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ACS Appl. Mater. Interfaces, **Just Accepted Manuscript** • DOI: 10.1021/acsami.7b14086 • Publication Date (Web): 07 Nov 2017

ACS Publications

ACS Applied Materials & Interfaces is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

Mussel-inspired Universal Bioconjugation of Polydiacetylene Liposome for Droplet-array Biosensors

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KEYWORDS. Polydiacetylene liposome, Dopamine, Mussel-inspired chemistry, Droplet-array Biosensor, and Vascular endothelial growth factor detection

ABSTRACT: Most solid-state biosensor platforms require a specific immobilization chemistry and a bioconjugation strategy separately to tether sensory molecules to a substrate and attach specific receptors to the sensory unit, respectively. We developed a mussel-inspired universal conjugation method that enable both surface immobilization and bioconjugation at the same time. By incorporating dopamine or catechol moiety into self-signaling polydiacetylene (PDA) liposomes, we demonstrated efficient immobilization of the PDA liposomes to a wide range of substrates, without any substrate modification. Moreover, receptor molecules having a specificity toward a target molecule can also be attached to the immobilized PDA liposome layer without any chemical modification. We applied our mussel-inspired conjugation method to a droplet-array biosensor by exploiting hydrophilic nature of PDA liposomes coated on a hydrophobic polytetrafluoroethylene (PTFE) surface, and demonstrated selective and sensitive detection of vascular endothelial growth factor (VEGF) down to 10 nM

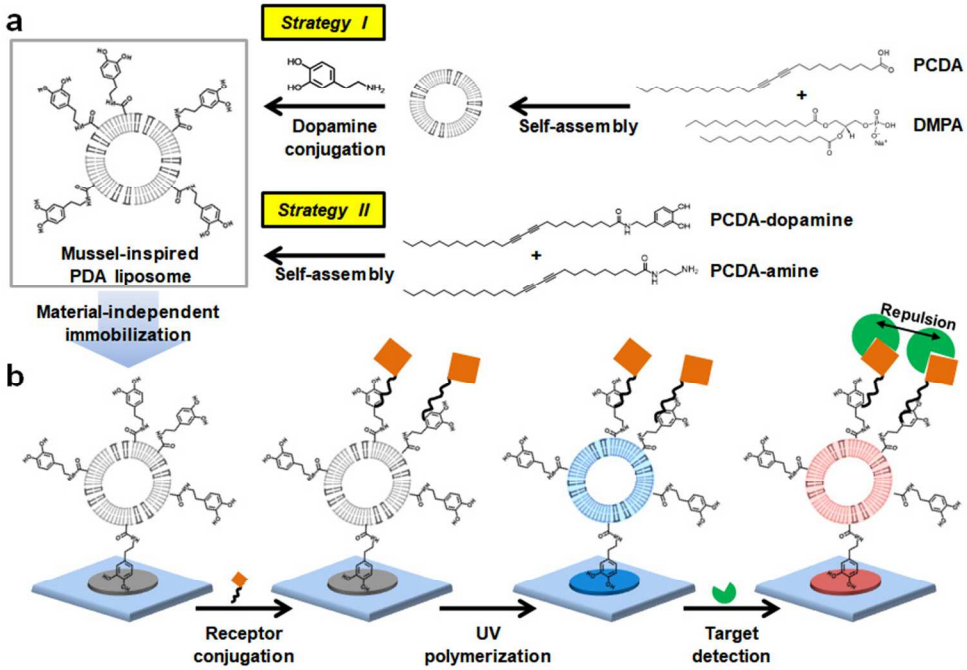
INTRODUCTION

Solid-state biosensor platforms have various advantages over solution-based counterparts, such as, easy handling during washing and buffer exchange steps, stable long-term storage, high throughput simultaneous multi-detection, and good portability.[1-9] To build a solid-state sensor, a signal generating unit should be firmly immobilized to a substrate and a receptor molecule or a specific functional group is to chemically tethered to the signal generating unit. These two conjugation steps require specific chemical modifications and optimization of the substrate, signal generating unit, and receptor molecule.[1-2]

Polydiacetylene (PDA) is an attractive conjugated polymer for colorimetric and self-signaling sensory applications due to its well-known mechanochromism.[10-12] When mechanically stressed by external stimuli, such as heat,[13-15] humidity,[16-17] metal ions,[4, 6, 8] chemicals,[7, 18-25] and biological molecules,[9, 26-31] PDA changes its color from blue to red due to the resulting distortion of its conjugated yne-ene main chain. In addition to this colorimetric signal, PDA also provides an additional fluorescence-based detection scheme because the red phase PDA emits red fluorescence while the original blue phase has no emission. Liposomes prepared by self-assembly of amphiphilic diacetylene monomers are the most commonly used form of PDA for sensor applications. The self-assembly leads to a close packing of the diacetylene monomers, and subsequent photo-polymerization via 254 nm UV light converts the well-packed monomers to blue PDA. A specific target molecule can be directly and selectively detected by PDA liposomes having a surface-tethered and rationally designed head group or receptor that has a high affinity to the target.

We previously reported various PDA liposome-based microarrays as a highly sensitive, selective, and universal optical biosensor platform.[4, 8, 27] We designed PDA monomers having a functional head group such as NHS-ester[4] and epoxy[8, 27] for i) immobilization of the liposomes to an amino-functionalized surface (e.g. aminosilane-coated glass), and ii) tethering of amine-containing specific receptors (e.g. DNA aptamers, peptides, and antibodies) to the liposome layer. However, these previous approaches

require a specific functional group on the substrate and the receptor before the covalent immobilization of the PDA liposomes to the substrate and the tethering of the receptors to the PDA layer, respectively. Although there are well-established functionalization and surface modification methods, such as silanization on a silicon or glass surface,[32] grafting on a polymer surface,[33] and thiol monolayer assembly on a gold surface[34], these methods are material-dependent and thereby applicable to a specific surface. Moreover, these methods are costly, time-consuming, labor-intensive and often require extensive optimization through multiple steps.



Scheme 1 A) Universal bio-conjugation of sensory PDA liposomes based on mussel-inspired catechol chemistry. B) Substrate-independent immobilization, chemical modification-free bio-conjugation of receptors, and self-signaling target detection of mussel-inspired PDA liposomes.

Certain mussel foot proteins in nature show powerful adhesion to any types of materials, even in wet condition.[35-40] The mussel adhesion is mediated by the abundant catechol and amine groups co-existing in the side chains of the protein.[37-39] Interestingly, catechol group is a versatile functional group that can form covalent bonds with amine,[36, 41-44] catechol,[41] thiol,[36, 41, 45] imidazole,[41, 46] hydrazine,[47] and others. Therefore, the catechol chemistry can covalently link various molecules having the above-mentioned functional groups, such as small chemicals,[47-48] polymer,[48] DNA[42] and protein[46], to mussel-inspired catechol-containing materials.

In this contribution, we present our development of incorporating mussel-inspired chemistry to the PDA system to devise convenient fabrication of PDA-based sensory systems. As illustrated in Scheme 1, we designed two mussel-inspired coating strategies to immobilize PDA liposomes material-independently without any surface pre-treatment. In the **Strategy I**, we adapted dopamine, a simple mimic of the mussel adhesive protein, into PDA liposome and in the **Strategy II**, catechol and amine head groups were incorporated into PDA liposome. The mussel-inspired stickiness plays dual roles; i) immobilizing PDA liposome on a substrate and ii) tethering receptor molecules to the immobilized PDA liposome layer. We also constructed PDA-based droplet-array devices by applying the developed strategies to a hydrophobic PTFE (polytetrafluoroethylene) membrane. In this droplet-array device, aqueous solution is spontaneously confined, due to the surface tension, within the hydrophilic PDA liposome spots on the hydrophobic PTFE.[49-52] These devices are a promising tool for rapid and convenient diagnosis since complex and expensive additional components, such as a micro-pump, are not necessary.

RESULT AND DISCUSSION

Coating of PDA Liposome By Strategy I. Dopamine having both catechol and amine groups is self-polymerized on various surfaces in an oxidative environment (e.g. mild basic aqueous solution).[35-36, 41] It has been reported that various materials co-dissolved with dopamine can be immobilized onto various surfaces via polydopamine formation.[48, 53] We developed the polydopamine-based PDA coating method by achieving covalent connection of dopamine to PDA liposomes. As illlustrated in Scheme 1, we assembled PDA liposome composed of 4:1 (molar ratio) mixture of PCDA (10,12-pentacosadiynoic acid, a PDA monomer) and DMPA (1,2-dimyristoyl-sn-glycero-3-phosphate, a phospholipid). The purpose of DMPA insertion is to achieve an enhanced sensitivity by increasing the flexibility of the PDA packing in the liposome.[27, 29] The PDA liposomes were then conjugated with dopamine by means of the carbodiimide chemistry (EDC coupling) between the carboxylic acid of the PDA liposome and the amine of dopamine. The conjugation was carried out at pH ~ 5.6 to increase the reaction rate as well as to prohibit undesired dopamine polymerization. Unbound dopamines were not removed but used for subsequent polydopamine formation. After the conjugation, the pH of the reaction mixture was adjusted to 8 to oxidize both PDA-bound and unbound dopamines for the PDA immobilization on a substrate via polydopamine formation.

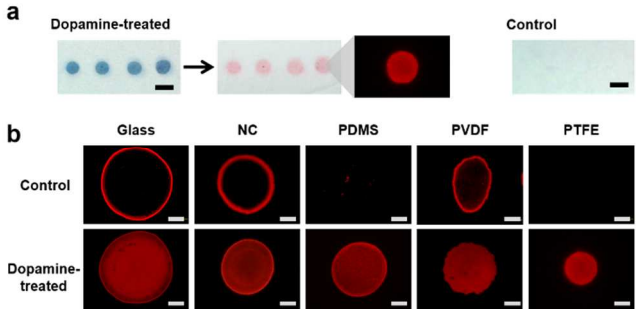


Figure 1 A) Camera and fluorescence images of the PDA liposome spots coated on a PTFE surface with and without dopamine treatment before and after heat treatment (Scale bar: 1 mm). B) Fluorescence images of the PDA liposome spots coated on various substrates (Scale Bar: 250 μm). The hydrophobicity of the surfaces increases from left to right, determining the spread of aqueous PDA liposome solution on the substrates.

Figure 1A shows the micro-spotted PDA liposomes with and without the dopamine treatment on a pristine PTFE membrane. Even on the inert and hydrophobic PTFE surfaces, the formed PDA liposome pattern shows clear initial blue color and color change from blue to red upon heating (90 °C, 5 min) while dopamine-free PDA liposomes could not be coated on the PTFE surface. The red fluorescence from the heat-treated PDA liposome implies that while the dopamine modification provides facile immobilization of PDA liposomes on the solid substrate it does not disrupt self-assembled PDA liposome and inherent self-signaling property of the PDA. We further confirmed successful dopamine-assisted coating of the PDA liposomes on various common substrates including glass, polydimethylsiloxane (PDMS), nitrocellulose (NC), and polyvinyli denefluoride (PVDF). Glass is the representative substrate for microarray system, PDMS is the most widely used material for microfluidic devices, and NC and PVDF membranes are widely used in dot or western blotting. As shown in Figure 1B, high quality red fluorescence spots on the various surfaces were achieved by microspotting. Surprisingly, the spot of PDA-dopamine liposome does not show the coffee ring phenomena and thus is uniform, especially on hydrophobic surfaces such as PDMS, PVDF, and PTFE whereas the dopamine-free PDA liposomes results in either no-coating or poor quality spots having the coffee ring. We believe that dopamine induces crosslinking among PDA liposomes, as well as the tether the PDA liposome on the substrate (Figure S1). Therefore dopamine-treated PDA liposomes in the solution were aggregated each other after long overnight incubation at pH 8 (Figure S2). Through such multiple binding, more PDA liposomes can be tethered efficiently and uniformly on a substrate like other researchers found that crosslinking between PDA liposomes improved the coating uniformity.[30] It is also known that hydrophobic surfaces produce more uniform coating by suppressing the coffee ring effect due to the reduced evaporation-induced flow to the contact line.[54]

We also observed that the coated spot size decreased as the hydrophobicity of the substrate increased when the same volume of PDA liposome was deposited. For examples, the diameter of the PDA spot on PTFE was about 0.5 mm while the one on the glass was about 1.1 mm even though twice larger volume was spotted on PTFE (0.1 μl was spotted on the glass and 0.2 μl on PTFE). It is related to the static water contact angle of the surfaces: glass (below 41°) < NC (82°) < 90° < PDMS (108°) < PVDF (128°) < PTFE (132°) (Figure S3). This phenomenon implies that the more liposomes can be concentrated on a hydrophobic substrate via the microspotting. Therefore, we concluded that the most hydrophobic PTFE membrane would be the most suitable substrate for further experiments.

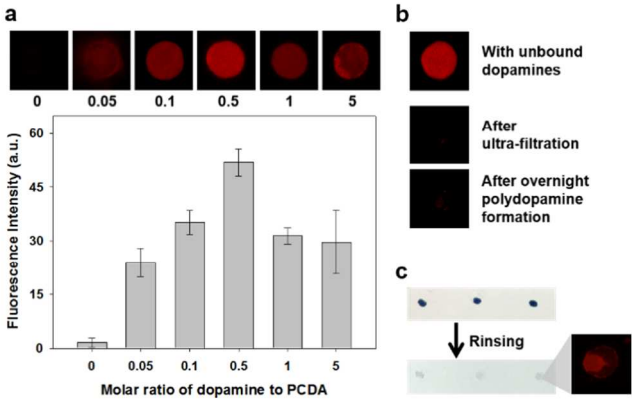


Figure 2 A) Fluorescence images of the dopamine-treated PDA liposome spots having various concentrations of dopamine and their corresponding fluorescence intensity. B) Fluorescence images of the dopamine-treated PDA

liposome spot with or without unbound dopamines. For the bottom two images microspotting was performed after the unbound dopamines were removed by ultra-filtration or polydopamine formation by overnight incubation. C) Camera and fluorescence images of the dopamine-treated PDA liposome pattern without EDC coupling between dopamine and PDA liposome.

The effect of dopamine concentration on the quality of PDA liposome coating was also investigated. PDA liposomes conjugated with various molar ratios of dopamine to PCDA were microspotted on a PTFE surface, polymerized, and heated subsequently. The red fluorescence images of the resulting PDA liposomes were captured and the fluorescence intensity was measured (Figure 2A). In the range of low dopamine molar ratio from 0 to 0.5, the PDA fluorescence became more intense as the molar ratio increased, implying that dopamine mediates the coating process. The PDA fluorescence is, however, significantly reduced at the molar ratios above 1. Moreover, when the molar ratio is 5 the coatings of PDA liposome are not uniform. These reduced and non-uniform coatings are likely resulted from that the excessive binding of dopamine to the diacetylene liposome perturbs the packing of PDA liposomes and causes massive aggregation in the solution. The role of unbound dopamines and carbodiimide chemistry in the coating process was also investigated. The fluorescence intensity of the PDA liposome coating is significantly reduced when the unbound dopamines are removed by ultra-filtration or polydopamine formation in the solution is excessively proceeded before the spotting (Figure 2B). Per our calculation based on the UV/Vis measurement, 64.9 % of dopamines were conjugated with PDA liposome when the PDA liposomes were reacted with 0.5 molar ratio of dopamine by EDC coupling. It is reasonable to expect that unbound dopamines significanlty promote the immobilization of the PDA liposomes through polydopamine formation (Figure S1). We also noticed that the dopamine-modified PDA liposomes tended to clog the filter during ultra-filtration. Therefore, we were convinced that direct spotting of the PDA liposomes without removal of the unbound dopamine was the optimum condition for high quality PDA spot formation. In addition, if there is no EDC coupling, the most of immobilized PDA liposomes were rinsed away due to the weak interaction between PDA liposomes and polydopamine as we anticipated (Figure 2C).

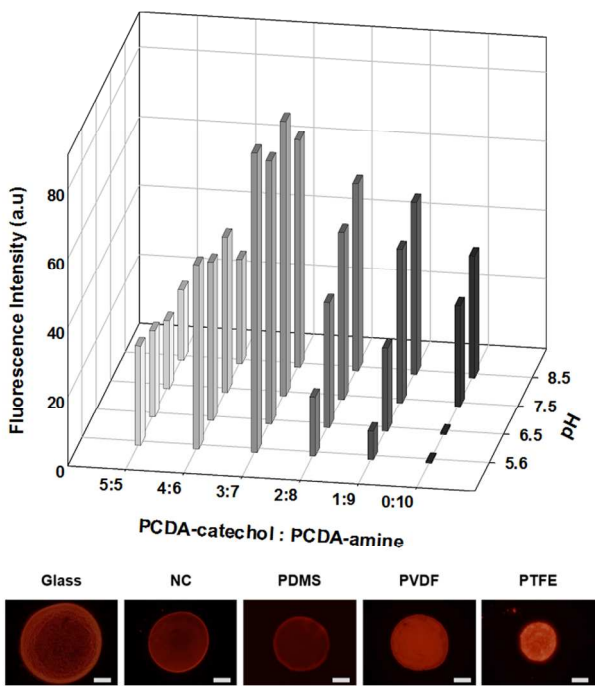
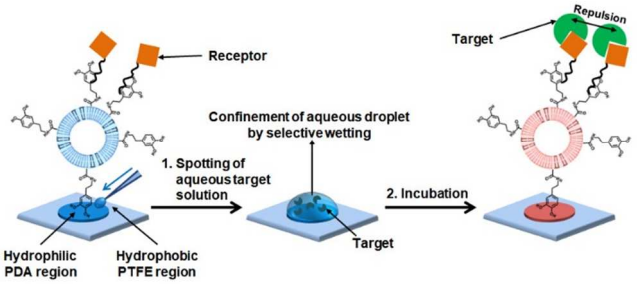


Figure 3 A) Fluorescence intensity of the PDA liposome spots prepared at various pH and mixing ratios of PCDA-catechol to PCDA-amine. The corresponding fluorescence microscope images are presented in Figure S5. B) Fluorescence images of the PDA liposome spotted on various substrates by means of Strategy II (Scale Bar: 250 μm). The hydrophobicity of the surfaces increases from left to right, determining the spread of aqueous PDA liposome solution on the substrates.

PDA liposome coating by Strategy II. In addition to the successful immobilization of PDA liposomes by Strategy I, we further developed a simpler coating strategy (Strategy II) by co-assembling diacetylene molecules having catechol (PCDA-catechol) and amine group (PCDA-amine), respectively. The synthetic procedures are described in Supporting Information. We found that the diacetylene liposomes were not stably assembled at higher than 6:4 molar ratios of PCDA-catechol to PCDA-amine while photopolymerization became inefficient at molar ratios higher than 4:6 judging from the quality of the developed blue color of resulting PDA (Figure S4). We tested the immobilization efficiency of the PDA liposomes assembled from 0:10, 1:9, 2:8, 3:7, 6:4, to 5:5 ratio of PCDA-catechol to PCDA-amine on a PTFE surface. We also confirmed the effect of pH from 5.6, 6.5, 7.5 to 8.5 (Figure 3A and Figure S5). At the optimized ratio of 3:7, the PDA liposomes were attached efficiently in the broad range of pH from 5.6 to 8.5. We also verified that the optimized PDA liposome can be efficiently tethered on various substrates including glass, polydimethylsiloxane (PDMS), nitrocellulose (NC), and polyvinylidenefluoride (PVDF) (Figure 3B).

Application to Droplet-Array Sensor. Excellent quality large-area coating (Figure S6A) and micro patterning (Figure S6B) of PDA liposomes were also achieved by means of the developed strategies. A solvent-assisted capillary force lithography is an useful technique for residual-layer-free micro pattering.[55-59] We moved forward to the development of a droplet-array optical biosensor device with the high compatibility of our mussel-inspired PDA liposome with various substrates and coating techniques (Scheme 2).



Scheme 2 Schematic illustration of target detection by mussel-inspired PDA liposome-based droplet-array biosensor.

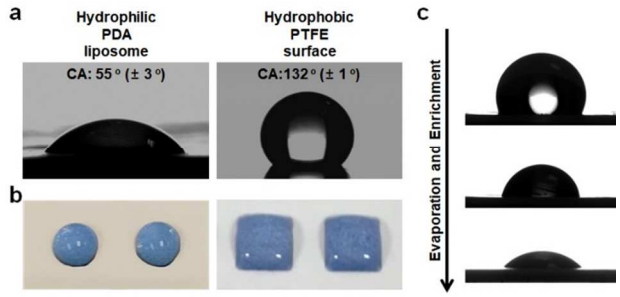
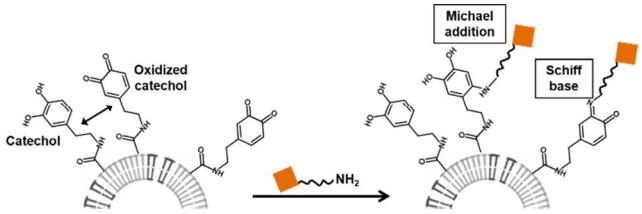


Figure 4 A) Static water contact angle on the coated mussel-inspired PDA liposome layer and a bare PTFE surface. B) Camera images of the spontaneously formed selective wetting on circular (D: 2 mm) and square (L: 1 cm) shapes of the mussel-inspired PDA-liposome coating formed on the PTFE surface. C) Camera images of the evaporation of the selectively-wetted and pinned analyte droplet on the PDA pattern. Evaporation in this fashion will concentrate the analyte within the solution droplet.

We first measured the static contact angles of the mussel-inspired PDA coating formed on the PTFE membrane surface. The mussel-inspired PDA liposome coating had a hydrophilic contact angle of 55°, whereas the contact angle of the hydrophobic PTFE was 131° (Figure 4A). The surface of PDA liposome is hydrophilic because its self-assembled polar head groups are presented on the liposome surfaces and their hydrophobic long alkyl chains are hided under the head groups. We further tested the selective-wetting behavior of hydrophilic mussel-inspired PDA liposome spots coated on the hydrophobic surfaces. Figure 4B shows circular and square shapes of PDA-liposome patterns which are entrapping water droplets by surface tension generated at the boundary between the hydrophilic PDA spot and the hydrophobic PTFE surrounding. It is also worthy to note that the entrapped droplet was gradually evaporated while being pinned within the boundary (Figure 4C). Therefore, we anticipated an increase in sensitivity from our selective-wetting device because the target molecules in the analyte droplet will be uniformly concentrated on the sensory PDA spot through evaporation.

VEGF Detection. A good sensor must have specificity toward a target molecule, which can be achieved by rational receptor design and selection. The identified receptor should be chemically attached to the sensory system. For the PDA sensor platform, receptors are tethered to the PDA liposome surface. We investigated whether the developed mussel-inspired bioconjugation strategies allow efficient receptor tethering to the PDA liposome without any further chemical treatment and the mechanical stress induced by specific interactions between the surface bound receptor and target molecules would be effectively transferred through the mussel-inspired adhesion layer to the conjugated yne-ene backbone of PDA. We applied our system to VEGF (vesicular endothelial growth factor) detection by tethering a bio-receptor on the mussel-inspired PDA liposome. VEGF is an important biomarker because it regulates angiogenesis of endothelial cells and is over-expressed when tumor cells grow and metastasize.[60] Through a literature survey, we found an ssDNA aptamer (5'-TGTGGGGGTGGACGGGCCGGGT AGA-3') that recognizes VEGF selectively by forming the G-quadruplex structure.[60] We loaded the amine-modified ssDNA aptamers onto the spots of PDA liposome coating. The amine-modified aptamers were conjugated to the surfaces of the pre-coated PDA liposomes via catechol chemistry: Michael addition or Schiff base formation between amine and catechol (Scheme 3).



Scheme 3 Binding mechanism of an amine-containing bio-receptor to the mussel-inspired PDA liposome through the catechol chemistry (Michael addition and Schiff base formation).

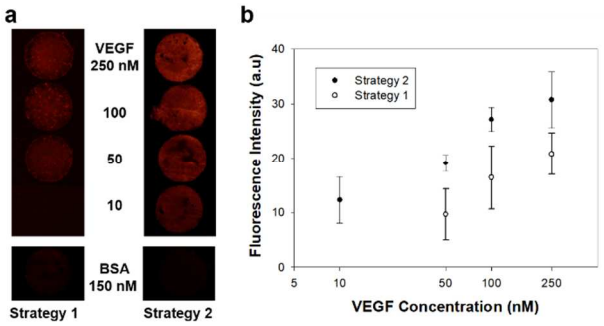


Figure 5 A) Fluorescence microscope images of the mussel-inspired PDA liposome arrays conjugated with VEGF ap-

tamer after 1 h incubation with various concentrations of VEGF solution and 10 μg/ml of BSA, and B) corresponding relative fluorescence intensity

We first examined fluorescence intensity of the resulting PDA liposome spots after being exposure to various concentrations of VEGF for an hour at RT. As shown in Figure 5, the red fluorescence intensity of the PDA spot increased gradually as the VEGF concentration increased. The detection limit obtained from the **Strategy II** was 10 nM while the **Strategy I** could provide the detection limit of 50 nM. Since the **Strategy I** allows polymerization of unbound dopamine, thicker adhesion layer will be formed on the PDA liposme surface. Therefore, mechanical stress produced by the target recognition will be less effectively transferred through the thick adhesion layer to the conjugated yne-ene backbone located at the PDA interior. This result is consistent with the well-established fact that the optical signal intensity drops as the distance between bioreceptor and PDA backbone increases.[24] The aptamer-tethered PDA coating did not shows any fluorescence response to BSA (bovine serum albumin) as an negative control, confirming the specificity of the system.

CONCLUSION

In summary, we developed convenient solid-state sensor array fabrication strategies by adapting the strong adhesion property of the mussel protein to tether PDA liposome to a variety of substrates and facilely bioconjugate DNA aptamer as a receptor to the sensory PDA layer. Dopamine is incorporated with the diacetylene liposomes or catechol/amine-attached diacetylenes are self-assembled into well-defined PDA liposome. The integrated mussel-inspired chemical moieties provide both excellent immobilization of the PDA liposome to various solid substrates and efficient tethering of receptor molecules to the immobilized PDA liposome. We applied mussel-inspired coating methods combined with self-signaling PDA liposomes to droplet-array device development for VEGF detection. The mechanical stress induced by the specific interactions between VEGF aptamers and VEGF is effectively transferred to the PDA backbone and produces an optical sensory signal. Detection limit of 10 nM is achieved. We envision that the developed mussel-inspired approaches can be readily applied to convenient immobilization of many other nanoobjects such as nanoparticles, nanowires, as well as facile development of variety of PDA-based sensor systems.

ASSOCIATED CONTENT

Supporting Information

The detailed synthetic procedures of PCDA-amine and PCDA-catechol, experimental procedures for the immobilization and bioconjugation, characterization methods, and supporting figures supplied as Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interest

ACKNOWLEDGMENT

We acknowledge the financial support from National Institute of Health (NIH R56 CA203290-01) and MCubed 2.0 from University of Michigan. Initial development of the project was supported by Agricultural Biotechnology Development Program, Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea (314013-3), the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2016R1D1A1B03936364), and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI16C1013).

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