PIP$_2$ liposome microarray after 20-minute reaction with 50 μM concentration of neomycin and non-aminoglycosidic antibiotics at 37 °C. B) Fluorescence intensities and corresponding microscope images of the PDA-PIP$_2$ liposome microarray after 20-minute incubation with a mixed solution of 50 μM of non-aminoglycosidic antibiotics having or without having 1 μM of neomycin at 37 °C.
3.4 Summary

We developed a PDA-based biomimetic colorimetric sensory system for the detection of aminoglycosidic antibiotics by adapting the interaction between PIP₂ lipids and aminoglycosidic antibiotics into our PDA sensor design. Binding of aminoglycosidic antibiotics to the PDA-PIP₂ liposome imposes stress to the conjugated yne-ene PDA backbone and produces an ensuing color change and develops fluorescence emission as a dual sensory signal. The most commonly used neomycin has the high cation density among the aminoglycosidic antibiotics. Hence, the designed PDA-PIP₂ liposome microarray showed the best detection limit of 61 ppb for neomycin. The developed PDA-PIP₂ liposome microarray also displayed good sensitivity toward aminoglycosidic antibiotics. Non-aminoglycosydic antibiotics such as penicillin G, oxytetracycline, and sulfamethazine did not cause any false signaling nor hindered the detection capability of the PDA-PIP₂ liposome microarray for neomycin. We believe that the presented biomimetic sensory design principle can be readily applicable to the detection of various other biomolecules and the investigation of bio-interfacial phenomena.
Chapter 4  Janus-compartmental Microbead Having Polydiacetylene Liposome and Magnetic Nanoparticle for Visual Lead (II) Detection

4.1 Introduction

Polydiacetylene (PDA), a kind of conjugated polymer, has been attractively applied to various sensory systems due to their unique colorimetric and fluorescent signal generation property. When exposed to specific external stimuli such as heat[9-10], mechanical stress[11], metal ions[12-20], chemicals[15, 21-24], biomolecules[25-26], and bacteria[27-29]. PDA can changes its color from blue to red, and the red PDA generates red fluorescence, realizing the self-signaling and dual detection system. The signal generation mechanism of PDA is believed to distortion of the conjugated yne-ene main chain of PDA by mechanical stress from external stimuli. Representative preparation method for sensory PDA material is liposome assembly utilizing amphiphilic PDA monomers with following advantages: i) the amphiphilic PDA monomers in liposome can be well-ordered in such a cooling
process for UV-induced topochemical polymerization which readily results in conjugated blue PDA, and ii) Owing to the structural nature of liposome, sensory interactions between PDA and target can be occurred with high affinity through well-oriented head groups of PDA or inserted specific amphiphilic molecules.

Though the liposome form of PDA can be directly used in solution, there are some limitations in solution-type PDA liposome sensor: i) long term storage of liposomes is difficult because of inherent aggregation,[25] ii) buffer exchange or washing steps (e.g. dialysis, centrifuge) for removing unbounded probe or target is skillful and can cause the damage of liposomes, and iii) sensitivity is lowered due to homogeneous dispersion of targets in solution. Therefore, as the post-processing, the immobilization of PDA liposomes into or on solid substrate have been researched such as microarrays,[18-19, 24, 32], thin films,[22, 28, 33] microfibers,[17, 34-35] or microbeads[36-38]. In this regard, our group previously reported a kind of PDA microbeads system which utilizing calcium-crosslinked alginate hydrogel microbeads embedding sensory PDA liposomes[36]. Our PDA microbead system realized enhanced sensitivity, selective multi-target detection, and long-term storage of liposomes.

In this contribution, we improved our previous PDA microbeads system to Janus-compartmental microbeads having two divided phases of sensory PDA
liposomes and magnetic nanoparticles for useful bead handling in sensory experiments. As shown in Figure 4.1 A), the magnetic field was able to stir the Janus microbeads with enhancing mass transport, and thus increased the sensitivity. In addition, concentration of Janus microbeads through magnetic field realized the convenient washing of unreacted molecules and change of buffer or solvent. The Janus microbeads were fabricated through calcium-crosslinking of alginate solutions having sensory PDA liposomes and magnetic nanoparticles, using simple homemade microbeads generator operated by centrifugal force (Figure 4.1 B)).[36, 79-80]

Second, we applied our Janus alginate microbeads system to lead (II) detection with utilizing our above mentioned developments (Figure 4.1 C)). Water or land contamination by lead is a concerned problem, because lead is a poisonous heavy metal ion which can accumulate in animal or human bodies, and damages nervous system, kidney, bone, or other tissues.[81-82] Various methods for lead (II) detection have been developed using gold nanoparticles,[83-85] photonic crystals,[86] fluorophores,[87] and polymers.[14-15, 88] In parallel to studies about the detection methods, absorbents such as alginate,[89-94] polyphenolic compounds,[89, 95] and chitosan.[89, 96] have been also researched for removal of lead (II) or other heavy metal ions in the contaminated water or land. Especially, alginate has been widely used as an low cost adsorbent in usually the form of ca-
crosslinked microbeads because alginate has abundant carboxylic groups which have inherent high affinity to heavy metal ions.[89-94] The smart systems integrating sensory materials and absorbents have great potentials to detect and remove heavy metal ions at a same time.[92, 97] The sensory materials in the absorbent can detect heavy metal ions or self-indicate the degree of adsorption of heavy metal ions. In such smart systems, sensory materials should maintain their sensory property in the matrix of absorbents. Therefore, we expected that our Janus microbeads consisting of alginate matrix and lead (II) sensitive PDA liposomes become the smart system having dual functions, detection and adsorption. For the lead (II) detection, we designed a novel PDA liposomes (PDA-DPGG liposome) consisting of PDA monomers (10,12-pentacosadiynoic acid) and lipids (1,2 dipalmitoyl-sn-glycero-3-galloyl,DPGG)[98] which is selective probe to detect lead (II). In detail, the galloyl groups in the DPGG are known to be bound with lead (II) ions with high affinity, forming the phenolic metal complex.[83-85] As anticipated, the lead (II) ions exerted stress on the conjugated main chain of PDA in our designed PDA-DPGG liposome, and thus generated a sensory sharp color change and red fluorescence. The sensory response of our PDA-DPGG liposome to lead (II) was retained in the alginate matrix of the Janus microbeads with enabling convenient lead (II) detection and removal applications.
Figure 4.1 A) Magnetic manipulations of Janus microbeads in sensory processes: i) bead stirring for enhanced reaction and ii) bead concentration for solvent exchange or washing. B) Fabrication of sensory Janus-compartmental microbeads having PDA liposomes and magnetic nanoparticles. C) Target (lead (II) ions) detection using the Janus microbead having PDA liposomes and magnetic nanoparticles.
4.2 Materials and Methods

Materials

Chemicals such as a PDA monomer, 10,12-pentacosadiynoic acid (PCDA), magnetic nanoparticles (Fe₃O₄, 50 ~ 100 nm), lead(II) ion (chloride salt), other heavy metal ions (chloride salt), sodium alginate, calcium chloride, solvents and buffers were purchased from Sigma-Aldrich Chemicals. A lipid for lead (II) detection, 1,2-dipalmitoyl-sn-glycero-3-galloyl (DPGG), was ordered from Avanti Polar Lipids.

Assembly of PDA-DPGG Liposome

PDA liposomes having DPGG lipids (PDA-DPGG liposome) were assembled by the following injection method. PCDA and DPGG were dissolved in 100 µL Tetrahydrofuran and the mixture solution was injected to 10 ml 5 mM HEPES buffer pH 7.4. The total concentration of PCDA and DPGG (4:1 molar ratio) is 1 mM. The liposome solution was 120W probe-sonicated for 10 minutes and filtrated through a 0.8 µm cellulose acetate syringe filter. The liposome solution was stored at 5 °C overnight before use.
Surface Treatment of Magnetic Nanoparticle

Before the mixing with the alginate solution, the surfaces of magnetic nanoparticles are treated with citrate ions for preventing interactions with carboxylate functions of alginate.[99-100] 0.2 g Fe₃O₄ magnetic nanoparticles in 10 ml D.I. water containing 0.2 g citric acid is heated to 95 °C during 2 hours and precipitated in acetone at 25 °C.

Fabrication of Janus Microbead Having PDA Liposome and Magnetic Nanoparticle

This procedure was based on our previous reference utilizing the ionic crosslinking of alginate hydrogel.[36] As shown in Scheme 1B, the centrifugal microbead generator is consists of two syringes having 100 μL alginate solutions embedding PDA liposomes and magnetic nanoparticles separately. The alginate solution for PDA phase was made by mixing the 4 wt% alginate solution and 1 mM PDA-DPGG liposome solution (1:2 volume ratio) while the alginate solution for magnetic phase is mixture of the 4 wt% alginate solution and the prepared magnetic nanoparticle solution (1:2 volume ratio). Embedded PDA-DPGG liposomes were previously polymerized by 254 nm 1 mW/cm² UV irradiation for 5 minutes, because its blue color enables facile visualization of the homogeneous mixing to the alginate
solution. (Or the PDA liposomes in Janus microbeads were polymerized after the bead fabrication by 254 nm UV irradiation for 5 minutes.) The ends of two syringe needles (25 Gauge) are attached to each other, and the alginate solutions in syringe are injected to CaCl$_2$ solution (2.5 wt%) dropwise by 100 G centrifugal force for 5 minutes in lab-scale centrifuge. The fabricated Janus beads were hardened in the CaCl$_2$ solution for additional 20 minutes and washed three times by D.I. water with the magnetic concentration. The Janus microbeads are stored 5 °C before use.

**Lead (II) and Heavy Metal Detection with PDA-DPGG Liposome Solution**

For confirming the colorimetric response of PDA-DPGG liposome to lead (II) and heavy metal ions in solution, 0.66 mM PDA-DNA liposome solution was polymerized by 254 nm 1 mW/cm$^2$ UV irradiation for 5 minutes and 0.26 mM heavy metal ion solution was introduced into the solution. After 1 hour incubation, Camera image was obtained and UV/Vis adsorption spectra were taken from PerkinElmer Lambda 45 UV/Vis spectrometer. Fluorescence spectra were also obtained on PTI QuantamasterTM spectrofluorometer.
Lead (II) Detection with Janus Microbead

For detection tests, 8 mg of wet Janus microbeads (approximately 100 microbeads) were incubated with various concentrations of lead (II) solutions during 1 hour with or without magnetic stirring (1000 rpm). Optical and fluorescence microscopic images were obtained on Olympus BX 71 microscope or Nikon eclipse Ti microscope.

Lead (II) Removal with Janus Microbead

To confirm the lead (II) removal by alginate matrix of Janus microbeads, 8 mg of Janus microbeads were incubated with 1 mM lead (II) solution with 1000 rpm stirring. The supernatant solutions were collected at various time intervals, and the lead (II) concentrations of the solutions were measured by Shimadzu ICPS-7500 ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometer). The exact numbers of used microbeads were counted after the removal experiment and the adsorbed lead (II) ions (nmol) per hundred beads were calculated.
4.3 Results and Discussion

4.3.1 Sensory Behaviors of Janus Microbead

The fabricated Janus microbeads showed clear compartmentalization of PDA liposomes and magnetic nanoparticles (Figure 4.2 A)). The size of the microbeads was about 500 μm and uniform. In the alginate hydrogel matrix of the Janus microbeads, the PDA liposomes maintained their blue color and colorimetric property, showing the blue-to-red transition on heating (70 °C, 5 minutes). The red phase of PDA liposomes also emitted clear red fluorescence. In addition, the PDA liposomes in the microbeads stably retained their blue color over 30 days of storage.

Due to the embedded magnetic nanoparticles, the Janus microbeads also showed actuating responses on the magnetic field. The microbeads are concentrated by static magnetic field (Figure 4.2 B)) and stirred by rotating magnetic field (Supporting Information Video). In addition, the existence of magnetic field had no effects on the color and stability of PDA liposomes, In the general biosensor or assay experiments, these magnetic actuations are routinely used because the concentration enables the easy washing of unreacted molecules, or change of buffer or reactants, and the stirring realizes faster reaction.[101-103] Therefore, our developed Janus
microbeads system can be usefully applied to the assay systems with the label-free sensory property of PDA liposomes.

A notable advantage of compartmentalization of PDA liposomes and magnetic nanoparticles in the Janus microbeads is the clear detection of the red fluorescence of PDA by the magnetic field. As shown in the Figure 4.2 C), when the magnetic field did not existed, the black magnetic nanoparticle were tend to mask the red fluorescence emitted from PDA liposomes. On the other hand, by applying the vertical magnetic field to the light path of the fluorescence microscope (thus, z-direction to the microscope), the clear images of PDA fluorescence were achieved. In detail, due to the attraction to the vertical magnetic field, the Janus microbeads are rotated until the part of magnetic nanoparticles was heading toward the top. Thus, the parts of magnetic nanoparticles did not exist in the light path and therefore whole circle images of the parts of PDA liposomes were obtained. In this regard, all the fluorescence images of Janus microbeads for the following sensory experiment are obtained on the magnetic field, called “detection mode”.
Figure 4.2 A) Optical and fluorescent microscope images of Janus microbeads according to the phases of embedded PDA liposomes (Scale bar: 500 μm). B) Concentration of Janus microbeads by magnetic field. C) Magnetic manipulation of Janus microbead for clear fluorescence detection. (Red phase of PDA liposomes in the Janus microbead were converted from blue phase of PDA liposomes through 5 minutes heating at 70 °C)
4.3.2 Lead (II) Detection with PDA-DPGG Liposome Solution

To direct the developed Janus microbeads to the heavy metal detection system, we designed a novel PDA liposome including DPGG lipids which is known to have metal binding property, as shown in Figure 4.3. The galloyl group, which is the head group of DPGG lipid, can form the phenolic metal complexes with heavy metal ions and therefore have been used in heavy metal removal applications.[95, 104-105] Furthermore, the galloyl groups have been reported as a high sensitive and selective lead (II) probe in gold nanoparticle sensors because, among heavy metal ions, the lead (II) ion can makes strong complex with multiple galloyl groups, enabling various inter or intramolecular interactions for signal generation.[83-85] With expecting that the inter/intramolecular interaction between lead (II) and galloyl groups can also generate the sensory signal in PDA system,[15] we co-assembled DPGG lipids into a PDA liposome (PDA-DPGG liposome). In comparison to other present PDA heavy metal sensors,[13-14, 16, 18, 20] our present method did not require any complex synthesis of PDA monomers or further modification of PDA liposomes with heavy metal specific probe.

The colorimetric response, UV/Vis adsorption spectra and PL spectra of PDA-DPGG liposome after 1 hour incubation with various metal ion solutions are
confirmed as shown in Figure 4.4. As we anticipated, the PDA-DPGG liposome showed sharpest color change from blue to red and emission of strong red fluorescence about the lead (II) solution. In addition, there are a little color change from blue to violet and weak red fluorescence about the zinc (II) and cadmium (II), which seemed to be generated by the interaction between PCDA (PDA monomer) and the metal ions.[14, 17] Calcium (II), copper (II), mercury (II) did not make any noticeable color change.
Figure 4.3 Assembly of PDA-DPGG liposomes and lead (II) ion detection.
Figure 4.4  A) Optical images of PDA-DPGG liposomes (0.66 mM) after 1hr incubation of in 0.26 mM lead (II) and other heavy metal ion solutions and corresponding B) UV-Vis spectra and C) PL spectra.
4.3.3 Lead (II) Detection with Janus Microbead Embedding PDA-DPGG Liposome

Based on the above results in the solution, we have applied the lead (II)-sensitive PDA-DPGG liposome to the developed Janus microbead system for the more convenient and practical lead (II) detection and applications. As expected, the sensory response of our PDA-DPGG liposome to lead (II) was maintained in the alginate matrix of the Janus microbeads. In addition, we confirmed the effect of magnetic stirring on signal generation of the PDA liposomes in Janus microbeads (approximately 100 microbeads) as shown in Figure 4.5. On 1 hour incubation with 1 mM lead (II) solution, the fluorescence intensity of PDA-DPGG liposome parts in the stirring Janus microbeads was approximately 1.5 times greater than the ones in stagnant microbeads. Stirred Janus microbeads also had more uniform intensity with reduced standard deviation of the graph. It can be explained that the stirred microbeads have increased opportunities to bind with lead (II) ions with the enhanced mass transports.[102]

We further conducted a study about the relation between fluorescence intensity of the Janus microbeads (approximately 100 microbeads) and various concentrations of lead (II). The fluorescence intensity of the liposome part became
stronger as the lead (II) concentration increased (Figure 4.6 A)). We could develop a good correlation between the fluorescence signal intensity and the lead(II) concentration as plotted in Figure 4.6 B) and the detection limit of the present system is 0.1 mM (~ 20.7 ppm).
Figure 4.5 A) Fluorescence microscope images of Janus microbeads embedding PDA-DPGG liposomes after 1 hr incubation in 1 mM lead (II) solution with or without stirring and B) corresponding fluorescence intensity.
Figure 4.6 A) Fluorescence microscope images of Janus microbeads embedding PDA-DPGG liposomes after 1hr incubation in various concentrations of lead (II) solution with stirring and B) corresponding fluorescence intensity.
4.3.4 Lead (II) Removal with Janus Microbead Embedding PDA-DPGG Liposome

Alginate is well-known low cost adsorbent due to their abundant carboxylic groups which have high affinity to heavy metal ions.[89-94] In detail, alginate has the affinity to divalent metal ions (including heavy metals) as following order: Pb 2+ > Cu 2+ > Cd 2+ > Ba 2+ > Sr 2+ > Ca 2+ > Co 2+, Ni 2+, Zn 2+ > Mn 2+. [106] Based on these knowledge, we confirmed the lead (II) adsorption by the alginate matrix of our Janus microbeads embedding lead (II) -sensitive PDA-DPGG liposomes. As shown in Figure 4.7, about 450 nmol of lead (II) ions were removed by hundred Janus microbeads during 2hrs. According to our approximation, hundred Janus microbeads included 1.10 μmol of alginate monomeric units (Each alginate monomeric units has a carboxylic groups), 50.5 nmol of citrate coated on the magnetic nanoparticles (Though citrate has three carboxylic groups, it is supposed that part or whole of carboxylic groups are bound to the surface of the nanoparticle, blocking the aggregative interaction between the nanoparticle and alginate), and 5.34 nmol lipids (PCDA and DPGG). Therefore, it was concluded that the lead (II) adsorption by Janus microbeads are mainly dependent to the alginate matrix.

Our suggested approach for the integration of sensory material (PDA-DPGG
liposome) and adsorbent (alginate) has following advantages; i) in a label-free manner, the beads can indicates the lead (II) concentration in the environment or the degree of lead (II) adsorption in the bead, ii) there is no chemical modifications of the adsorbent which can reduce the removal property of the adsorbent, and iii) magnetic separation is also possible. In the future work, the specific PDA monomers which have a reversible colorimetric property[107] can be applied to this system, with enabling the reusable adsorption and detection.
Figure 4.7 Kinetics of lead (II) ion removal by Janus alginate microbead to the 1 mM lead (II) solution.
4.4 Summary

Janus-compartmental alginate microbeads having two divided phases of sensory PDA liposomes and magnetic nanoparticles were fabricated by simple centrifugal microbead generator for facile sensory applications. The Janus microbeads maintained sensory properties of PDA liposomes, and were manipulated by magnetic field usefully in the sensory process such as bead concentration for washing and solvent exchange, stirring, and detection. Janus microbeads successfully applied to the lead (II) detection with newly designed PDA liposomes having lead (II)-sensitive lipid, DPGG. Binging of lead (II) to the galloyl head group of DPGG in PDA-DPGG liposome caused the distortion of conjugated yne-ene main chain of PDA and therefore PDA changes its color from blue to red color and emits red fluorescence. The suggested Janus microbeads system has remarkable potentials to the convenient label-free detection of lead (II) with removal function of alginate to lead (II). Furthermore, we expect that the present developments can be applicable to other sensory systems and environmental applications.
Chapter 5   Conclusion and Future Work

5.1 Conclusion

We have constructed various polydiacetylene (PDA)-based biosensors with utilizing the self-signaling mechanochromism of PDA. Our approaches for design of PDA-based biosensors are mainly based on two keywords, bioinspiration and multiscale. As the bioinspired design, we exploited liposome form of PDA materials which have structural similarity to cell membrane in nature. In addition, through multiscale design from molecular regulation about sensory properties of PDA liposomes to microfabrication of integrated microarrays and microbeads, the inherent disadvantages of nanoscale PDA liposomes, such as low sensitivity and stability, were solved. In detail, we rationally designed the polydiacetylene-phospholipid liposomes for enhanced stability and sensitivity, and achieved detections of neomycin and lead (II) ions. Furthermore, we applied to sensory PDA liposomes to microarrays and microbeads with achieving high sensitive detection, stable storage of liposomes, and facile sensor (assay) processes. We expected that our present methods can be applied to developing various PDA-based biosensor systems for bioanalytes.
detection.

In the future, due to their simple self-signaling property, the PDA-based biosensor systems have great potentials to practical use ranging from biological laboratory, hospital, and farm to our home health care. There are, however, several obstacles for the commercialization of PDA-based biosensors. First, the current detection limit of PDA-based sensors is still higher than the one of Enzyme-Linked ImmunoSorbent Assay (ELISA), which is the most popular biosensor process today. Though the PDA-based biosensors are much simpler and more user-friendly than ELISA, the PDA biosensors should have higher sensitivity for replacing ELISA and satisfying recent demands for early detection of cancer, Alzheimer or other diseases. For the purpose, novel types of PDA materials should be researched on the fundamental understandings of forces generated by molecular interactions. Studies about immune cells, which are ultra-sensitive to antigens, will be also helpful because the sensitive immune process were occurred through cellular membranes, thus natural original of liposomes.

Second, simplified fabrication of PDA-based biosensor systems is required because the fabrication process tend to damage the PDA liposomes, and thus reduced the sensitivity and stability. Concretely, PDA-based biosensors have been generally constructed by immobilization of PDA liposomes on solid substrates or into the solid
matrix. The process for immobilization usually requires surface modification of substrate or PDA liposomes and the surface modification needs skillful and complex steps and has limitations to materials. In addition, stable and strong combination of PDA liposomes and bioreceptor is also a problem. In this regard, we suggested a simple method for preparing PDA liposome coatings as a future work in the following paragraphs. Our developing method is inspired by strong-adhesion property of mussel protein in nature and is expected to realize simple and material-independent PDA liposome coatings, and strong tethering of bioreceptor to PDA liposomes.
5.2 Future Work: Mussel-inspired Polydiacetylene Liposome Coatings for Facile Biosensor Chip Development

5.2.1 Introduction

Polydiacetylenes are attractive materials for sensory applications through their self-signaling property, called mechanochromism. PDA materials have been generally applied to biosensory applications as bioinspired liposome form, with utilizing the amphiphilicity of PDA monomers. Though the liposome type of PDA can be directly used as solution-type sensory system, the post-processing, thus the immobilization of PDA liposomes into or on solid substrate has been researched for more convenient sensory systems. Representative examples are microarrays,[18-19, 24, 32], thin films,[22, 28, 33] microfibers,[17, 34-35] or microbeads[36-38]. Such post-processing of the PDA liposomes, however, has a lot of obstacles for practical uses because it needs complex and multiple fabrication steps which requires delicate skills and deteriorates the stability of liposomes. In detail, for immobilization of PDA liposomes to substrates, at least two step of surface modification process is needed: one is for the substrate and the other one is for PDA liposomes. It is also noted that the surface modification process is quite limited to specific surfaces such as silicon-
based substrates (e.g. glass, silicon), gold, or polymers having rich functional groups due to the confinement in surface chemistry. Furthermore, the tethering of bio-receptors to PDA liposomes is also another difficult step requiring thorough consideration of the surface chemistry.

In nature, mussel protein shows the remarkable adhesion property to the substrate material-independently, even in wet condition. Catechol and amine groups on the protein results in such adhesion protein and therefore dopamine, a small molecule having both catechol and amine group, have attracts attentions in view of bioinspiration. P. B. Messersmith and coworkers have been reported that dopamine can coated on various substrates, metal, ceramic and polymer by self-polymerized to polydopamine [108]. In addition, they also showed that the remaining catechol groups of dopamine can be used for addition tethering of proteins and DNAs having amine or thiol groups[108-110]. H. Lee and coworkers also developed remarkable microfluidic systems exploiting hydrophilic dopamine coatings on hydrophobic surfaces [111-112].

In this regard, we present a facile method for preparing PDA liposome coatings inspired by the strong adhesion property of mussel protein in nature. We designed the PDA liposome conjugated with dopamine (PDA-dopamine liposome) with expecting following advantages: i) material-independent coating of PDA liposomes to substrate without difficult surface modification, ii) universal tethering of
the receptors to PDA liposome surface through the catechol groups of dopamine.

Furthermore, we are planning to fabricate surface tension-confined microfluidic PDA biosensor based on hydrophilic PDA-dopamine liposome coating on hydrophobic surface.
Figure 5.1 Schematic illustrations about A) mussel-inspired PDA dopamine liposome, B) its assembly process, and C) fabrication process for biosensor chip.
5.2.2 Materials and Methods

Materials

Chemicals such as a PDA monomer, 10,12-pentacosadiynoic acid (PCDA), dopamine, solvents and buffers were purchased from Sigma-Aldrich Chemicals. A phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) was ordered from Avanti Polar Lipids. PVDF and PTFE membranes with 0.45 μm pore purchased from Chmlab and NC membrane with same dimension purchased from Millipore. Before the experiments, all the buffers are purged by N₂ gas for preventing dopamine oxidation.

Fabrication of PDA-Dopamine Liposome

PDA liposomes having PCDA and DMPA lipids were assembled by thin film method. PCDA and DMPA were dissolved chloroform and totally evaporated by N₂ gas in a 20 ml vial. Dried lipid films hydrated by 10 ml 5 mM HEPES solution (pH 5.6) at 80 °C. The pH should be 5.6 for preventing undesired oxidation of dopamine. The total concentration of PCDA and DMPA (4:1 molar ratio) is 1 mM. The liposome solution was 120W probe-sonicated 80 °C for 15 minutes and filtrated through a 0.8 μm cellulose acetate syringe filter. The liposome solution was stored at 5 °C overnight.
before use. For conjugation of dopamine, the PDA liposomes were reacted with 0.5 mM EDC(1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and dopamine during 3hrs. After the reaction, unreacted molecules in the PDA liposome solutions were ultra-filtrated by Sartorius Vivaspin 2 MWCO 300000 and the PDA liposomes were suspended in 5 mM HEPES buffer pH 8. The liposome solution was stored at 5 °C and used in 2 days.

Material-independent Coating of PDA-Dopamine Liposome

For fabrication of microarray, PDA-dopamine liposome solution was spotted to the surface of the hydrophilic glass, nitrocellulose (NC) membrane, hydrophobic PDMS, PVDF membrane, and PTFE membrane by a microspotter (Aurora Versa Minispotter) and incubated overnight at 5 °C. Spotting volumes are 100 nl for hydrophilic surfaces (glass, nc membrane) and 200 nl for hydrophobic surface (PDMS, PVDF membrane, PTFE membrane). It is note that a half of liquid volumes are spotted on the hydrophilic surface due to the wide liquid spreading. For large-area coating, the glass slide (2.5 cm X 7.5 cm) was dipped into the PDA liposome solution and incubated overnight at 5 °C. After the coating, the substrate were washed by HEPES buffer thoroughly and dried by N₂.
5.2.3 Results and Discussion

We confirmed the material-independent coating of PDA-dopamine liposomes to various substrates such as glass, PDMS, nitrocellulose (NC) membrane, PVDF membrane, and PTFE membrane. As shown in Figure 5.2 A), high quality of red fluorescence spots were achieved by microspotting, indicating that the dopamine moieties on PDA-dopamine liposomes have maintained their inherent adhesion. The presented coating method did not make any coffee-staining phenomena, in comparison to control experiment, and shows uniform coating even in inert and hydrophobic PTFE surfaces. The size of spots is varied according to the substrate materials and tends to be reduced as the hydrophobicity of surface increased. It is noted that the contact angle of surface in this experiment increased as following order: glass (<10°) < nitrocellulose (40°) < 90° < PDMS (108°) ~ PVDF (110°) < PTFE (140°). It is explained that liquid spots containing the liposomes has smaller contact areas on hydrophobic surfaces. In parallel, the large-area coating of PDA-dopamine liposomes is also possible on glass substrate with high uniformity, as shown in Figure 5.2 B)

Furthermore, we tested the wetting behavior of hydrophilic PDA-dopamine liposome spots coated on the hydrophobic surfaces. PDA-dopamine liposomes spots
on hydrophobic PDMS surface were used as a model. As shown in Figure 5.3. The PDA-liposome spots entrapped water droplets by surface tension generated at the boundary between hydrophilic and hydrophobic regions. This surface tension-confined water trapping can be applied to constructing two-dimensional microchannels, such as paper microfluidic channel, which is driven by surface tension.
Figure 5.2 A) Red fluorescence images of PDA-dopamine liposome spots on various substrates. Inserted images are the blue PDA-dopamine liposome spots taken by camera. B) Red fluorescence image of large-area PDA-dopamine liposomes coating on a surface of glass slide. (Scale bar: 250 μm)
**Figure 5.3** Water trapping by hydrophilic PDA-dopamine liposome spots on hydrophobic PDMS surface.
5.2.4 Summary

We developed a facile method for preparing PDA liposome coatings inspired by the strong adhesion property of mussel protein in nature. We realized the material-independent coating of PDA liposomes by conjugation of dopamine to PDA liposome surface. As well as this strong material independent coating, we anticipate that the catechol groups on PDA-dopamine liposomes can make tethering to the receptors for sensory applications. Eventually, we are aiming to suggesting a microfluidic PDA biosensor utilizing hydrophilic PDA-dopamine liposome coating on hydrophobic surface.
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국문 초록

본 논문은 폴리다이아세틸렌 고분자를 이용한 바이오센서를 멀티스케일 및 자연모사 설계를 통하여 제작하는 방법들에 관한 연구이다. 분석 물질이 폴리다이아세틸렌과 결합할 때 생기는 힘에 의해 폴리다이아세틸렌의 색이 청색에서 적색으로 변하고, 적색의 형광이 발생되는 기계적 섹션이 현상을 바이오센서에 적용하였다.

자연모사 설계로서 양친성의 폴리다이아세틸렌 단량체로부터 자가 조립되어 제조된 폴리다이아세틸렌 리포좀을 바이오센서 연구에 활용하였다. 리포좀의 구조가 자연계의 세포막의 구조와 유사한 점을 착안하여, 세포막을 이루는 주요 구성 물질인 인지질을 리포좀에 삽입하였으며, 그 결과는 크게 두 가지로 나눌 수 있다. 첫째, 높은 전하의 머리 구조와 적절한 전이 속도를 가지는 인지질을 폴리다이아세틸렌 리포좀에 삽입하여, 센서의 감도와 안정성의 증가를 이루었다. 아울러 서로 다른 전하의 머리 구조와 전이 속도를 가지는 인지질들에 대한 비교 실험을 통해서, 인지질의 삽입이 리포좀의 크기, 모양, 마 유연성 같은 기계적인 물성을 조절하여 감도와 안정성을 향상시킴을 밝혀내었다. 둘째, 아미노글리코사이드 계열의 항생제와 납 이온과 결합할 수 있는 인지질들을 각각 삽입하여, 위 물질들을 높은 감도와 분명함으로 감출해 냄 수 있는 폴리다이아세틸렌 리포좀을 제조하였다. 그 중 아미노글리코사이드 항생제의
김출은 실제 자연계의 세포막과 항생제 사이에서의 일어나는 상호작용을 모사한 것이라는 점에서 의의가 있다.

나아가, 위에서 자연모사 설계를 통해 제작한 폴리다이아세틸렌 리포즘들에 멀티스케일 설계를 적용하여 마이크로 어레이 및 마이크로 비드 형태의 바이오 센서 소자를 제작하였다. 첫째, 폴리다이아세틸렌 리포즘이 코팅된 마이크로어레이를 제작하여, 아미노글리코사이드 항생제 중 네오폴리신을 61 피피비의 낮은 검출 한계와 높은 선별성으로 검출해 내었다. 둘째, 폴리다이아세틸렌 리포즘과 자성 나노 입자가 알지넷 하이드로겔 내부에 아누스 형태로 구획화된 마이크로 비드를 마이크로 유체 시스템을 통해 제작하였다. 자성 나노 입자가 도입된 아누스 마이크로 비드를 자기장을 통해 조작하여 센서 실험 과정을 간편화하였으며, 센서의 감도를 향상시켰다. 여기에 남아온 검출용 폴리다이아세틸렌 리포즘을 아누스 마이크로 비드에 도입하여, 남아온의 검출 및 알지넷 하이드로겔의 성질을 통해 남아온 세균까지 모두 가능한 지능형 시스템을 제작하였다. 마지막으로, 자연계에서 높은 점착력을 가지는 홍합단백질을 모사하여, 간단한 과정으로 폴리다이아세틸렌 리포즘을 표면에 코팅할 수 있는 방법을 제안하였다. 홍합단백질의 점착력과 관련된 화학 구조들을 가지는 저분자 물질 중 하나인 도파민을 폴리다이아세틸렌 리포즘에 결합하여 표면에 코팅한 결과, 표면의 재료에 상관없이 균일하고 높은 품질의 코팅을 얻을 수 있었다. 이 방법을 이용하여, 소수성의 표면에 친수성의
폴리다이아세틸렌 리포즐을 코팅하였고, 그 결과 표면장력에 의한 마이크로 유체 액적 패턴을 만들 수 있었다. 이는 종이 및 멤브레인을 이용한 마이크로 유체 기반 바이오센서 소자 제작에 활용될 수 있다.

주요어: 바이오센서, 멀티스케일, 자연모사, 기계적색전이

폴리다이아세틸렌, 리포즐, 자가조립, 인지질
마이크로어레이, 마이크로비드, 마이크로/나노 공정, 마이크로 유체역학,
항원, 항체, 아미노글리코사이드 계열 항생체, 납 이온,
홍합 단백질, 도파민

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감사의 글

우선, 지난 대학원 생활 동안 저를 지도해주셨던 서갑양 교수님께 진심으로 감사를 드립니다. 지금은 비록 저희의 결을 떠나셔서 안타깝게도 본 학위 논문을 전해 드릴 수 없게 되었지만, 교수님께서는 여전히 세계 문헌하고 따뜻한 버림목이 되어주시며, 훌륭한 연구자의 모범을 보여주시고 계십니다.

그리고 제가 어려움 없이 학위 논문을 잘 마무리 할 수 있도록 아낌없이 도와주신 전우리 교수님께도 깊은 감사를 드립니다. 아울러 심사 과정 동안 기꺼이 시간을 내어 주시고, 세심하게 조언과 지도를 해주신 최만수 교수님, 김호영 교수님, 김진상 교수님, 그리고 괴문규 교수님께도 머리 숙여 깊이 감사드립니다. 또한 김도년 교수님께도 배려에 감사를 전하고 싶습니다.

저와 함께 연구하고 늘 저를 격의 없이 도와주시는 분들께도 감사를 드립니다. 먼저 김기성 박사님과 정호섭 박사님, 그리고 김진상 교수님의 따뜻한 조언과 가르침이 본 학위 논문을 완성하는 데에 큰 밑거름이 되었습니다. 그리고 지난 5 년간 가족보다 더 오랜 시간을 함께 보냈었던 멀티스케일 자연모자 시스템 연구실 식구들에게도 진심 어린 감사와 함께 앞으로는 좋은 일들만 있기를 기도합니다. 아울러, 낯선 분야를 연구하게 된 저를 꼼꼼하게 도와주신 이지석
박사님과 성백이형을 비롯한 미시간대 재료공학부 김진상 교수님 연구실
분들에게도 감사의 인사를 드립니다. 그리고 깊게나마 함께 생활하였던 멀티스케일
의기계공학 연구실 식구들 모두 감사합니다.

가족들, 언제나 저를 믿어주시고 목록이 옹원해주시는 아버지와 어머니, 그리고
동생에게도 이 자리를 떠나 평소에는 잘하지 못했던 깊은 감사의 말을 전하고
싶습니다. 연구가 바쁘다는 평계로 자주 찾아 빼지 못했지만, 부모님의 말없이는
현신이 있었기에 본 학회 논문을 완성할 수 있었습니다.

끝으로 저에게 연구의 즐거움을 주시고, 부족한 제가 본 걸 Isl을 빼들 수 있도록
은혜를 주신 하나님께 감사 드립니다. 저의 앞으로의 연구와 삶이 제가 아닌
하나님의 영광을 드리네는 것이 될 수 있도록 저를 계속 인도하여 주시길 기도
드립니다.

감사합니다.

강도현 드림.

2014년 1월 23일

관악 남성대에서.