Polydiacetylene Liposome Microarray Toward Influenza A Virus Detection: Effect of Target Size on Turn-On Signaling

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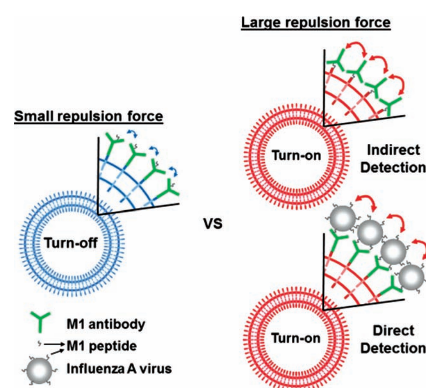
Target size effect on the sensory signaling intensity of polydiacetylene (PDA) liposome microarrays was systematically investigated. Influenza A virus M1 peptide and M1 antibody were selected as a probe–target pair. While red fluorescence from the PDA liposome microarrays was observed when the larger M1 antibody was used as a target, when the same M1 antibody was used as a probe to detect the smaller M1 peptide sensory signal did not appear. The results reveal that the intensity of the PDA sensory signal is mainly related to the steric repulsion between probe–target complexes not the strength of the probe–target binding force. Based on this finding, we devised a PDA sensory system that directly detects influenza A whole virus as a larger target, and confirmed the target size effect on the signaling efficiency of PDA.



1. Introduction

The emergence of the influenza A virus pandemic in 2009 aroused an increasing need for its rapid and sensitive detection.[1] There are two ways to identify the influenza A virus infection. The first one is a direct detection method, such as recognizing peptides or proteins originated from the influenza A virus, or sensing the virus itself. For an alternative indirect detection strategy, produced antibodies by influenza A virus infections are identified by using enzyme linked immunosorbent assays (ELISA) or real time-polymerase chain reaction (RT-PCR). However, because these methods are expensive and require long sample preparation and operation, a cheaper and more rapid diagnostic test system is strongly desired.

Polydiacetylene (PDA) has been attractive for sensory applications due to its unique optical property; PDA undergoes colorimetric transitions by various external stimuli, such as temperature,[2] pH,[3] mechanical stress,[4] and receptor–ligand interactions.[5] The thermochromism and pH-based colorimetric transitions have been attributed to conformational changes in the head group hydrogen bonding and alkyl side chain packing of PDA molecules by an external stimulus.[6] We have developed PDA-based sensors using the intermolecular force that receptor–ligand interactions produce, as an external stimulus, i.e., a DNA sequence or a phospholipid as a selective probe for the detection of a target molecule such as K+,

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Hg^{2+}, and aminoglycosidic antibiotic.[7] The steric repulsion between the bulky probe–target complexes induced the perturbation of the conjugated ene-yne backbone of PDA, resulting in a colorimetric transition from blue to red and red fluorescence development as well. However, even though systematic investigation has been made to build better understanding about the effect of the analyte size on the signaling property of PDA, the mechanisms for the color change in the presence of biological probe–target interactions at the surface of PDA are not fully understood.[8] For example, how microorganism induces the color change of PDA has been debated through various hypotheses, such as conformational change of PDA backbone via the insertion of proteins into the bi-layer of PDA liposomes or cleavage of PDA lipids by an enzyme.[6a] Therefore, to design sensitive and practically applicable biosensors, developing better understanding about the critical factors causing the colorimetric transition under biological probe–target interactions is very important.

In this contribution, we demonstrated a rapid and sensitive PDA-based microarray sensor for influenza A virus detection. We chose the pair of influenza A virus M1 peptide-M1 antibody as a probe–target model. Due to easy tethering of biomolecules such as peptide or antibody at the surface of PDA liposomes, we designed and analyzed direct and indirect detection strategies by switching the role of M1 peptide and M1 antibody as a probe molecule or a target molecule. By using the same pair but only switching their role in the detection system, we could keep the same paring affinity and therefore expound the target size effect on the turn-on signaling of PDA-based sensory systems. For the development of a real detection system, we incorporated 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPA) phospholipid into the PDA liposome in order to give better mobility to the PDA backbone in the liposome, which as a consequence improved the sensitivity of the PDA microarray for influenza A H1N2 virus detection.

2. Experimental Section

2.1. Materials and Methods

All solvents were purchased from Sigma–Aldrich. 10,12-pentacosadiynoic acid (PCDA) was purchased from GFS Chemicals. N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were obtained from Acros Organics. 2-(2-aminoethoxy)ethanol, epibromohydrin, sodium hydride, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), phosphate buffered saline (PBS), and Tween® 20 were purchased from Sigma–Aldrich. 1,2-dimyristoylsn-glycero-3-phosphate (DMPA) were purchased from Avanti Polar Lipids. The frame-seal slide chambers for the liposome immobilization was obtained from Bio-Rad.

Influenza A virus M1 peptide (Matrix Protein 1 peptide, purity >90%, SIIPSGPLK, Mw 911.1) was purchased from AnaSpec. Mouse monoclonal to influenza A virus M1 antibody and NP antibody were obtained from Abcam. Influenza A H1N2 virus (swine/Korea/H1N2) was supplied by Animal Plant and Fisheries Quarantine and Inspection Agency (QIA).

The synthesis of PCDA derivatives was described in our previous work.[7b] The PDA monomers were characterized with 1H NMR spectra (500 MHz) using Varian Inova 500 instrument. UV–Vis absorption spectra were obtained by Varian Cary 50 UV–Vis spectrophotometer. Fluorescence images were taken by Olympus BX51 with DP71 fluorescent microscope. Evaluation of the fluorescent dot intensity was carried out by using Image J software.

2.2. Preparation of PDA Liposome Solution

PDA liposome solution was prepared by the injection method. PCDA-Epoxy and PCDA (4:1 molar ratio) were dissolved in 0.2 mL of tetrahydrofuran. The homogeneous solution was injected into a 20 mL of 5×10^{-3} M HEPES buffer at pH 8 and subsequently dispersed in a bath sonicator for 10 s to produce the final concentration of the liposome of 0.5×10^{-3} M. After filtration through a 0.8 μm cellulose acetate syringe filter, the resulting PDA liposome solution was stored at 5 °C at least 2 h for the next immobilization step.

2.3. Fabrication of PDA Liposome Microarray

An amine-modified glass slide was prepared similarly through the literature procedure.[7b] Glass slides were cleaned with chloroform, acetone, and 2-propanol for 3 min each. The pre-cleaned glass slides were then sonicated in sulfuric acid containing NOCHROMIX. After thorough rinse with deionized water and dry, the glass slides were stirred in a 2 wt% 3-aminopropyltriethoxysilane toluene solution using an orbital shaker for 1 h and afterward baked at 115 °C for 30 min. The glass slides were sonicated in toluene, toluene: methanol (1:1), and methanol for 3 min each to remove any unbound silane monomer.

We devised PDA liposome microarrays using two strategies. First, as illustrated in Scheme 1 of Figure 2, PDA liposome was immobilized onto the prepared amine glass by means of 20 min incubation and by successive thorough rinse with 5×10^{-3} M HEPES buffer at pH 8.0 followed by additional rinse with deionized water. Onto the immobilized PDA layer, a probe solution (M1 peptide or M1 antibody) was microarrayed using a manual microarrayer (VP 478A, V&P Scientific) and incubated at 5 °C for overnight. After washing with 0.2% (v/v) PBST (0.2% Tween 20 in 1 x PBS) and deionized water, the probe-tethered PDA slides were treated with ethanolamine to block the unreacted area. The prepared PDA microarrays were photopolymerized by 254 nm UV light for 30 s right before the detection tests. Second, as depicted in Scheme 2 of Figure 2, PDA liposome was microarrayed onto the amine glass using the manual microarrayer for a 20 min incubation period. It was then rinsed with 5×10^{-3} M HEPES buffer at pH 8.0, and washed with deionized water. Then, a probe solution (M1 peptide or M1 antibody) was covered onto the prepared PDA layer and the slides were stirred using an orbital shaker at room temperature for 1 h and left at 5 °C for overnight. After washing with 0.2% (v/v)

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PBST and deionized water, respectively, the probe-tethered PDA slides were treated with ethanolamine to block the unreacted area. The prepared PDA microarrays were photopolymerized by 254 nm UV light for 30 s right before the detection tests.

2.4. Detection Tests

To investigate the selectivity and sensitivity, the PDA liposome microarrays were incubated with a target solution (M1 peptide, M1 antibody, NP antibody, or influenza A H1N2 virus) at room temperature for 1 h.

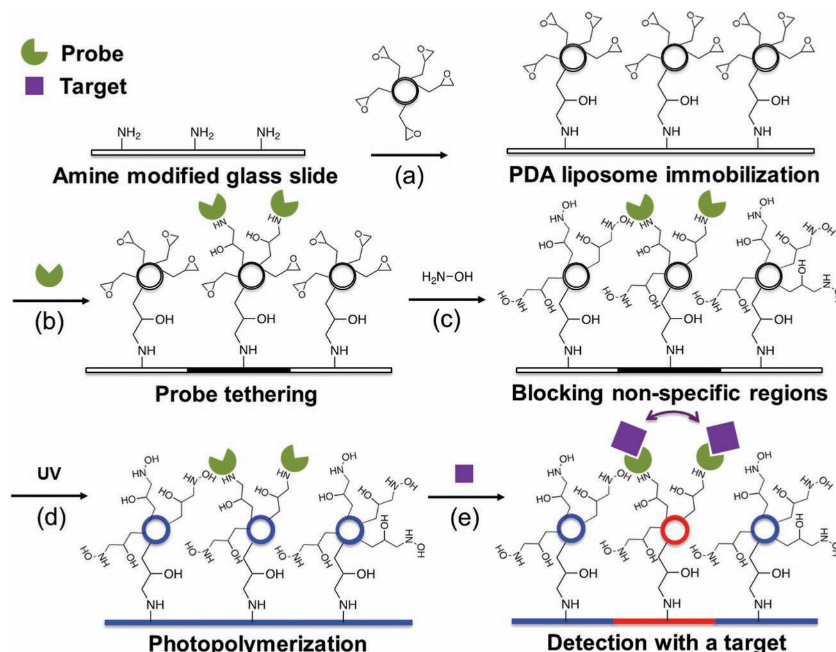
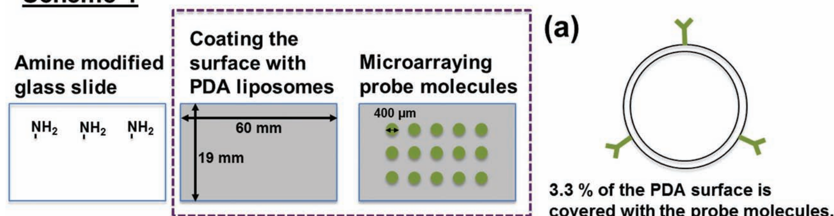


Figure 1. Schematic illustration of the PDA liposome microarray fabrication. (a) Immobilization of PDA liposomes onto an amine-modified glass slide, (b) probe molecule tethering, (c) blocking the probe-free area with ethanolamine to prevent non-specific binding, (d) photopolymerization, and (e) detection with a target. Arrow presents repulsion between adjacent probe–target complexes.

3. Results and Discussion

We investigated how the known specific interaction between the M1 peptide and the M1 antibody[9] can be rationally combined with our PDA liposome-based sensory system to detect influenza A virus. We prepared PDA liposome microarrays for influenza A virus detection as schematically illustrated in Figure 1. The bare glass (60 mm × 19 mm) was silanized with aminopropyltriethoxysilane to modify the surface to have primary amine groups. The epoxy functionality was used for the probe tethering as well as the liposome immobilization. Through optimization study, the 4:1 molar ratio of PCDA–Epoxy to PCDA was established to provide the most stable and sensitive detection. The liposome was covalently bound on the amine glass by amine-epoxide reaction. The M1 antibody was used as a probe molecule for M1 peptide detection while the M1 peptide was utilized as a probe for M1 antibody detection. After tethering the probe molecules (M1 antibody or M1 peptide) as circular dots having diameter of 400 μm, with ethanolamine we blocked the area where probe molecules are not present in order to avoid any signal generation by non-specific binding.

First, as illustrated in the Scheme 1 of Figure 2, we fully covered the amine-modified substrate with the PDA liposome. Then, the probe molecules were microarrayed onto the PDA liposome layer. This afterward-tethering strategy of probe molecules provides convenient spotting of various probe molecules on the microarray surface, rendering high-throughput detection possible.[7b] After 30 s of 254 nm UV irradiation, we observed blue color development confirming successful photopolymerization of the PDA liposomes on the microarray. We anticipated that the probe–target binding events at the PDA liposome surface produced red emission due to the induced stress by the formation of bulky probe–target complex. However, we could not observe noticeable signals regardless



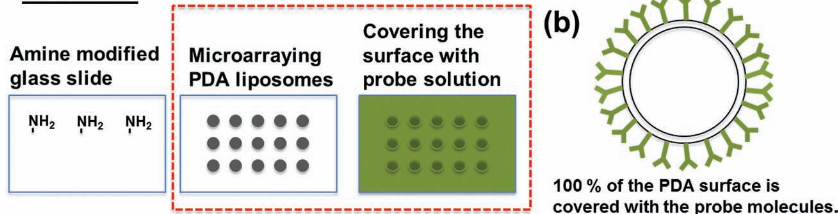


Figure 2. Schematic illustration of PDA liposome microarray fabrication protocols for controlling the number of probes tethered to the PDA liposome surface. (a) PDA liposome containing 3.3% probe molecules, (b) PDA liposome fully tethered with probe molecules.

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of whether the M1 peptide was used as a probe or a target.

Our calculation implied that the number of probe molecules actually tethered at the PDA liposome surface was too few to form good enough complex formation for efficient steric perturbation of the PDA conjugated backbone. To quantity the probe molecules at the PDA surface, we first calculated the number of epoxy groups at the PDA surface available for the reaction with probe molecules (M1 antibody and M1 peptide). Since we used 0.5×10^{-3} M PDA liposome solution consisting of PCDA-Epoxy and PCDA at 4:1 molar ratio, the 0.5×10^{-3} M PDA liposome solution is equivalent to 400×10^{-6} M of epoxy groups. We covered 300 μL of the PDA liposome solution onto the amine glass slide for immobilization, which means that 0.12 μmole of epoxy groups is available in the volume. Through the immobilization step, 45% of the initial PDA liposome bound on the glass slide, which was calculated by comparing the UV-Vis absorption intensity at 648 nm of the initial PDA liposome solution before the immobilization and the unbound PDA liposome solution after the immobilization. Then, the number of actually immobilized epoxy groups per glass slide should be 0.054 μmole (0.12 μmole x 0.45). Because the size of the glass slide was 60 mm x 19 mm and the diameter of the microarray dots where the probe molecules were microarrayed was 400 μm, the number of the immobilized epoxy groups per microarray dot was calculated to be 6 pmole. The concentration of the purchased M1 antibody was 6.67×10^{-6} M. Considering the spotting volume of 30 nL per spot by a manual microarrayer, 200 fmole M1 antibody is available to react with the immobilized epoxy groups within each microarray spot. Therefore, the calculation showed that at best only 3.3% epoxy groups on the tethered PDA liposome surface would react with M1 antibody (Figure 2a, 200 fmole antibody onto 6 pmole epoxy groups). Even though a more concentrated M1 peptide solution was commercially available, we used M1 peptide solution at the same concentration of 6.67×10^{-6} M for M1 antibody detection, in order to match the same probe molecule density on the PDA liposome surface. We did not observe any sensory signal generation in this case either.

To enhance the number of tethered probe molecules at the PDA surface, we used the strategy illustrated in the Scheme 2 of Figure 2. Instead of tethering PDA liposomes to fully cover the amine glass, PDA liposomes were spotted to form 36 microarray dots having an average diameter of 400 μm by means of a manual microarrayer. Then, the PDA microarray was covered with 300 μL of 1.2×10^{-6} M M1 peptide solution and enclosed under a sealing slide chamber. The amount of M1 peptide in the volume was calculated to be 1.67 times excess to the number of the epoxy groups per microarray dot. While the PDA microarray under the sealing slide chamber was stirred on an orbital shaker for 1 h, the epoxy group should have enough chances to react with the excess amount of the probe molecules. Hence, this strategy significantly increases the mole ratio of the available M1 peptide to the epoxy moiety at the PDA liposome surface, largely enhancing the number of expressed M1 peptides at the PDA liposome surface (Figure 2b). As the consequence of the increased number of probing molecules, larger number of probe–target complex is expected to form, and indeed we observed strong red emission from the microarrays upon incubating with M1 antibody (Figure 3a). We confirmed that the fluorescence signal was generated from the specific interaction between M1

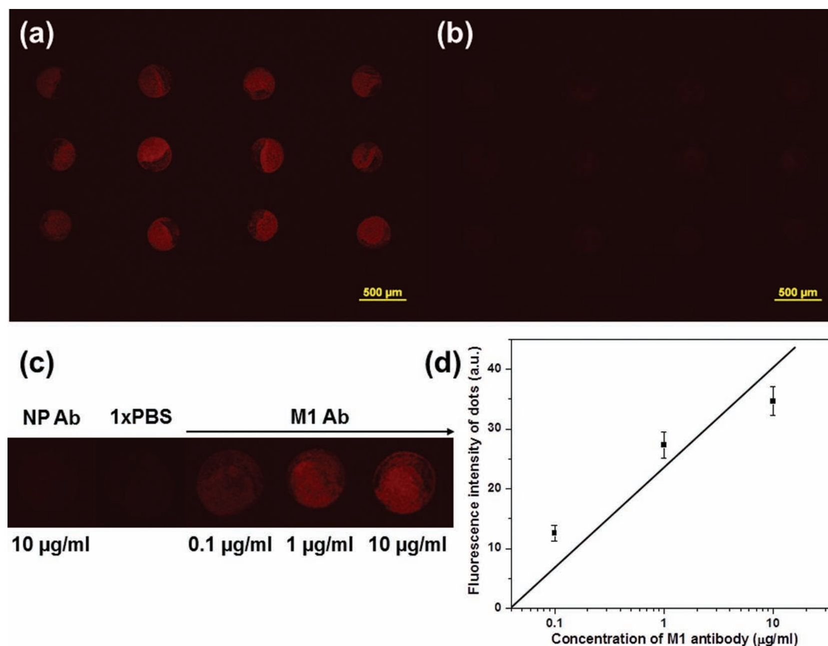

Figure 3. Fluorescent microscope images of PDA liposome microarray (a) having M1 peptide probes for the detection of M1 antibody (10 μg/mL), (b) having M1 antibody probes for the detection of M1 peptide, and (c) enlarged fluorescent microscope image of PDA microarray dots having M1 peptide probes after 1 h incubation with NP antibody, 1xPBS, and various concentrations of M1 antibody from 0.1 to 10 μg mL^{-1} at room temperature (excitation at 550 nm and a emission filter with 600 nm cutoff were used) (d) correlation curve between fluorescence intensity of dots and concentration of M1 antibody. Dot intensity is calculated as numerical values from the dot images by using ImageJ software. Each point and error bar represent a mean value and a standard deviation, respectively.

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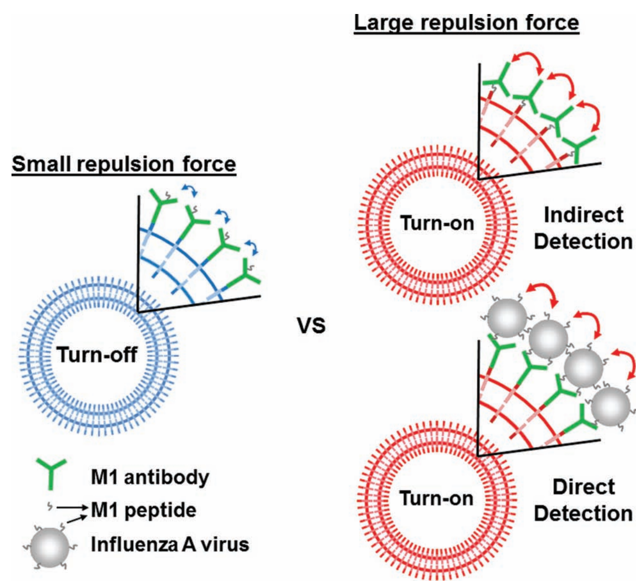

Figure 4. Schematic illustration of the target size effect on turn-on signaling of the PDA liposome microarray.

peptide and M1 antibody because we could not observe any noticeable signal when NP antibody was incubated as a non-specific target (Figure 3c). We plotted the fluorescence emission intensity of the microarray against the M1 antibody concentration as shown in Figure 3d. As one can see from the correlation curve, a quantitative analysis of M1 antibody is possible.

When M1 antibody was used as a probe for the detection of M1 peptide by means of the same second strategy, on the contrary, we could not observe noticeable sensory signal generation (Figure 3b). From this unexpected result, we postulated the target size effect on turn-on signaling of the PDA liposome microarray. The M1 peptide is a nonapeptide having an estimated length of a few nm ($\overline{M}_w$: ≈1000) at most, and the M1 antibody is thought to have a hydrodynamic diameter of 7–10 nm ($\overline{M}_w$: ≈150,000).[10] As depicted in Figure 4, the small target, M1 peptide, cannot produce large enough steric repulsion to warrant a sensory signal even after being captured by the M1 antibody probe having a much larger size. However, the large target, M1 antibody, can produce the red fluorescence signal because once being captured by the densely packed M1 peptide probes, the captured M1 antibodies will generate large enough steric repulsion at the PDA liposome surface. In this study, we could make a very important finding that the intensity of the PDA sensory signal is mainly related to the steric repulsion between probe–target complexes not the strength of the binding force between a probe and its target.

Based on this finding, we anticipated that influenza A virus can be directly detected by PDA liposome having M1 antibody probes because the whole virus is larger than M1 antibody. As shown in Figure 5a, indeed red dots appeared on the PDA liposome microarray after incubation with H1N2 viruses, which confirms the target size effect on the turn-on signaling of PDA. We could achieve the detection limit of 2^2 HAU from PDA liposome consisting of PCDA-Epoxy and PCDA (4:1 molar ratio) while conventional influenza A virus kits have the general detection limit of 0.1–0.5 HAU.[11] To further improve the sensitivity of the PDA liposome microarray, we incorporated a phospholipid, DMPA, into the PDA liposome to provide more mobility and ensuing easier perturbation of PDA backbone by probe–target complex formation and repulsion. It is well known that incorporating non-polymerizable phospholipids into PDA liposome weakens the intermolecular packing of PDA monomers by lowering the hydrogen bonding strength among their side chains.[12] As shown in Figure 5b,

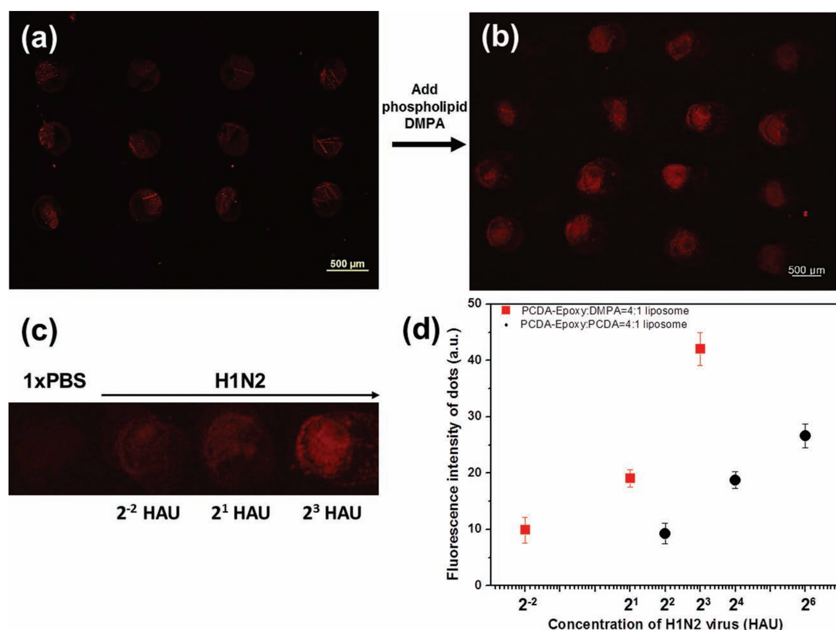

Figure 5. (a) Fluorescent microscope images of PCDA-Epoxy:PCDA (4:1) liposome microarray having M1 antibody probes for the detection of H1N2 virus (2^4 HAU), (b) Fluorescent microscope images of PCDA-Epoxy:DMPA (4:1) liposome microarray having M1 antibody probes for the detection of H1N2 virus (2^3 HAU), and (c) enlarged fluorescent microscope image of the PCDA-Epoxy:DMPA (4:1) liposome microarray dots after 1 h incubation with 1xPBS and various concentrations of H1N2 virus from 2^{-2} to 2^3 HAU at room temperature (excitation at 550 nm and a emission filter with 600 nm cutoff were used), (d) Correlation between fluorescence intensity of dots and concentration of H1N2 virus. Dot intensity is calculated as numerical values from the dot images by ImageJ software. Each point and error bar represents a mean value and a standard deviation, respectively.

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when we used PDA liposome of PCDA-Epoxy and DMPA (4:1 molar ratio) the brighter red emission appeared at 2^3 HAU of H1N2 viruses. We ultimately reached the detection limit of 2^{-2} HAU from the PDA liposome microarray, which is comparable to the detection limit conventional influenza A virus kits can provide. The quantitative correlations between the emission intensity of the PDA microarrays and the concentration of influenza A virus H1N2 are shown in Figure 5d.

4. Conclusion

We systematically studied the effects of the target size on the turn-on signaling of PDA sensory systems for the detection of biological molecules based on the intermolecular interactions between a probe molecule and its target. The interaction between the M1 peptide and the M1 antibody of influenza A virus was rationally coined into a PDA sensor design for direct and indirect detection of influenza A virus. By using the same pair but only switching their role as a probe or a target in the detection system, we could keep the same paring affinity and therefore unquestionably examine the target size effect. While the larger M1 antibodies produced red fluorescence emission upon binding with densely packed M1 peptides at the PDA liposome surface, the smaller M1 peptides could not generate any noticeable signal when they bound to tightly packed M1 antibodies. When the probe density at the PDA surface was low, we could not observe any sensory signal generation from the PDA microarray regardless of the role of M1 antibody and M1 peptide. These results clearly revealed that the PDA sensory signal is mainly from the steric repulsion between probe–target complexes not the strength of the probe–target binding force. Based on the finding, we developed PDA microarray for direct detection of influenza A virus. The PDA liposome microarrays having densely packed M1 antibody probes and co-assembled phospholipids sensitively detected influenza A virus with the detection limit of 2^{-2} HAU. The demonstrated target size effect can be readily applicable to various PDA-based biosensor designs and developments.

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