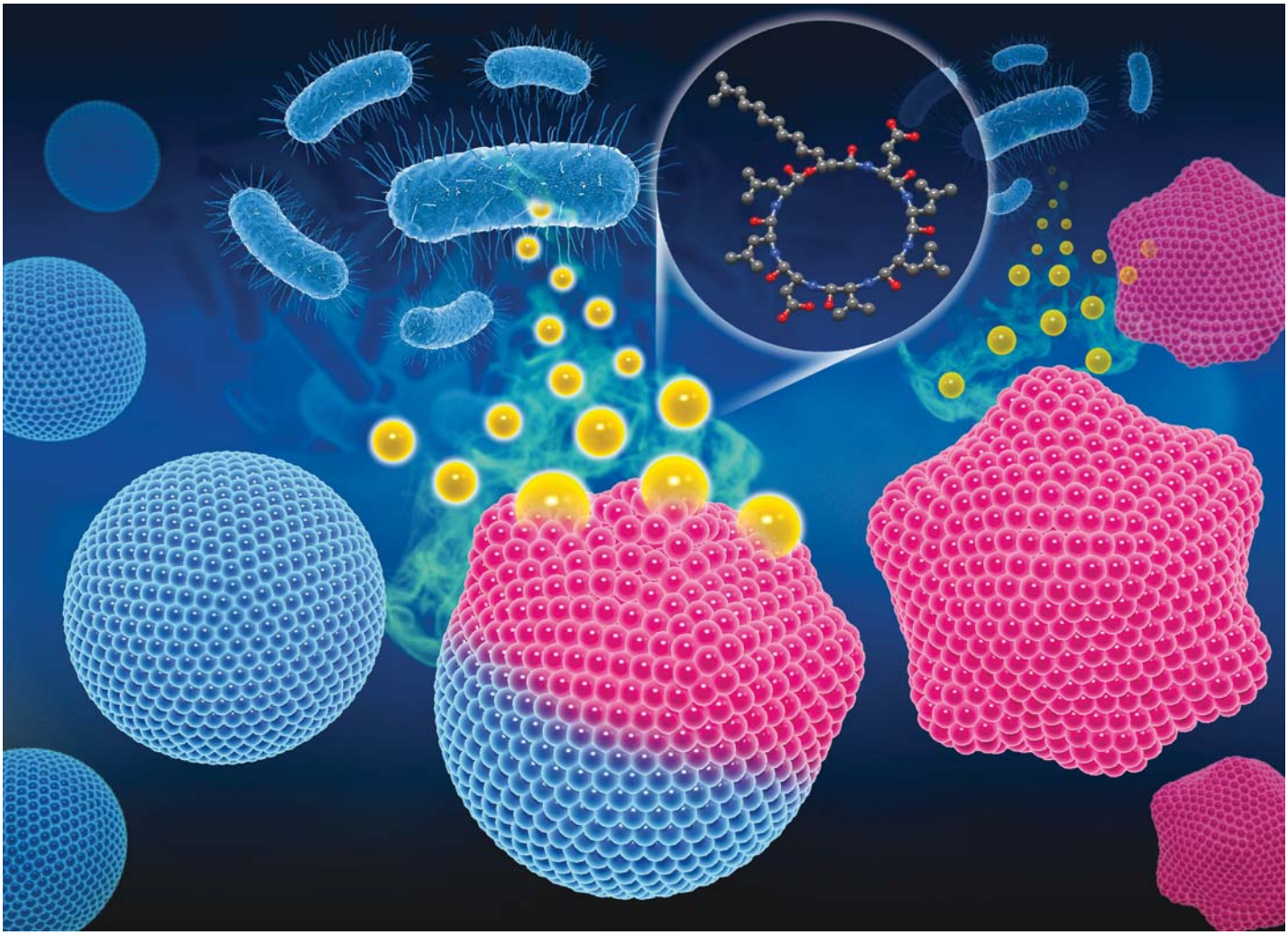

Showcasing research from Professor Kangwon Lee's lab, Program in Nano Science and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul

Label-free bacterial detection using polydiacetylene liposomes

Here, we synthesized an amine functionalized PDA liposome as a model system to present a simple, alternative method for detecting bacteria by selectively capturing the released species from bacteria.

As featured in:

See David J. Mooney, Kangwon Lee *et al.*, *Chem. Commun.*, 2016, **52**, 10346.

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Cite this: *Chem. Commun.*, 2016, **52**, 10346

Received 14th April 2016,
Accepted 8th July 2016

DOI: 10.1039/c6cc03116a

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Label-free bacterial detection using polydiacetylene liposomes†

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Polydiacetylene (PDA) liposomes were prepared to selectively capture target released from bacteria. Specific interplay between released-surfactin and PDA resulted in a conformal change in the structure of PDA, highlighting the potential of indirect interactions between bacteria and PDA in the construction of new label-free bacterial sensors.

Polydiacetylene (PDA) has received intensive attention among the various amphiphilic materials due to its high-responsiveness to light, heat, and mechanical pressure.[1–16] PDA monomers contain hydrophobic tails that form a conjugated p-orbital backbone when they are polymerized *via* photoinduction into liposomes. A significant UV absorption shift from the blue phase (650 nm) to the red phase (540 nm) and the emergence of fluorescence at 530 nm can simultaneously occur through the conformal change of the backbone induced by external stimuli.[1–6] These different methods of measuring conformational changes in the liposome caused by external stimuli have made PDA liposomes the target of many studies surrounding sensors.[1–16]

In addition to the external stimuli that lead to non-specific colorimetric responses, specifically-designed ligands have been introduced at the terminal of PDA liposomes for detecting specific types of biomolecules/bacterial strains/ions with high-sensitivity.[7–16] For example, inspired by specific binding between tryptophan/tyrosine residues and carbohydrates, Rangin *et al.* have shown the potential of amino-acid-functionalized PDA liposomes for detecting lipopolysaccharide on the membrane of Gram-negative bacteria.[7] In another route, Lee *et al.* developed guanine-rich ssDNA tethered PDA sensors for detecting potassium ions *via* electrostatic interaction between guanine and potassium ions.[11] All of these works suggest that understanding the interplay between PDA and sensed substances is a prerequisite for the design of new highly specific and sensitive PDA sensors.

With bacteria being a concern in various fields, a method for rapidly detecting and identifying certain strains is of interest.[15,16] By attaching different functional groups to the PDA liposomes, numerous efforts have been made to maximize their specificities in sensing different types of bacteria.[15,16] However, to date, most of the studies were focused on designing receptors that can specifically bind to the bacterial strain itself, and consequently little information about the effect of chemicals released from bacteria on the chromatic change of PDA was revealed.

Herein, we developed an amine-functionalized PDA liposome as a platform system for detecting bacteria *via* specific interplay between released chemicals from bacteria and the PDA liposome (Fig. 1). The liposomes were formed by self-assembly of a mixture of two types of diacetylene monomers with different lengths of amine ligands. The assembled liposome has gaps along the

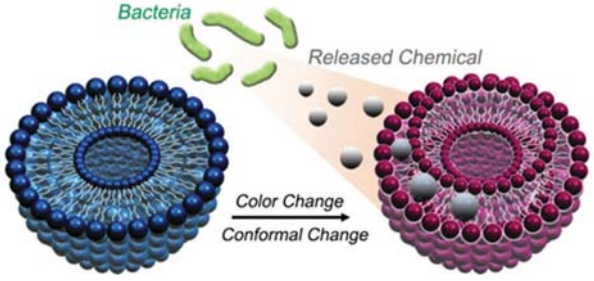

Fig. 1 Bacteria-sensing mechanism of our PDA using released chemical from bacteria.

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† Electronic supplementary information (ESI) available: Experimental details of liposome synthesis, characterization, first-principles calculation methods, and supplementary movie legends. See DOI: 10.1039/c6cc03116a

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length of its hydrophilic exterior, ensuring better adherence and reaction to other compounds.[5] The assembled liposome was then subjected to UV irradiation at 254 nm for cross-linking (Fig. S1, ESI†). A color change from colorless to blue was observed, confirming the successful polymerization process with ene–yne alternating conjugation chains.[2,6] When the dark blue solution was heated, a significant color change to red was subsequently observed, further confirming their well-assembled structure.[2,6] Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analyses showed that each liposome was approximately 30–80 nm in size (Fig. S2, ESI†).

The ability of the synthesized PDA liposomes to function as a sensor for bacteria was examined by detecting chromatic responses to different types of bacterial strains. Three bacterial strains were selected based on their structure and secretion of chemicals: *Bacillus subtilis* SSB466, the surfactin-less strain of the Gram-positive bacterium; *Bacillus subtilis* NCIB3610, the surfactin-producing strain of the Gram-positive bacterium; and *Pseudomonas aeruginosa* 14 (PA14), a Gram-negative bacterium. For observing the color changes induced by bacteria, PDA liposomes were incorporated into Luria-Bertani-agar (LB-agar) and UV irradiated for 1 min. The different strains were then grown on the irradiated LB-agar that had been incorporated with PDA liposomes. After 16 h of incubation at 37 °C, the NCIB3610 led to the most visible color change, from blue to purple, among the three groups whereas no noticeable change was observed after SSB466 and PA14 exposure (Fig. 2A–D).

For quantitative evaluation of the chromatic behavior, we monitored the change in UV-absorbance at 550 nm (red phase) and 645 nm (blue phase). As a first step, we compared the colorimetric change between NCIB3610 and SSB466 groups over incubation time (Fig. 2E). For both strains, increases in UV absorbance at 550 nm were observed with a concurrent decrease at 645 nm over the bacterial incubation time. However, NCIB3610 showed a considerably higher colorimetric change as compared to SSB466; UV absorbance at 550 nm increased approximately 0.2 after 24 h of growth for NCIB3610 while it increased only by 0.1 after 24 h of growth for SSB466. Along with the change in UV absorbance, the fluorescence of the NCIB3610 group recorded at 590 nm showed an approximately two-fold increase compared to that of the SSB466 group, similar to the absorbance results (Fig. 2F). It should be noted that the small color changes in SSB466 and PA14 groups may be mainly due to the temperature increase during the incubation of bacteria.[2,6] Indeed, a control group where no bacterial solution was added also exhibited a similar, small increase in fluorescence comparable to the SSB466 group due to the increase in temperature from 4 °C at 0 h to 37 °C after 1 h (Fig. 2F).

Despite the distinct colorimetric changes between the two groups, both groups had similar bacterial growth rates, based on the amounts of bacteria measured by optical microscopy. This result indicated that the color change in the PDA-LB-agar could have resulted from an indirect interaction between bacteria and PDA. It is worthwhile to note that the UV absorbance and fluorescence change after incorporating a 10-fold diluted NCIB3610 bacterial solution also exhibited an approximately 10% decrease from that of the non-diluted group, further implying that the mechanism for color change in PDA is not a result of the direct adsorption of bacteria onto the PDA liposome (Fig. 3A and B).

The implication of an indirect interaction between bacteria and PDA liposomes was further verified by comparing the color changes of NCIB3610 and PA14-incorporated PDA-LB-agar. Even though the bacterial growth in PA14 was more prolific, the surrounding gel exhibited more color change towards purple for the NCIB3610 group (Fig. 2B and C). If direct bacteria–PDA interaction were the dominant mechanism for color change, the PA14 group would have shown more dramatic shifts in color compared to the NCIB3610 group.

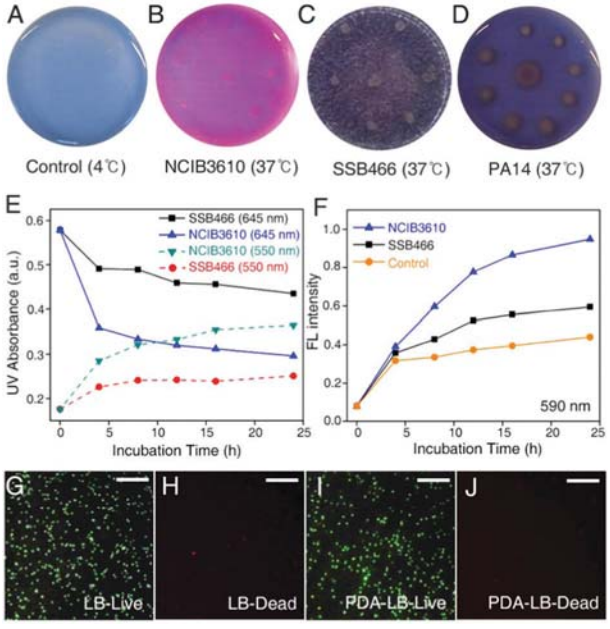

Fig. 2 (A–D) Photometric change in PDA-LB-agar plate after incubation of various bacterial strains for 16 h. (E) UV/Vis and (F) fluorescence spectrum change of NCIB3610 and SSB466 incorporated PDA-LB-agar plate over incubation time of bacteria. (G–J) Live-dead assay for LB agar control and PDA-LB-agar plate (scale bar = 10 μm).

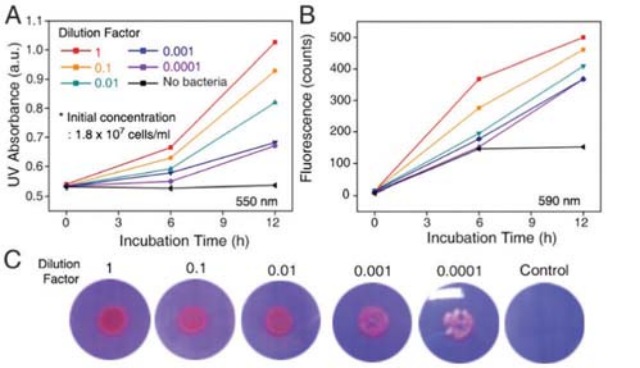

Fig. 3 (A) UV/Vis and (B) fluorescence spectrum with various initial bacterial concentrations as a function of incubation time. (C) Photographs of PDA-LB-agar with different bacterial concentrations.

Bacterial viabilities in PDA-LB-agar and pristine LB-agar were investigated using a Live/Dead assay, where live cells and dead cells exhibit green and red fluorescence, respectively (Fig. 2G–J). After 12 h of growth, negligible amounts of dead cells were found in both samples. Quantitative evaluation for live cells in LB-agar and LDA-LB-agar exhibited similar absorbance (0.563 for PDA-LB-agar, 0.602 for LB-agar).

The detection limit of PDA-LB-agar was evaluated by varying the initial concentration of the bacterial strains (Fig. 3A–C). Based on the assumption that OD_{600nm} of 1.0 equals to 8.0×10^8 cells per ml of bacteria, we prepared different bacterial solutions with concentrations ranging from 1.8×10^7 cells per ml to 1.8×10^3 cells per ml. 10 μl of bacterial solutions were added to PDA-LB-agars and the corresponding UV absorbance and fluorescence changes were measured after 6 h and 12 h of cultivation. Even in the groups where 1.8×10^3 cells per ml of bacterial solution was added, distinguishable changes in UV absorbance and fluorescence were observed compared to the control group where no bacterial solution was added. For the bacterial strains that do not secrete surfactin, negligible colorimetric change was noted even at a much higher bacterial concentration of 1.8×10^7 cells per ml. The high sensitivity of our PDA liposomes toward NCIB 3610 is notable.

Considering that the distinct difference between NCIB3610 and SSB466 is the presence and absence of surfactin-releasing ability, we tested whether surfactin itself triggered a color change in the PDA liposomes. Surfactin is a bacterial cyclic lipopeptide which has the surfactant ability due to its amphiphilic nature (Fig. 4A).[17,18] Interestingly, the simple addition of a 0.2 mM of surfactin solution induced significant color change of a 0.2 mM of PDA solution in phosphate buffer saline within 30 s (Fig. 4B and Fig. S3 and S4, ESI†). For a more detailed investigation of the effect of surfactin on the color change in PDA, different concentrations of surfactin, from 0.016 mM to 0.143 mM, were added to the 0.2 mM of PDA solutions (Fig. 4C). An increase in the concentration of surfactin up to 0.071 mM led to an apparent color change from visible blue to purple in the PDA solution, similar to the shift in color of PDA-LB-agar after incorporation of NCIB3610 (Fig. 2B). Further addition of surfactin resulted in complete color change toward red, clearly indicating the dominant mechanism for color-change resides in surfactin.

Time-lapse fluorescence images from the time of addition of the NCIB3610 clearly demonstrated that the color change in the PDA liposome is triggered by a specific interplay between surfactin and PDA (Fig. 4D and Movie S1, ESI†). After a NCIB3610 containing solution was dropped onto PDA incorporated LB-agar, fluorescence rapidly emerged in the region of the droplet, as expected. Noticeably, even in the region where bacteria did not exist, significant fluorescence was detected, comparable to the region where the bacterial solution was added. Finally, 24 h after the addition of bacterial solution, the entire PDA-LB-agar showed fluorescence (Movie S1, ESI†).

The selectivity of our PDA liposome towards surfactin was tested with 16 different targets, including the various surfactants, simple anions, and fatty acids (cetyl trimethylammonium bromide (CTAB), sodium dodecyl benzenesulfonate (SDBS), F^-, Cl^-, Br^-, I^-, SO_4^{2-}, CO_3^{2-}, NO_3^-, HPO_4^{2-}, SCN^-, CH_3COO^-, $C_3H_7COO^-$, $C_5H_{11}COO^-$, $C_7H_{15}COO^-$, and $C_9H_{19}COO^-$), by adding 0.1 mM of the targets to the 0.2 mM of PDA solution. We found that simple anions and short-chain fatty acids did not trigger any colorimetric changes in the PDA liposomes. In contrast, as the chain length increased, the anionic targets (SDBS, $C_7H_{15}COO^-$, and $C_9H_{19}COO^-$) induced slight colorimetric changes in the PDA liposomes. However, no changes in the PDA liposomes were observed after incorporation of the cationic surfactant, CTAB, which has a long carbon chain in its chemical structure (Fig. 5A).

It should be noted that surfactin-incorporated PDA solution exhibited significantly higher colorimetric changes compared to other groups (Fig. 5). UV absorbance at 550 nm increased approximately 0.8 after incorporation of 0.1 mM of surfactin solutions whereas 0.1 mM of SDBS, $C_7H_{15}COO^-$, and $C_9H_{19}COO^-$ solutions induced only 0.3, 0.1, and 0.3 increases in the absorbance of PDA solution, respectively (Fig. 5B). In line with the

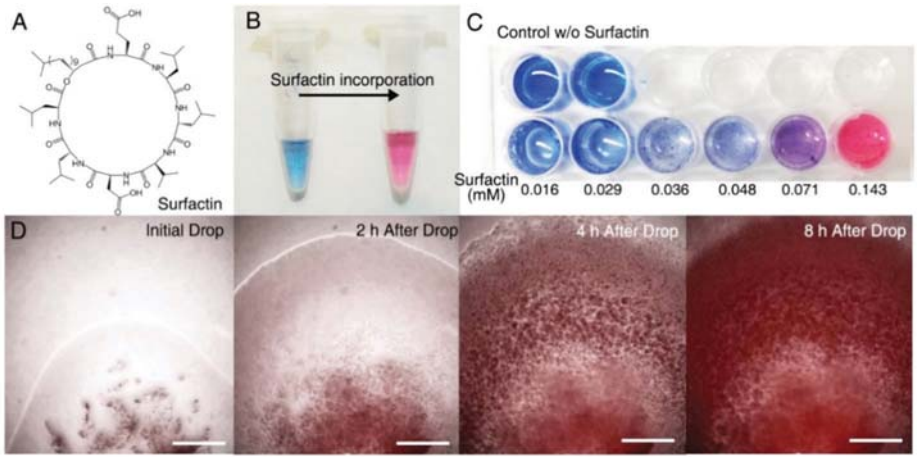

Fig. 4 (A) Chemical structure of surfactin. (B) Representative image for surfactin-induced color change in PDA solution. (C) Surfactin-concentration dependent color change in PDA solution. (D) Time-lapse images of PDA-LB-agar after an initial drop of NCIB3610 containing solution was placed. In the region where bacteria colonized, a consistent color change was observed (scale bar = 500 μm).

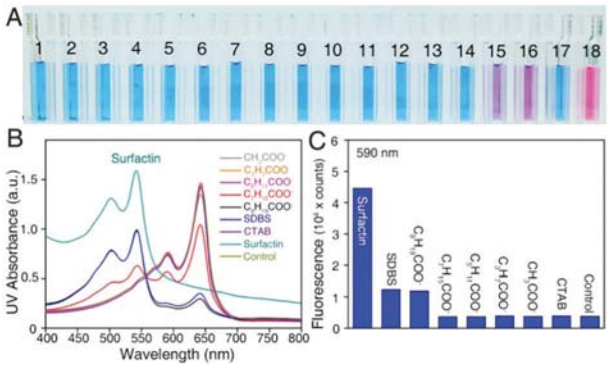

Fig. 5 (A) Colorimetric responses of PDA solution towards various targets (1: control, 2: NaF, 3: NaCl, 4: NaBr, 5: NaI, 6: Na_2SO_4, 7: Na_2CO_3, 8: $NaNO_3$, 9: Na_2HPO_4, 10: NaSCN, 11: CH_3COONa, 12: C_3H_7COONa, 13: $C_5H_{11}COONa$, 14: $C_7H_{15}COONa$, 15: $C_9H_{19}COONa$, 16: SDBS, 17: CTAB, and 18: surfactin) (B) UV/Vis spectrum and (C) fluorescence of 0.2 mM of PDA solution after incorporating 0.1 mM of the target species.

changes in UV absorbance, the fluorescence of the surfactin-incorporated PDA solution was approximately three-times higher than that of SDBS and $C_9H_{19}COO^-$ – incorporated groups, similar to the absorbance results (Fig. 5C). The high selectivity of PDA solutions towards surfactin was further confirmed by adding different concentrations of the targets from 0.02 mM to 0.1 mM to the PDA solutions (Fig. S5, ESI†).

We considered possible interactions between surfactin and amine-functionalized PDA, based on their chemical and physical properties. It has been reported that surfactin is negatively-charged due to its two charged groups (L-Glu[1] and L-Asp[5]) in cyclic heptapeptide.[17,18] Considering the pK_b values of primary amines ($\sim$3.3), the terminal amine group on the PDA is expected to be positively-charged (NH_3^+) at neutral pH, and consequently the ammonium cation in PDA can electrostatically interact with the negative charges of surfactin.[9,10] Moreover, the fatty acid chain in surfactin can enhance the binding-affinity by van der Waals interactions with carbon-backbones in PDA.[17,18] Indeed, theoretical calculations in previous studies showed that surfactin can penetrate into charged bilayer membranes with its amphiphilic nature.[17,18] Moreover, it was reported that anion surfactants, such as sodium dodecyl carboxylate, can trigger chromatic changes in positively-charged PDA by ion–ion interactions.[9,10] In this regard, we believe that surfactin has a profound effect on the conformational change in the PDA liposome, causing the blue to red color change. Indeed, our first-principles calculations indicated that the electronic structure and the resulting absorption spectrum of the amine-functionalized PDA could be altered as the torsion angle between two neighboring diacetylene monomers is changed (Fig. S6 and S7, ESI†). Although the exact binding sites between surfactin and amine-functionalized PDA and the resulting degree of torsion angle remains unclear, we believe that specific interaction between surfactin and amine-functionalized PDA, such as ionic interactions and van der Waals interactions, lead to a change in the torsion angle between two PDA monomers and alters the absorption spectrum.

In conclusion, we found that amine functionalized PDA liposomes can be used to detect surfactin and identify specific types of bacteria, through combined experimental and theoretical considerations. Introducing simple amine-modified PDA liposomes as a model system, we found the pivotal role of bacteria-secreted molecules on chromatic changes in PDA and envision that indirect sensing mechanisms can be an alternative route for sensing bacteria with high selectivity and sensitivity.

This work was supported by the research resettlement fund for the new faculty of SNU, a grant of the SNU invitation program for distinguished scholar, the KIST project (2E262 30), and the BASF research initiative at Harvard University.

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