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Design of a simple paper-based colorimetric biosensor using polydiacetylene liposomes for neomycin detection†

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We developed a paper-based analytical device (μPAD) combined with self-signaling polydiacetylene (PDA) liposomes for convenient visual neomycin detection. The simple dot array type of μPAD was fabricated by the wax printing technique, and the PDA liposomes in the aqueous solution were facilely immobilized onto the hydrophilic dot region of the paper substrate. We found that, when the PDA liposomes were inserted to the paper matrix, the stability of the PDA liposomes can be significantly enhanced by adding a hydrophilic reagent such as polyvinyl alcohol and glycerol to the liposome solution. In particular, polyvinyl alcohol (PVA) provides the best stabilization among the various hydrophilic reagents tested in this contribution, and the enhanced stability sharply increased the sensitivity of the PDA liposomes in the paper matrix. Based on the above results, we successfully detected neomycin through both naked-eye observation and fluorescence measurement of PDA signals. The detection limit was 1 ppm and was selective to non-aminoglycoside antibiotics.

Introduction

Paper is an attractive solid support for various bioassays because paper is not only easy to use, cheap, portable, and disposable, but it can also absorb aqueous solution containing analytes instinctively by natural capillary action. Moreover, paper enables convenient naked-eye based colorimetric detection, providing a white background for sharp contrast. In this regard, the G.M. Whiteside group invented a microfluidic paper-based analytical device (μPAD) to realize facile and inexpensive point-of-care kits, especially for impoverished areas in developing countries.[1–4] The μPAD utilizes hydrophilic parts surrounded by a hydrophobic barrier as fluidic channels or reservoirs, handling the aqueous solution by spontaneous selective wetting without any external power source such as a micropump. Wax printing is a representative method to form such a hydrophobic barrier by using two unsophisticated setups, a wax printer and a hot plate (or oven).[4,5] The wax printer deposits the hydrophobic wax onto the top surface of the desired region of the paper substrate, and then the hot plate induces the coated wax to fill the full thickness of the desired region by thermal reflow, resulting in uncontaminated pristine hydrophilic parts. Through two such simple steps, rapid (<1 h), low-cost, and scalable production of μPAD can be achieved.

Polydiacetylene (PDA) is an ideal signal transducer for μPAD in terms of the facile production method using photopolymerization and the unique self-signalling by dual absorbance and fluorescence responses. When closely packed, the transparent diacetylene monomers can be converted into PDA having a blue colour and no fluorescence after several minutes of UV (254 nm) irradiation even from a compact handheld lamp (~4 W). More interestingly, such blue phase of PDA can change its colour to red and emit red fluorescence in response to various external stimuli such as heat,[6,7] mechanical stress,[8] and receptor–ligand interaction.[9–21] The dual responses have been attributed to the conformational change of the π-conjugated main chain of PDA by external stimuli. It is remarkable that, as a generic biosensor platform, PDA

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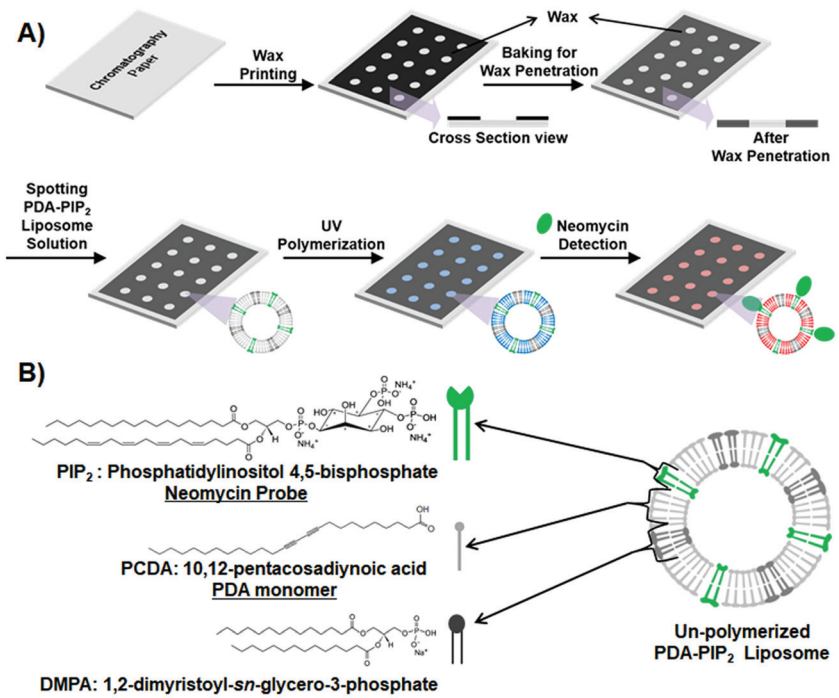
materials can detect numerous analytes such as metal ions,[12,15,19,20] small organic molecules,[9,13,14] proteins,[16,17,21] viruses,[17] and bacteria[10,11] through the receptor–ligand interaction. For such biosensor applications, the liposome type of PDA material prepared from amphiphilic diacetylene monomers is commonly used because the outer head groups can be efficiently exploited as specific receptors to capture the bioanalytes in aqueous solution. In detail, the head group of diacetylene monomers or co-assembled non-diacetylene lipids can be rationally modified to include the specific receptor, achieving the selective detection.

Though some PDA sensors have been constructed on simple paper supports such as strips,[20,22–24] there is still lack of studies to realize systems integrating the PDA liposomes and μPAD. In general, the current paper-type PDA sensors do not combine with the PDA liposomes but the PDA crystals prepared from drop-casted diacetylene monomers.[20,22–24] However, the drop-casting method uses organic solvents which can damage the hydrophobic barriers of the μPAD, and the large amount of head groups becomes buried inside the PDA film, lowering the efficiency of target capturing. A few PDA liposome-based sensors have been fabricated on a paper substrate but highly-concentrated PDA liposomes (>4 mM) are inserted in such sensors to obtain an intense signal.[25,26] Such high concentration of the PDA liposome is likely to deteriorate the sensitivity by inducing the aggregation of the liposomes and lowering the molar ratio of target to sensing unit, as compared to the PDA liposome concentration (0.5–1 mM) for conventional glass-type PDA sensors (*e.g.* microarray).[12,13,15]

In this contribution, we developed an array type of μPAD incorporating the sensory PDA liposomes. The dot array μPAD was fabricated by the wax printing method, and the PDA liposomes were immobilized onto the hydrophilic circular dots of the μPAD, as illustrated in Scheme 1A. In addition, we especially demonstrated that the PDA liposomes can be stably loaded into the paper substrates in the aid of hydrophilic reagents such as polyvinyl alcohol (PVA). The hydrophilic reagent helps the inserted diacetylene liposomes to maintain their closely-packed assembly during water (solvent) evaporation, resulting in intense blue PDAs after photo-polymerization. Therefore, even lower concentration (0.5 mM) of the PDA liposomes was successfully integrated to the paper substrate, and showed the clear colorimetric responses achieving naked-eye detection.

Neomycin, an aminoglycoside class of antibiotics, is usually prescribed by veterinarians to treat diverse bacterial infections of food-producing animals. However, the accumulated neomycin in animal derived foods (*e.g.* milk, egg, and meat) is a potential threat to human health as it causes damage to organs (ear, kidney and nervous system), allergy, and the emergence of super bacteria.[27,28] In this regard, we previously reported a highly-sensitive neomycin sensor by designing a bio-inspired PDA liposome.[13] As illustrated in Scheme 1B, the neomycin-sensitive PDA liposome (PDA-PIP_2 liposome) consists of three lipids, PCDA (10,12-pentacosadiynoic acid, a PDA monomer), PIP_2 (L-α-phosphatidylinositol-4,5-bisphosphate), and DMPA (1,2-dimyristoyl-*sn-glycero*-3-phosphate). The PIP_2, lipids existing in cellular membranes,



Scheme 1 A) Schematic illustration of μPAD fabrication by wax printing and neomycin detection by PDA-PIP_2 liposomes immobilized onto the μPAD and (B) components of the neomycin-sensitive PDA-PIP_2 liposomes.

contains a head group having specificity to neomycin.[29] DMPA is inserted for enhanced stability and sensitivity of the PDA liposomes.[13,16] As anticipated, our new μPAD combined with the PDA-PIP_2 liposome achieves sensitive neomycin detection by both naked-eye observation of the colour change and fluorescence measurement.

Materials and methods

Materials

Whatman™ cellulose chromatography paper 1 CHR was used for the fabrication of the paper-based microfluidic biosensor. A PDA monomer, 10,12-pentacosadiynoic acid (PCDA), and hydrophilic reagents such as polyvinyl alcohol (PVA, M_w 85 000–124 000, 99% hydrolysed), polyethylene glycol (PEG, M_w 600), and glycerol were purchased from Sigma-Aldrich Chemicals. Neomycin, other antibiotics, solvents, buffers, and other chemicals were also purchased from Sigma-Aldrich Chemicals. Phospholipids, L-α-phosphatidylinositol-4,5-bisphosphate (PIP_2) and 1,2-dimyristoyl-*sn-glycero*-3-phosphate (DMPA) were purchased from Avanti Polar Lipids.

Assembly of neomycin-sensitive PDA-PIP_2 liposomes

Neomycin-sensitive PDA-PIP_2 liposomes were assembled by our previously developed method.[13] PCDA, PIP_2 and DMPA lipids were dissolved in chloroform and mixed at a molar ratio of 7 : 2 : 1. After evaporating the chloroform thoroughly by N_2 blowing, the lipids were suspended in 2.5 ml of 0.1× PBS at 80 °C. The total concentration of the lipids was 1 mM. The suspension was sonicated by using a 120 W probe-sonicator at 80 °C for 10 minutes and filtered through a 0.8 μm cellulose acetate syringe filter. The cloudy diacetylene liposome solution was stored at 5 °C for 24 h before use.

Fabrication of dot array μPAD by wax printing

The pattern consists of hollow circles (diameter: 2.5 mm, spacing: 7.5 mm), which were drawn by using the Microsoft PowerPoint 2007 program, and then printed onto the chromatography paper with black wax by using a Xerox ColorQube 8870 printer. The patterned paper was baked on a hot plate at 125 °C for five minutes to fill the full thickness of the printed region with the melted wax.

Loading of PDA liposomes onto dot array μPAD in the aid of hydrophilic reagents

1 mM diacetylene liposome solution and 5 wt% PVA solution were mixed at a 1 : 1 volume ratio and 2.5 μl of the mixed solution were spotted onto the hydrophilic dot region of the paper-based microfluidic device. In the case of PEG and glycerol addition, the same concentration (5 wt%) of the solution was also prepared and used. After an hour of incubation for the evaporation of the solvent (water), the PDA liposomes in the paper microfluidic dot array were polymerized by 254 nm UV irradiation for one minute. The fabricated devices were stored in a Petri dish sealed with parafilm and aluminium foil at 5 °C until further use. The absorption spectra were recorded by using a USB 4000 spectrometer.

Neomycin detection

For detection tests, the dot array μPAD containing PDA liposomes was incubated with various concentrations of neomycin solutions for 1 hour at 37 °C. The temperature was chosen to increase the interaction between neomycin and PIP_2.[13,29] Fluorescence microscopy images were obtained on a Nikon eclipse Ti microscope. Fluorescence intensities were measured by using the image processing software ImageJ (National Institutes of Health). Relative fluorescence intensity (RFI) was calculated by the following equation: $RFI = (I - I_0)/I_0$, where I_0 and I are the initial and final fluorescence intensity.

Results and discussion

Wax printing of dot array μPAD

We chose the wax printing technique to develop the array type of μPAD because the technique is not only rapid and convenient but also yields the pristine hydrophilic parts uncontaminated with unremovable chemical residues. The pristine parts having no auto-fluorescence by residues enable the fluorescence-based detection of analytes with sharp contrast, as compared to other techniques such as photolithography.[2] The μPAD consisting of the hydrophilic circular dots was fabricated by a sequential process within an hour, as shown in Scheme 1A. The diameter of the circular dot was designed to be 2 mm since such diameter is sufficiently large to detect targets clearly with the naked eye, despite using a small amount of PDA liposomes in the post-processes. In the wax printing, the final area of the hydrophilic wax-free region is unavoidably shrunk as compared with the initial blue print because the deposited wax spreads to the lateral direction, as well as the vertical direction, during the baking process.[5] By considering such size reduction, we designed the pattern having hollow circles with 2.5 mm diameter, and printed the pattern onto the chromatography paper with black wax. Black wax was chosen owing to its low auto-fluorescence providing zero background for fluorescence-based detection.[5] As anticipated, the array type of μPAD having about 2 mm diameter of the wax-free regions was successfully produced after the baking process (Fig. 1A).

Instability of PDA liposomes in the dot array-type μPAD

We first tried to integrate the μPAD with PDA by loading the non-polymerized diacetylene liposome solution having no additives onto the hydrophilic region of the paper substrates. The aqueous solution containing PDA liposomes was well confined and absorbed in the hydrophilic region by natural surface tension. After the complete evaporation of the solvent (water), we exposed the liposomes in the paper substrates to 254 nm UV light to initiate the polymerization of diacetylene. However, despite the prolonged UV irradiation (>10 minutes),

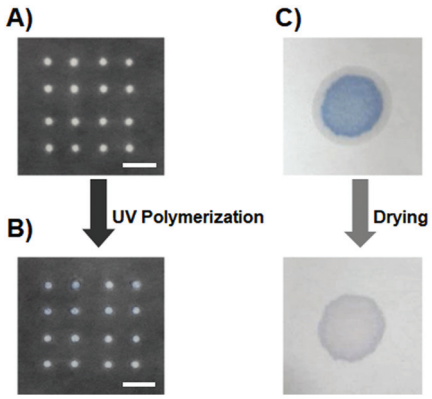


Fig. 1 (A) Camera image of dot array μPAD (dot diameter: 2 mm) having spotted PDA liposomes in the dot region (scale bar: 1 cm), (B) faint blue color of PDA liposomes in the paper substrate after UV polymerization, and (C) blue-to-red color change of pre-polymerized PDA liposomes by drying the paper substrate.

the liposomes in the paper substrates show a faint blue color which indicates poor polymerization (Fig. 1B).

For the stable polymerization to the intense blue color of PDA, close packing of the diacetylene monomers is necessary.[16] In this regard, we doubt that the packing of the diacetylene lipids was disrupted by certain external stress during the drying process and then poorly polymerized. To confirm the origin of the poor polymerization, the pre-polymerized blue PDA liposome was used as a colorimetric indicator analogous to the non-polymerized diacetylene liposome because the blue-to-red colour change of PDA, and thus the distortion of the conjugated main chain, means the disrupted packing of the diacetylene monomeric units.[16] After spotting the solution containing the blue PDA liposomes onto the pristine paper substrates, we observed that the PDA liposomes changed their colour from blue to red, displaying disordered packing by the evaporation of the solvent, as anticipated (Fig. 1C). It can be explained that, as the water evaporates, the hydrophobic interactions between the lipids are weakened while the interaction between the lipid and the paper matrix is strengthened due to the decrease of the shield effect by water molecules, thereby inducing the perturbation of the packing. Therefore, we are convinced that a method which can preserve certain amounts of water in the paper substrates is necessary to combine the PDA liposomes with μPAD.

Enhanced stability of PDA liposomes in μPAD by the addition of hydrophilic reagents

We expected that the addition of hydrophilic reagents can stabilize the immobilized PDA liposomes by preserving a certain amount of water in the paper substrates and physically inhibiting the direct interaction between the diacetylene lipid and the paper matrix after the drying process. To confirm our hypothesis, the non-polymerized diacetylene liposome solutions were mixed with various hydrophilic reagents such as polyethylene glycol (PEG), glycerol, and polyvinyl alcohol (PVA), and spotted onto the hydrophilic region of the μPAD. After an hour of the drying process, the diacetylene liposomes immobilized onto μPAD were polymerized by 254 nm UV irradiation for one minute (Fig. 2). As anticipated, the PDA liposome mixed with PVA or glycerol shows the intense blue colour indicating stable polymerization. The increase of the absorption peak at about 650 nm also indicates that the blue colour of the PDA liposomes was stably formed by PVA or glycerol, as compared to the PDA liposome without additives. It is also worth noting that the stabilized PDA liposomes by the addition of PVA or glycerol maintain the blue color in the paper substrate during 30 days of storage at 5 °C (Fig. S1†). The addition of PEG did not stabilize the PDA liposomes even though PEG is also a hydrophilic reagent. It is believed that the highly-concentrated PEG by the drying process rather deteriorated the stability of the PDA liposomes.[30]

Effect of PVA concentration on the stability and sensitivity of PDA liposomes in μPAD

We chose PVA due to its best performance to stabilize PDA liposomes among the above-mentioned hydrophilic reagents. It is also remarkable that PVA has non-biofouling property due to its hydroxyl side groups, which is necessary in biosensor applications. We investigated the effect of PVA concentration on the polymerization of PDA in the μPAD as shown in Fig. 3. In the range from 0 to 5 wt% PVA, the blue colour of PDA became more intense and the absorption peak (at 650 nm) increased, as the PVA concentration increased. It can be explained that the larger amount of PVA traps more water and blocks the interaction between the liposome and the paper

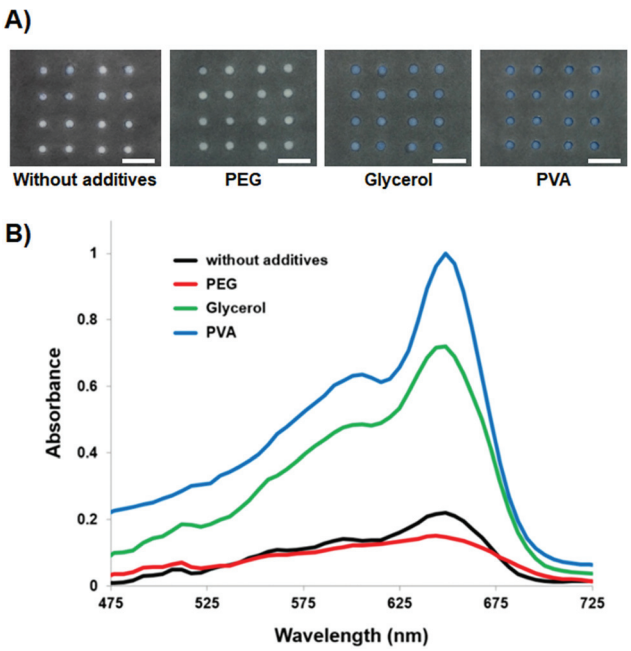


Fig. 2 (A) Camera images of PDA liposomes immobilized onto μPAD with 5 wt% of various hydrophilic reagents (scale bar: 1 cm) and (B) the corresponding UV-Vis spectra.

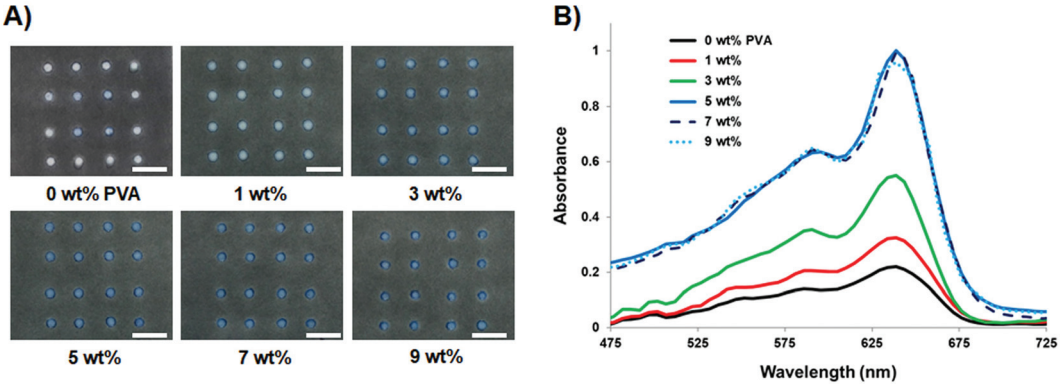


Fig. 3 (A) Camera images of PDA liposomes immobilized onto μPAD with various concentrations of PVA (scale bar: 1 cm) and (B) the corresponding UV-Vis spectra.

matrix more efficiently, thereby enhancing the stability of the PDA liposomes in the paper substrates. In the range above 5 wt% PVA, the absorption peak did not increase anymore, which means that the PDA was fully stabilized and polymerized.

We further explored the effect of PVA concentration on the sensitivity of PDA. The neomycin-sensitive PDA-PIP_2 liposome was mixed with 0, 1, 3 or 5 wt% PVA and loaded onto the μPAD. After polymerization, the liposomes were exposed to 100 ppm of neomycin for 1 h at 37 °C. Fig. 4 shows that the fluorescence intensity of the PDA liposomes in the μPAD became stronger as the PVA concentration was increased. In addition, a uniform fluorescence signal was obtained at 5 wt% of the high PVA concentration. It is reasonable to anticipate that the sensitivity and signal uniformity were enhanced at a higher concentration of PVA due to the increased stability of the entire PDA liposomes immobilized onto the paper substrate.

Neomycin detection by paper-based microfluidic dot array

By using the optimized 5 wt% PVA concentration, we prepared the μPAD combined with the neomycin-sensitive PDA-PIP_2 liposomes to study the detection limit of our system. The μPAD was incubated with various concentrations of neomycin solution for 1 h at 37 °C. As anticipated, the PDA liposomes emitted stronger red fluorescence as the neomycin concentration was increased (Fig. 5A). The detection limit was 1 ppm and we obtained a good linear correlation between the neomycin concentration and the fluorescence intensity. We also detected neomycin with the naked eye, as well as by fluorescence-based detection (Fig. 5B). The PDA liposomes in the μPAD changed their colour from blue to violet or red upon exposure to various concentrations of neomycin. The PDA liposomes showed a violet colour at 1 and 10 ppm of neomycin and presented a red color above 50 ppm. When compared with our previous PDA liposome-based microarray system (detection limit: 61 ppb, incubation time: 20 min), the present paper-based system is less sensitive and slow. This is because, to achieve equipment-free naked eye detection, we immobilized about 80 times more PDA liposomes onto the current system

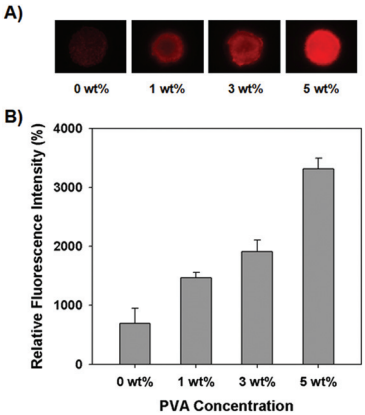


Fig. 4 (A) Fluorescence microscopy images of the PDA liposomes in the μPDA *versus* PVA concentration after 1 h of incubation with 100 ppm neomycin solution at 37 °C, and (B) the corresponding fluorescence intensity (n = 5).

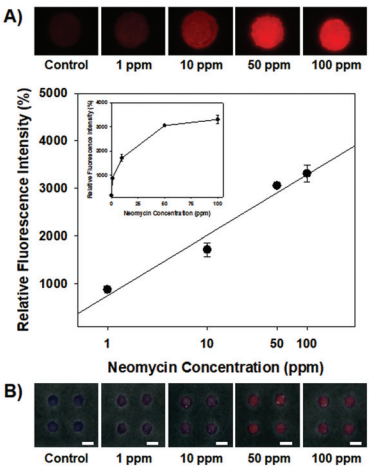


Fig. 5 (A) Fluorescence detection of various concentrations of neomycin by μPAD combined with PDA-PIP2 liposomes and the corresponding relative fluorescence intensity (n = 8) and (B) naked-eye detection of neomycin after 1 h of incubation at 37 °C (scale bar: 2.5 mm).

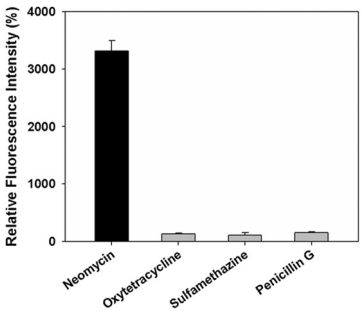


Fig. 6 (A) Selectivity of μPAD combined with PDA-PIP2 liposomes to 100 ppm of neomycin and other antibiotics after 1 h of incubation at 37 °C (n = 8).

than the ones for the previous microarray system (while about 30 nl of 0.5 mM PDA liposome solution were used for one spot of the PDA microarray, 2.5 μl were used for one spot of the current system). Therefore, as the molar ratio between the targets (neomycin) and the signal generating units (PDA liposome) was decreased, the sensitivity was lowered. This current detection limit of our present system is, however, still sufficient to detect the residue tolerance of neomycin defined by the WHO and FAO (1.5 ppm in milk). In addition, it is reasonably expected that the current incubation time can be reduced by the integration with well-established microfluidic devices such as a microfluidic concentrator in our future studies.[31]

We further conducted the selectivity test by exposing the μPAD to 100 ppm of other antibiotics such as oxytetracycline, sulfamethazine, and penicillin G. The PDA liposome in the μPAD did not show any fluorescence or colour change to the other antibiotics (Fig. 6).

Conclusions

We developed a μPAD combined with sensory PDA liposomes for facile and inexpensive biosensor applications. The hydrophilic circular dot array was fabricated on the chromatography paper by wax printing, and then the neomycin-sensitive PDA liposomes were embedded onto the hydrophilic region. We also demonstrated that the PDA liposomes in the paper substrates can be stabilized, showing intense blue colour, by the addition of hydrophilic reagents such as PVA. By combining the μPAD with the neomycin-sensitive PDA-PIP2 liposomes, we detected neomycin in both ways: measurement of the fluorescence signal and naked-eye detection of the blue-to-red colour change, and the detection limit was 1 ppm. Our system also had good selectivity to other antibiotics such as oxytetracycline, sulfamethazine, and penicillin G. We are convinced that the presented method can be readily applicable in the development of various other paper-based analytical devices using PDA liposomes.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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