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Research Article

Amplifying the Sensitivity of Polydiacetylene Sensors: The Dummy Molecule Approach

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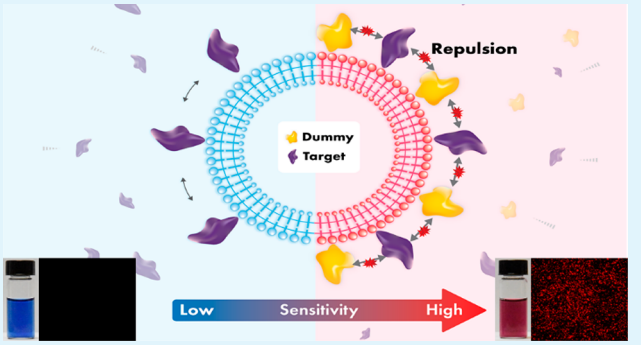
ABSTRACT: There is an increasing need for fast and accurate assessment of various health conditions, where polydiacetylenes (PDA), having unique stress-sensitive optical properties, have great potential. When the conjugated backbone of PDA is disturbed by steric repulsion between the receptor−target complexes formed at the PDA surface via specific recognition events, the bandgap of PDA increases and produces color change and fluorescent emission as a dual sensory signal. However, this detection mechanism suggests an intrinsic sensitivity limit of PDA platform because unless adjacent receptors are occupied by target molecules no signal is anticipated. A novel approach to improve the sensitivity and limit of detection of PDA sensors has been developed by preoccupying the surface of PDA liposomes with an optimized amount of artificial target molecules named as dummy molecules. The sensitivity and limit of detection (LOD) showed large improvement by the surface-bound dummy molecules. In addition, the dummy strategy was synergically integrated with another sensitivity enhancing method with a different working mechanism in a PDA sensor for Neomycin detection. When optimized, the LOD of the PDA sensor was improved to 7 nM from 80 nM of the control and the signal intensity increased consistently throughout the entire tested concentration range of the target Neomycin. Finally, the general applicability of the dummy strategy to other target molecules was successfully confirmed by implementing the dummy strategy in a PDA sensor for Surfactin detection.



KEYWORDS: *polydiacetylene sensors, colorimetric sensor, sensitivity enhancement, limit of detection, preoccupied dummy*

INTRODUCTION

Polydiacetylenes (PDAs) have gained much attention for their rapid and convenient colorimetric detection scheme for chemical and biosensing applications. The detectable optical property change as a sensory signal is originated from the distorted conjugated yne-ene main chain of PDA molecules induced by steric repulsion among the receptor−target complexes formed at the PDA surface via specific recognition events.[1−3] The distorted PDA backbone widens the bandgap of PDA and causes its absorption λ_{max} change from 650 to 540 nm, which appears as the color change from blue to red. In addition to the appearance color change, the red phase is fluorescently emissive at 530 nm. This unique self-signaling property combined with rational receptor design for specificity enables PDA to be a convenient and universally applicable optical sensor platform. PDA sensors have shown fast colorimetric and fluorometric dual signaling to various external stimuli such as chemicals,[4−10] biomolecules,[11−17] light,[18] heat,[19−21] and humidity.[22,23]

The convenient optical detection scheme and fast response time make PDA an attractive material for biomedical sensors to meet today's needs for rapid and equipment-free self-diagnosis at home. In recent years, the COVID-19 pandemic has amplified such needs at multiple levels from self-assessment for individuals to mass screening for the larger public. PDA biosensors have already been demonstrated for selective detection of Influenza A virus,[15] bacteria,[24] antibiotic,[7] and activated platelet.[25] However, the detection limit ranges widely from millimolar to nanomolar concentration, implying the need for sensitivity enhancement and reducing the limit of detection (LOD). We previously reported an effective strategy to achieve a lower LOD and higher sensitivity by coassembling a lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA), within the PDA liposome, which adds more fluidity to the self-assembled PDA liposomes making them more responsive to the molecular stress.[14] This lowered threshold stress results in a reduced number of the minimum target molecules needed for the signal generation.

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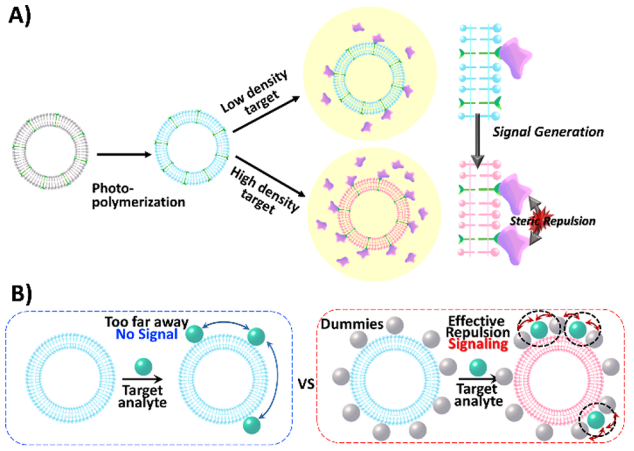
We investigated a new strategy to enhance the sensitivity of the PDA sensors by analyzing the fundamental reasons for the wide range of LOD. It is plausible that LOD largely depends on the relative size of target molecules to the receptor size because the sensory signal is generated by the steric repulsion among the formed receptor−target complexes. Therefore, as we previously demonstrated, if the target molecule is smaller than the receptor, no effective steric repulsion is anticipated, and consequentially, no signal would be generated.[15] Likewise, a more critical origin of the intrinsic sensitivity limitation of PDA sensors can be identified by analyzing the signal generation mechanism. The required steric repulsion for signal generation arises only if adjacent receptors are occupied by target molecules, which is unlikely, however, until a certain amount of target molecules are available as schematically illustrated in Scheme 1A. We envisioned that preoccupying some of the receptors with artificial target molecules named "dummy" would make otherwise required target bindings to adjacent receptors unnecessary. Here, we systematically investigated the optimum number density and size of dummy molecules for signal amplification by using phosphatidylinositol 4,5-bisphosphate (PIP2) and Neomycin as a model receptor and target pair. We also examined the general applicability of the dummy strategy by implementing the concept to a Surfactin detecting PDA system. We further demonstrated synergistic enhancement of PDA sensitivity by combining the dummy strategy with the lipid insertion method. The presented dummy approach is readily applicable to other PDA sensor designs and, in general, provide 1 order of magnitude improved sensitivity.

Scheme 1. (A) Schematic Illustration of the Working Mechanism of PDA Liposome-Based Sensor, and (B) Enhanced Signal Generating Mechanism by Means of Dummies



EXPERIMENTAL SECTION

Materials. We ordered 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) and L-α-phosphatidylinositol-4,5- bisphosphate (PIP2) from Avanti Polar Lipids. We purchased 2-Hydroxymethyl-18-crown-6, α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin from Sigma-Aldrich. Other chemical reagents were purchased from Sigma-Aldrich and directly used without further purification.

Fabrication of Polydiacetylene Liposome. The injection method was used to prepare the PDA liposomes. Constituent components of PDA liposome (PCDA, PCDA-EDA, PCDA-EDEA, PIP2, and DMPA) were dissolved in 300 μM DMSO at the molar ratios specified in Supporting Information (SI) Table S1 (their chemical structures are in Figures 1B and 4A, and in SI Figure S3).

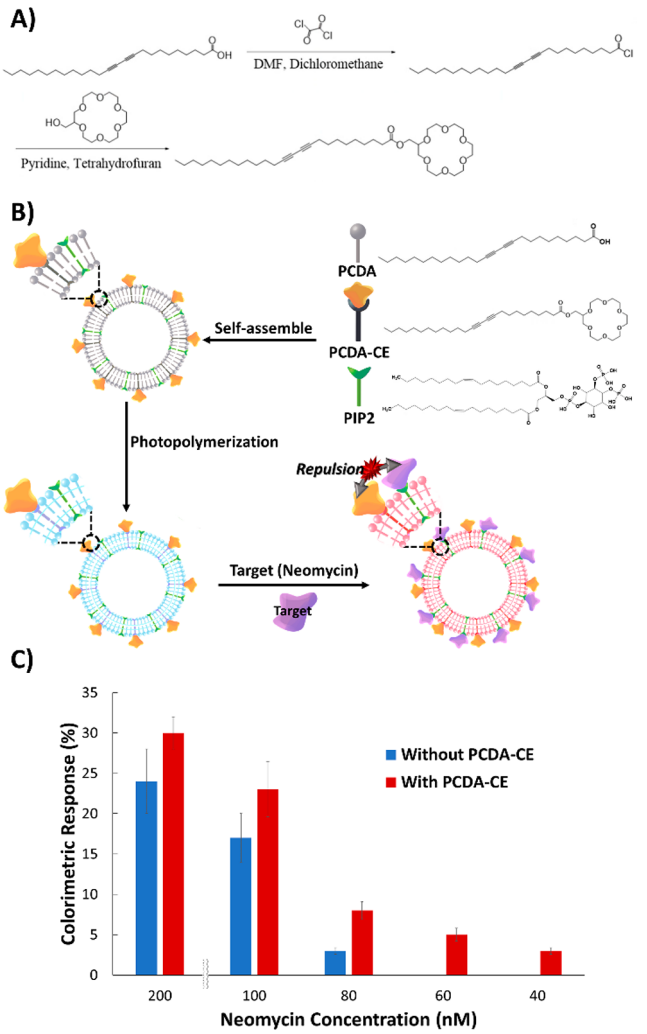


Figure 1. (A) Synthesis of PCDA-CE, (B) Schematic illustration of the PDA liposome-based Neomycin detection mechanism by the designed liposome including PCDA-CE dummy monomer. (C) Colorimetric response of PDA liposome solution after 20 min incubation with Neomycin.

The DMSO solution was injected into 20 mL of water followed by sonication at 90 °C by means of a 120 W probe sonicator for liposome self-assembly. After 20 min of sonication of the aqueous liposome solution at 0.5 mM, the solution was filtered through an 0.8 μm cellulose acetate syringe filter. The filtrate was stored overnight at 5 °C prior to using it for further experiments.

For the post attachment approach of dummy molecules, 5 °C overnight-incubated liposome solution was first UV-irradiated (1 mW/cm2, 254 nm) for 30 s for ene-yne conjugated backbone formation, which was confirmed by blue color generation as well as UV-absorption at 650 nm. To the 380 μL of photopolymerized PDA liposome solution, 20 μL aqueous solution of epoxy-functionalized dummy molecules at varying concentrations was then added and incubated for 12 h at room temperature. PDA microarrays were fabricated by microarraying the resulting PDA solution on amine-functionalized glass substrates.

Optical Signal Analysis of Polydiacetylene Liposomes. Aqueous solution of target molecules was added to the prepared PDA liposome microarrays. After 20 min of incubation followed by stringent rinsing, chromatic change in the PDA microarrays was examined with an optical fluorescence microscope (Olympus DP71).

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The quantitative absorbance and fluorescence of the PDA microarrays were measured using a spectrophotometer (Varian Cary 50 UV−vis spectrophotometer) and a fluorescence microplate reader (Horiba PTI QuantaMaster 400).

Computational Calculation. First, we performed quantum mechanical calculations, using Gaussian 16[26] for the molecules investigated in this work. We endeavored to better understand the molecular shape and volume of the molecules. We used B3LYP functional with 6-31G(d,p) basis set. To incorporate the solvent effects, we used the Polarizable Continuum Model (PCM) using the integral equation formalism variant (IEF-PCM).[27] All IEF-PCM calculations were performed using default settings of the Gaussian 16 program package. All geometries and free energies were calculated at 298.15 K. The optimized ground state geometries were obtained followed by an analysis of normal modes of atomic motion to confirm the stability of the optimized structures. Then the molecular volumes were calculated as implemented in Gaussian 16.

The optimized structures obtained from quantum mechanical calculations were then used to assess similarity between molecules. We used the USR (Ultrafast Shape Recognition)[28] function of Open Drug Discovery Toolkit (oddt)[29] for molecular shape comparison. USR accurately describes the shape of a molecule via a vector of geometrical descriptors. The comparison of molecular shape via these USR descriptors has been rigorously shown to be highly effective and efficient.

RESULTS AND DISCUSSION

Limit of detection (LOD) is the minimum concentration of target molecules required for a sensor to exhibit a detectable response. For PDA-based sensors, the LOD is dependent on the number of available target molecules as well as the target's molecular properties, because the sensors' working mechanism requires the conjugated PDA backbone to be strained by the steric repulsion among the captured target molecules at the PDA liposome surface. However, in some cases, the molecule of interest to detect is at the lower concentration than the LOD of PDA-based sensors, limiting the PDA sensors' practical applicability. In this study, we explored a strategy of decreasing the LOD by preoccupying the PDA liposome surface with designed dummy molecules (Scheme 1B).

The sensor performance was compared between PDA liposomes with and without the preoccupied dummy molecules. Neomycin was selected as the model target since we had previously developed a PDA sensor built on the well-studied specific interaction between Neomycin and PIP2.[7,30,31] As the first dummy molecule for the proof of concept study, 18-crown-6-ether (CE) was chosen considering that CE has a similar solubility in water, does not interact with Neomycin, and has a similar flexible structure to Neomycin. We later additionally studied three more different dummy molecules. To control the number of CE at the PDA liposome surface, initially we synthesized the CE-tethered diacetylene monomer (PCDA-CE) by attaching CE to 10,21-pentacosadiynoic acid (PCDA)[32] (Figure 1A and Supporting Information) and prepared PDA liposomes having varying ratios of PCDA, PCDA-CE, and PIP2 by controlling their mixing ratio (Figure 1B and SI Table S2).

However, we found that this preparation method caused an undesired consequence of reduced signal intensity because the liposome self-assembly was disrupted by the dummy molecule's bulky headgroup. As shown in SI Figure S1, as the amount of PCDA-CE increases in the mixture of PCDA-CE and PCDA, rapid reduction in the blue absorption intensity was observed, indicating unstable liposome packing and consequentially hindered photopolymerization. Nevertheless, the resulting PDA having the mixing ratio of PCDA:PDA-CE:PIP2 = 8:1:1 exhibited a lower LOD of 40 nM Neomycin compared to the control PDA liposome's 80 nM LOD without the dummy (Figure 1C). However, because we could not systematically study the dummy effect due to the self-assembly issue, we decided to attach the dummy molecules to preassembled PDA liposomes for further studies (Figure 2A).

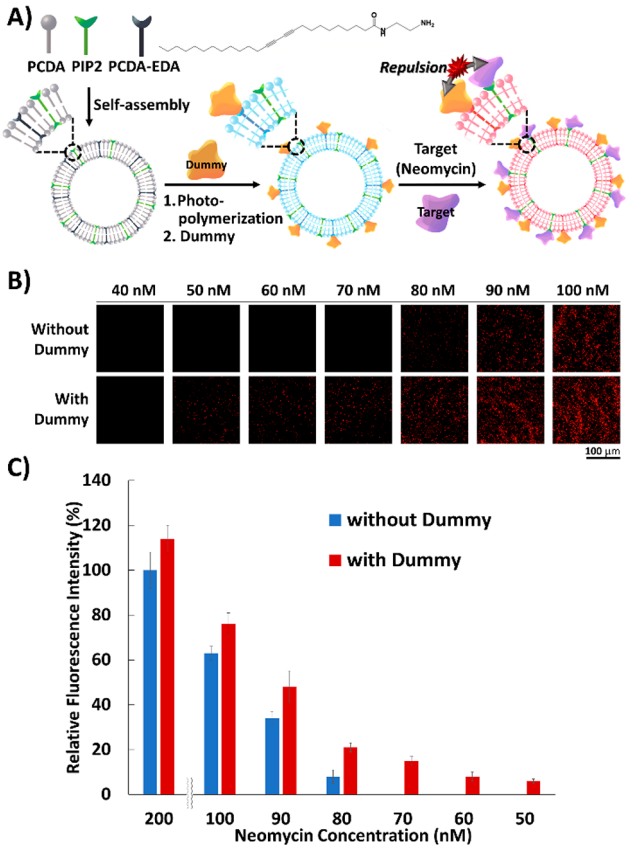

Figure 2. (A) Schematic illustration of the PDA liposome having the preoccupied dummy molecules for Neomycin detection with enhanced sensitivity, (B) Fluorescent microscope images, (C) Relative fluorescence intensity comparison between the PDA liposome with and without the CE dummy at the surface.

To attach the CE molecules to self-assembled PDA liposomes, the amine−epoxy reaction was utilized. First, *N*-(2-aminoethyl)pentacosa-10,12-diynamide (PCDA-EDA) monomer was prepared by reacting ethylene diamine with PCDA to introduce amine functionality.[24] The PCDA-EDA was then self-assembled with PCDA and PIP2 into liposomes at the molar ratio of PCDA:PCDA-EDA:PIP2 = 8:1:1. An epoxy functional group was introduced to CE by reacting CE with epibromohydrin[25] (SI Figure S2) and the resulting epoxy-functionalized CE was tethered to the diacetylene liposome having PCDA-EDA to prepare PDA liposomes having preoccupied dummy molecules (Figure 2A)

When the dummy molecules are attached to the surface of the PDA liposome, they can also generate optical signals if they occupy the surface too densely. In this case, the signals from the dummy themselves become noise background signal for the PDA sensor. Therefore, the dummy concentration should be in the range in which the dummy themselves are not generating optical signal. To avoid such background signal, we performed titration in order to identify a suitable dummy

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concentration range before applying the system to the Neomycin detection. SI Figure S2 shows that the maximum amount of dummy not to cause any background signal varies depending on the dummy molecule used: 5 μM for 18-crown-6-ether, 3 μM for α-cyclodextrin, 2 μM for β-cyclodextrin, and 1 μM for γ-cyclodextrin. Therefore, 1 μM turned out to be the highest dummy concentration for all four dummy molecules in order to avoid background signal by dummy itself. Accordingly, after self-assembly and photopolymerization, the PDA liposomes were incubated with 1 μM epoxidized dummy molecule for 12 h. The resulting liposome solutions were then exposed to various concentrations of Neomycin, the target molecule. While the LOD of the PDA liposome without the dummy was 80 nM, that of the PDA having CE dummy was lower at 50 nM (Figure 2B,C). Moreover, 2.6 times stronger fluorescent intensity was observed at 80 nM Neomycin concentration, implying that the sensitivity was also increased by preoccupying the liposome surface with CE dummy molecules (Figure 2C).

After confirming the improved sensor performance by the dummy approach, we investigated the size effect of dummy molecules since our previous results showed that a larger target molecule can induce more effective steric repulsion and consequentially better LOD than a smaller target.[15] Following the same logic, larger and compatible dummy candidates were selected for Neomycin by surveying molecules that satisfy the above-mentioned criteria (solubility, reactivity, and structure). After the initial screening, their molecular size was computationally calculated. α-cyclodextrin was chosen considering that it is significantly larger than CE but similar to Neomycin (SI Table S3). To attach the α-cyclodextrin dummy to the PDA surface the predetermined 1 μM concentration of α-cyclodextrin having epoxy functionality was used.

As shown in Figure 3A, the PDA liposomes with α-cyclodextrin dummy showed higher signal intensity and lower LOD for Neomycin detection compared to the PDA liposomes having CE dummy. We further investigated the size effect by additionally testing β-cyclodextrin and γ-cyclodextrin dummies which are larger than α-cyclodextrin (SI Table S3).

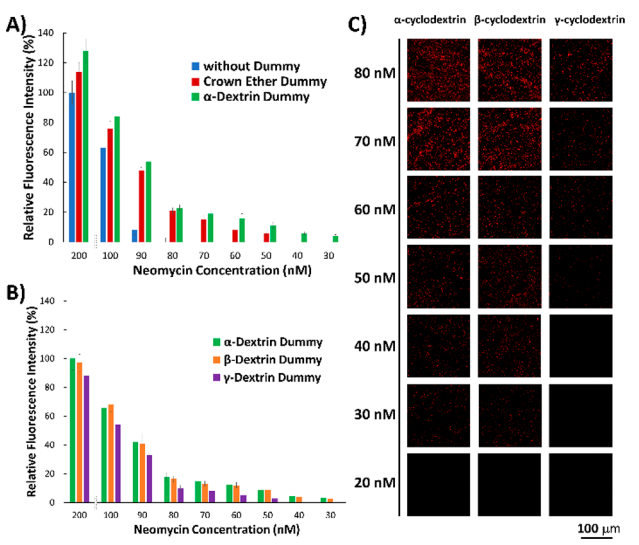

Figure 3. (A) Relative fluorescence intensity comparison among the PDA liposome without dummy, with CE, and with α-cyclodextrin dummy, (B) Relative fluorescence intensity, (C) fluorescence images comparison among the series of cyclodextrin dummies.

Interestingly, while the PDA liposomes having β-cyclodextrin dummy showed about the same sensitivity and signal intensity as the PDA liposomes with α-cyclodextrin dummy, the PDA liposomes with γ-cyclodextrin performed much worse as it showed lower sensitivity and signal intensity (Figure 3B,C). It is plausible that the much larger γ-cyclodextrin might sterically hinder the interaction between PIP2 and Neomycin or might be too large to be pushed effectively by Neomycin. These results imply that a dummy molecule having a similar form factor to the target molecule is the more effective one for the sensitivity enhancement.

We investigated a feasible synergic sensitivity enhancement effect by combining the dummy concept with the DMPA-based strategy in which coassembled phospholipids add more fluidity to the self-assembled PDA liposomes making them more responsive to the molecular stress.[14] Previously, by adapting this phospholipid-based enhancement strategy in PDA sensors we achieved higher sensitivity in the detection of Neomycin,[7] bovine viral diarrhea virus (BVDV),[14] and platelet activation.[25] First, a liposome solution containing DMPA was prepared at the mole ratio of PCDA:PCDA-EDA:DMPA:PIP2 = 6:1:2:1 followed by α-cyclodextrin attachment to the resulting PDA liposome surface by using 1 μM α-cyclodextrin (Figure 4A). The prepared PDA liposomes did not show red fluorescence upon photopolymerization, confirming that the distance between the tethered α-cyclodextrin is large enough not to produce steric repulsion among them and background signal, and were used for the sensitivity study.

As shown in Figure 4B, the synergy between the two sensitivity enhancement strategies further improved the LOD for Neomycin from 80 nM to 20 nM. Furthermore, the signal intensity increased at each level of target concentration (Figure 4C). These results imply that the devised dummy concept can be readily combined with other signal amplification strategies of PDA sensors, such as generating weak headgroup interaction[33] and amphiphilic length mismatch.[6]

The PDA liposome sensor performance was further optimized to achieve the highest signal intensity and lowest LOD for Neomycin by adjusting the amount of α -cyclodextrin. The maximum amount of α-cyclodextrin that can be added without disruption the liposome packing tuned out to be 3.4 μM. Above 3.4 μM, red fluorescence was observed right after the photopolymerization and even prior to Neomycin introduction, implying that the PDA packing was already distorted by excessive steric repulsion between the surface-bound α-cyclodextrin. As shown in Figure 5, when 3.4 μM of α-cyclodextrin dummy was used, higher signal intensity was observed consistently throughout the entire target concentration range. The LOD was as low as 7 nM.

Finally, we examined if the dummy strategy is generally applicable to other PDA detection scenarios by applying the dummy idea to a previously developed PDA sensor for Surfactin detection. The composition of the PDA liposome was PCDA-EDA:PCDA-EDEA:DMPA = 4:4:2 (SI Figure S3). The PCDA-EDA was used as the receptor for Surfactin binding through Coulombic interactions[24] as well as the functional group for attaching epoxy-functionalized cyclodextrins. As shown in Figure 6A, a similar sensitivity enhancement effect was observed, except that γ-cyclodextrin exhibited the best performance (22% increased signal intensity and 4 μM LOD), whereas α-cyclodextrin was the most efficient one for Neomycin detection. As discussed above, in this case it was also true that a dummy molecule having a similar form

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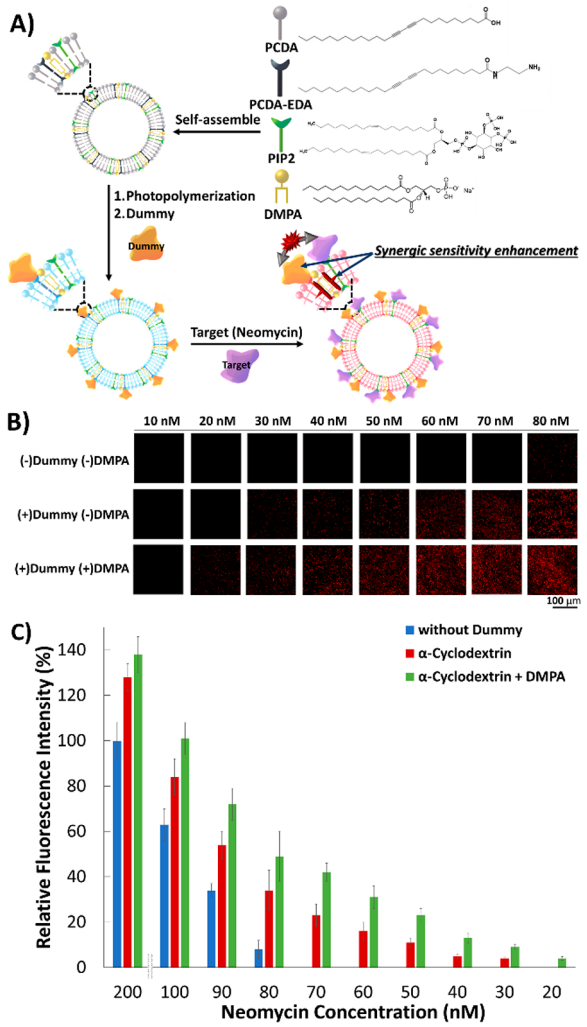

Figure 4. (A) Schematic illustration of the PDA liposome self-assembled with DMPA and having preoccupied dummy molecules for Neomycin detection with enhanced sensitivity. (B) Fluorescent microscope images, (C) Relative fluorescence intensity comparison among the PDA liposome without dummy, with α-cyclodextrin dummy, and with DMPA as well as α-cyclodextrin dummy.

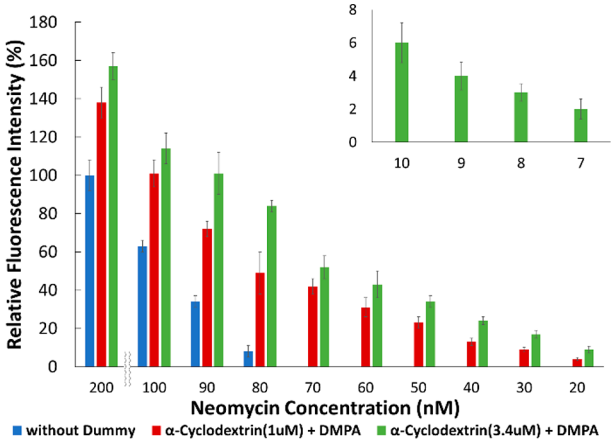

Figure 5. Relative fluorescence intensity comparison among the PDA liposome without dummy, with 1 μM α-cyclodextrin dummy and DMPA, and with 3.4 μM α-cyclodextrin dummy and DMPA.

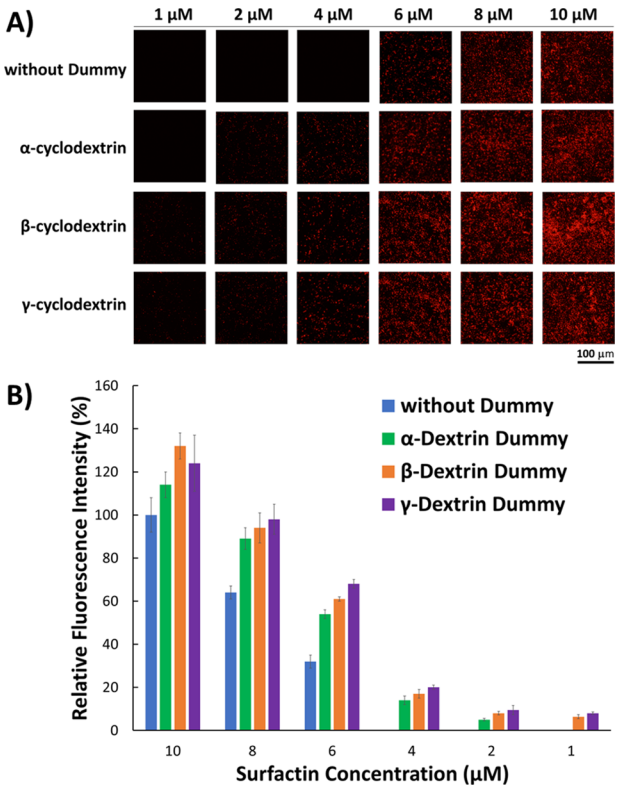

Figure 6. (A) Fluorescent microscope images, (B) Relative fluorescence intensity comparison among the PDA liposomes having the series of cyclodextrin dummies.

factor works best. As one can see from SI Table S4, γ-cyclodextrin has the closest molar volume with Surfactin and showed the best enhancement result. The results strongly suggest that the dummy strategy, when it combined with the lipid insertion strategy, provide 1 order of magnitude improved LOD and that when the form factor of the dummy matches well with that of the target, the largest sensitivity enhancement can be achieved.

CONCLUSION

We report a universally applicable strategy to improve the sensitivity and detection limit of PDA-based sensor by preoccupying the PDA liposome surface with artificial target molecules. By using Neomycin as a model target, and 18-crown-6-ether, α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin as the dummy, we found that a dummy having a similar form factor with the target molecule provides the largest signal enhancement effect. We applied the same dummy approach to a PDA liposome system for Surfactin detection and observed the same signal enhancement and improved LOD, which testifies that the dummy approach is generally applicable to the PDA sensory system. We also combined the dummy approach with another signal amplification strategy, lipid insertion approach, and achieved a synergic effect to accomplish 1 order of magnitude improved LOD. Therefore, the devised dummy strategy can be readily combined with other signal amplification methods for PDA sensors and further enhance the sensitivity and signal intensity.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c25066.

Additional experimental details: PCDA-CE synthesis route and characterization, UV−visible absorption spectra, photoluminescence spectra, and computational output (PDF)

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Author Contributions

D.S. and J.Kim conceived and designed the research. D.S. synthesized all chemicals, fabricated the liposomes and devices, and carried out the experimental measurements and analysis. R.A. and J.Kieffer contributed to the computational work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

A patent application has been filed based on the results presented in the paper.

The authors declare no competing financial interest.

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