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Zinc(II)-Dipicolylamine-Functionalized Polydiacetylene–Liposome Microarray: A Selective and Sensitive Sensing Platform for Pyrophosphate Ions

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Abstract: A microarray-chip assay system for the fluorescence detection of phosphate-containing analytes in aqueous media has been constructed from stimuli-responsive polymerized poly(diacetylene)-liposomes for the first time. Proper combination of the liposome components (Zn^{II}-dipicolylamine for phosphate binding and an amine-terminated component for anchoring the liposome onto an aldehyde-derivatized glass plate), has led to a microarray chip that selectively detects pyrophosphate, an important biomarker, over competing anions, such as phosphate and adenosine triphosphate, with nanomolar sensitivity. The chip-based assay shows advantages, such as high specificity and sensitivity, over solution-based assays that use the same liposomes, and over known homogeneous molecular sensing systems.

Keywords: liposomes · microreactors · pyrophosphate · sensors · zinc

Introduction

The development of a selective and sensitive detection method for pyrophosphate ions has been a subject of intense research effort because pyrophosphate ions are involved in a number of key biological processes, such as calcium–phosphate metabolism, adenosine triphosphate (ATP) hydrolysis, and DNA polymerization.[1] Among various approaches, fluorescence-sensing methods have received considerable attention owing to their versatile and sensitive features. In particular, fluorescent molecular probes based on zinc(II)-dipicolylamine (Zn(DPA)) ligands have received much attention[2] because the metal complexes show high affinity toward phosphate derivatives even in aqueous media, owing to the strong metal–phosphate coordination bonding. Although several notable fluorescence-sensing systems for pyrophosphate ions have been developed recently,[3] they are deficient either in the detection limit (usually at micromolar concentrations) or in their selectivity for pyrophosphate over competing derivatives such as phosphate and ATP. Furthermore, existing sensing systems are small-molecule-based probes, and thus operate only in a homogenous state. Heterogeneous sensing systems that contain multiple Zn(DPA) ligands remain to be explored even though they have potential for application in a chip-based assay. We became interested in such an "integrated" sensing system of Zn(DPA) ligands, which would facilitate a chip-based assay for phosphate-containing molecules. An interesting question that follows is whether the integrated Zn(DPA) system on a solid substrate would show sensing behavior similar to, or different from, that in solution.

The poly(diacetylene) (PDA) liposome system[4] would to be an ideal platform for introducing Zn(DPA) ligands onto a solid substrate with a signaling function. PDA liposomes are well known, as sensing platforms, for their stimuli-sensitive chromogenic and fluorogenic behavior. In solution, PDA liposomes show a color change from blue to red in response to various stimuli, such as heat and pH, ligand–receptor interactions, as the conjugation length of the polydiacetylene backbone is perturbed by the stimuli.[5] Importantly, the "red-phase" PDA liposomes show turn-on-type red fluorescence. The groups of Ahn and Kim fabricated PDA liposomes on solid substrates,[6] and demonstrated the potential of the liposome assembly on solid substrates as sensing platforms for the detection of cyclodextrins,[7a] protein–protein interactions,[7b] and ammonia.[7c] PDA–liposome-microarray systems with structurally more-elaborate ligands have appeared very recently that have demonstrated usefulness in

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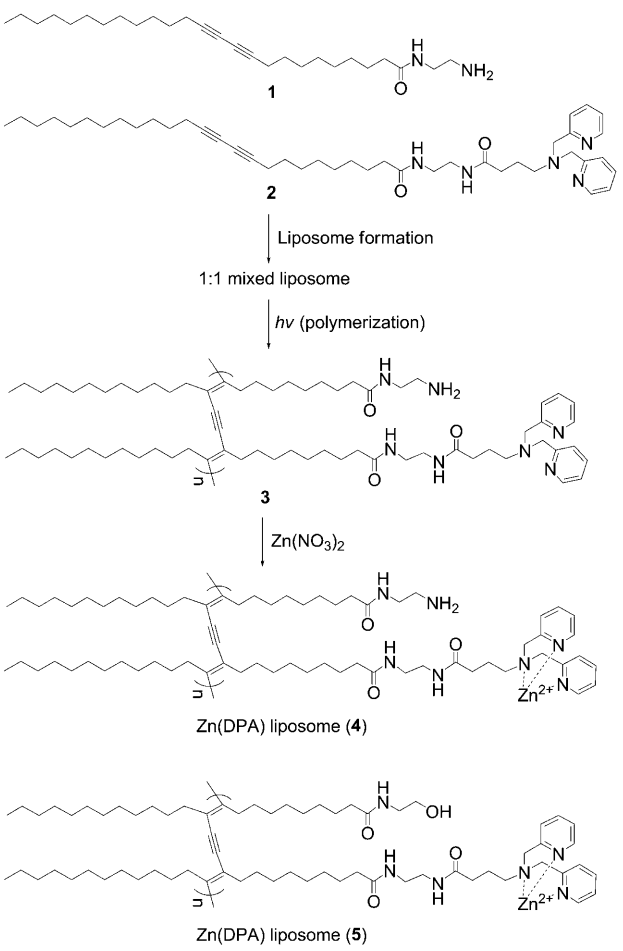
the detection of potassium ions,[8a] mercury ions,[8b] and anionic surfactants.[8c]

In our efforts to develop efficient molecular-sensing systems, particularly turn-on-type fluorescence-sensing systems for anions and metal cations,[9] we investigated microarray-chip systems based on PDA liposomes that allow use of fluorescence turn-on signaling for phosphate-containing molecules. Our goal is to develop chip-based-assay systems for analytes of biological interest, which might be developed into molecular-diagnosis tools for clinical purposes. Herein, we report the first chip-based fluorescence-sensing system for phosphate-containing molecules—specifically pyrophosphate ions—which shows unparalleled sensitivity and selectivity toward this important biomarker.

Results and Discussion

To realize a liposome-based assay system for phosphate-containing molecules, the liposomes must be constructed to function in solution and on a solid substrate. We have designed Zn(DPA)-containing liposome component **2** (Scheme 1) from diacetylenic amine **1**, which has two amide groups, and can thus provide two strands of intermolecular hydrogen bonds, and stabilize the liposome structure during fabrication. The synthesis of component **2** can be carried out using standard organic transformations. Details of these syntheses can be found in the Supporting Information.

Initially, we prepared a liposome solution solely from the Zn(DPA) component (**2**); however, the liposome system was unstable and readily underwent precipitation upon interaction with analytes. To solve the precipitation problem, we incorporated a second component as a dummy ligand for the solution assay, and as a linker for loading the liposomes onto a solid substrate. After several attempts, we found that component **1**, which contains an amine, fulfils all the requirements. We also found that introduction of the second component (**2**) was required for the detection of phosphate and pyrophosphate ions (see below), which indicates that the proper choice of liposome components can be a critical factor in realizing a desired functional liposome system.



Scheme 1. Synthesis of the polymerized dipicolylamine-containing liposome **3** from the liposome prepared from a 1:1 mixture of diacetylenic amine **1** and diacetylenic dipicolylamine **2**, and preparation of the corresponding polymerized Zn(DPA) liposome (**4**). Similarly, a liposome composed of a hydroxy-terminated component (**5**) has been synthesized. Only part of the chemical composition is shown for **3**, **4**, and **5**.

Thus, the formation of the mixed liposome has been optimized by changing the ratio of liposome components **1** and **2**; the optimal ratio for our purpose is 1:1. The mixed liposome thus prepared in solution was irradiated with UV light to obtain the corresponding polymerized liposome (**3**). **3** was treated with zinc nitrate to obtain the corresponding polymerized Zn(DPA) liposome (**4**) in solution, which was used for the solution study. The polymerized Zn(DPA) liposome solution was stable for months at 4 °C. The liposomes so prepared have size distributions in the range of 40–80 nm, as measured by scanning electron microscopy (SEM; see Figure 1) and dynamic light scattering analyses (see Figure S1 in the Supporting Information).

Sensing Studies in Solution

First, we evaluated the sensing behavior of **4** toward various anions such as chloride, bromide, nitrate, sulfate, perchlorate, azide, acetate, carbonate, phosphate, and pyrophos-

Abstract in Korean:

자극-감응성 디아세틸렌 리포솜 고분자로부터 수용액에서 인산 유도체를 형광 감지할 수 있는 마이크로어레이 칩 분석 시스템을 최초로 구현하였다. 리포솜 구성요소의 적절한 조합—인산 이온과 결합하는 Zn(II)-디피코릴아민 리간드와 그리고 알데히드 기로 처리된 유리판에 리포솜의 고정화를 위해서 아민 기를 가지는 두 구성 요소의 조합—을 통해서 중요한 생체 지표 화합물인 피로인산을 인산이나 ATP 와 같은 경쟁 화합물로부터 선택적으로 그리고 나노몰 농도 이하에서 검출할 수 있는 마이크로어레이 칩을 구성하였다. 이 칩 기반 분석법은 동일 리포솜을 이용한 용액상 분석법이나 기존의 균일상 분자 감지 계과 비교하여 높은 기질 선택성과 고감도를 보여 주었다.

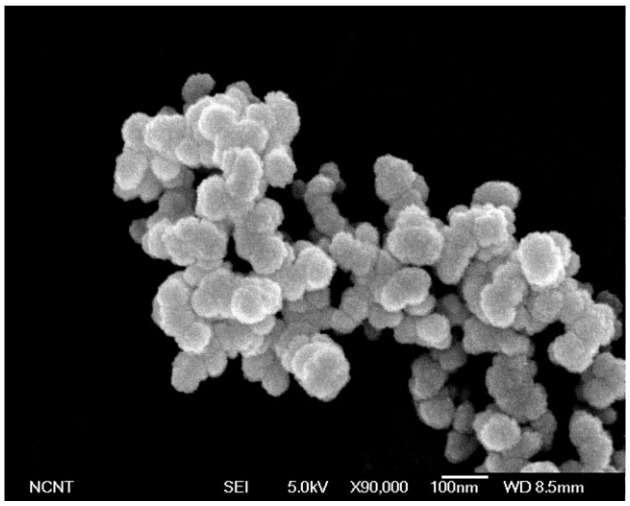


Figure 1. SEM images of the polymerized Zn(DPA) liposome (**4**) (× 90,000).

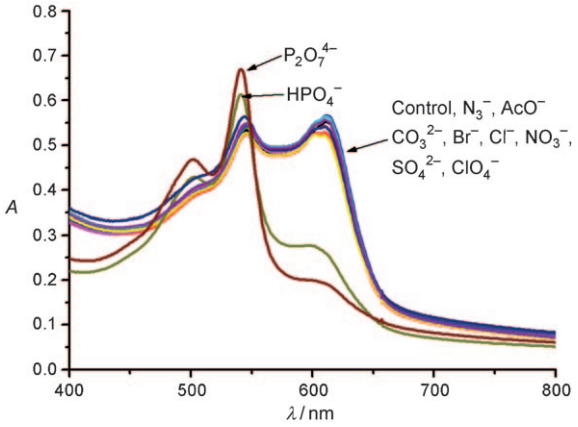


Figure 3. UV/Vis spectra of **4** (0.25 mM solution) upon addition of various anions ($P_2O_7^{4-}$, HPO_4^{2-}, control (**4** only), N_3^-, $CH_3CO_2^-$, CO_3^{2-}, Br^-, Cl^-, NO_3^-, and ClO_4^-) in a buffer solution (HEPES, pH 7).

phate (as their sodium salts). The sensing experiment was carried out by observing color and UV/Vis spectral changes in the liposome solution (1.0 mL, 0.25 mM, pH 7.0 HEPES buffer; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) upon addition of each anion solution (25 μL, 10 mM). The blue liposome solution became red–purple upon interaction with only phosphate or pyrophosphate; whereas no color change was observed with the other anions (Figure 2). The molecular interaction was complete

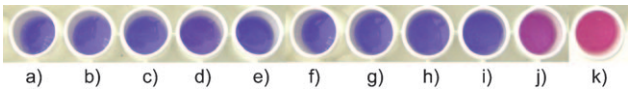


Figure 2. Photographs showing color changes of the Zn(DPA) liposome (**4**) solution (0.25 mM) upon addition of each anion as its sodium salt (0.25 mM, pH 7 HEPES buffer) after 30 min at 25 °C: a) buffer only, b) N_3^-, c) AcO^-, d) CO_3^{2-}, e) Br^-, f) Cl^-, g) NO_3^-, h) SO_4^{2-}, i) ClO_4^-, j) HPO_4^{2-}, and k) $P_2O_7^{4-}$ (pyrophosphate).

within 30 minutes, after which there was little change in absorbance or color. We also evaluated ATP and adenosine monophosphate (AMP), but these phosphate derivatives caused precipitation (even though color changes were observed), probably owing to the poor solubility of the liposomes bound with these anions. The color changes correspond to the spectral changes (Figure 3): the absorbance maxima at 602 and 612 nm, observed for the polymerized liposome solution, decreased drastically, whereas the absorbance maximum at 547 nm increased, to some extent, with a blueshift (541 nm). The observed analyte selectivity can be explained by evoking the strong affinity of the Zn(DPA) ligands toward phosphate and pyrophosphate anions. The color and thus absorbance changes observed suggest that the molecular interactions between the Zn(DPA) ligands and phosphate or pyrophosphate anions distort the polyenyne conjugation in the liposome.[4] The molecular interactions involving the Zn(DPA) ligands are further supported by the observation that the liposome prepared solely from **1** does not show any binding behavior (no color change) toward the phosphate anions; merely precipitation occurred.

A quantitative value for the extent of the blue to red color change is given by the colorimetric response,[7] which shows the high selectivity of the Zn(DPA) liposome **4** toward phosphate and pyrophosphate ions in solution (Figure 4).

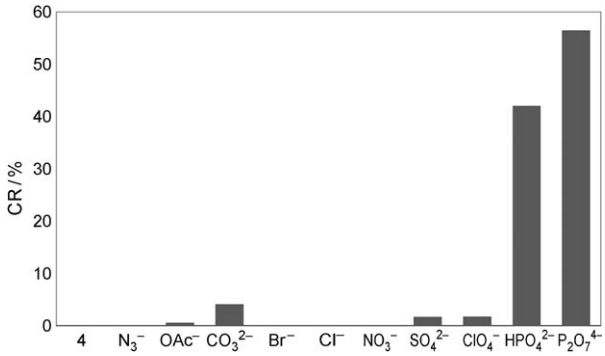


Figure 4. Colorimetric response (CR) of the Zn(DPA) liposome **4** solution toward various anions. $CR\% = [(PB_0 - PB_v)/PB_0] \times 100$, in which $PB = A_{blue}/(A_{blue}+A_{red})$, A is the absorbance at either the "blue" component in the UV/Vis spectrum (approx. 640 nm) or the "red" component (approx. 540 nm), PB_0 is the red/blue ratio of the control sample (before color change), and PB_v is the value obtained for the vesicle solution after the color change (A_{blue}: 540 nm, A_{red}: 612 nm).

Although several polydiacetylenic liposomes functionalized with different chemical ligands are known, this is the first example in which phosphate-binding Zn(DPA) ligands are incorporated into the polydiacetylenic liposome system to recognize and sense phosphate derivatives in solution.[10]

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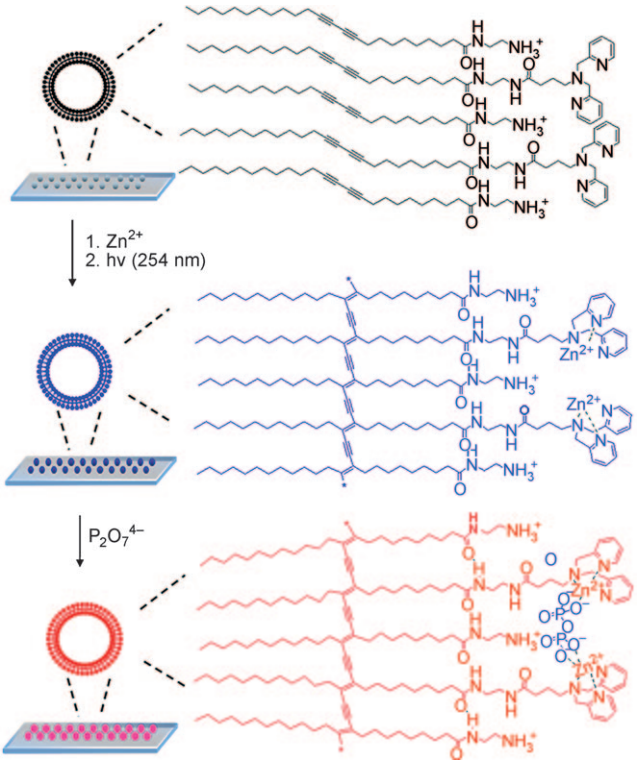
Sensing Studies on Microarray Chips

On the basis of the promising results from the solution study, we fabricated a microarray chip based on the Zn-(DPA) liposome. The preparation sequence is a little different from that followed for the preparation of the liposome solution. In this case, the 1:1 mixed liposome (from **1** and **2**) prepared in solution was spotted onto a glass substrate with terminal aldehyde groups, and then zinc-complex formation and polymerization between the diacetylene backbones were carried out. As has been previously observed,[6a] a blue to red color change was observed when the polymerized liposome (**3**), instead of the nonpolymerized liposome, was loaded onto the glass substrate through the aldehyde–amine reaction (imine formation). This means that chemical ligation of the polymerized liposome onto the glass substrate also causes distortion of the liposome backbone prior to interaction with analytes, which renders the chip system useless. Thus, we followed the previously reported sequence[6] to prepare the microarray chip as follows.

The liposome-microarray chip was prepared by noncontacted spotting of the liposome solution prepared from a 1:1 mixture of component **1** and **2** onto the aldehyde-modified glass slide (spot size ~200 nm in diameter). The spotted slide was stored at 80% humidity for 6 hours, and was then rinsed with deionized water and dried under a stream of nitrogen at ambient temperature. The incubation time was optimized by checking the fluorescence intensity for the final liposome chip prepared at various incubation times: if the incubation time was shorter than 6 hours, the fluorescence intensity was weaker; if it was longer (12, 24 h), there was little intensity change. The dried slide was dipped into a zinc nitrate solution (10 mM) for 30 minutes at room temperature, and was then rinsed with deionized water to remove uncomplexed zinc nitrates before being dried under a stream of nitrogen at ambient temperature. Finally, the slide was exposed to UV light (254 nm, 1 mW cm^{-2}) for 2 minutes to polymerize the diacetylene groups. This provided the desired blue-phase liposome-microarray chip (Scheme 2). The liposome-microarray chip shows the blue to red phase change upon addition of pyrophosphate ions (see below), which indirectly indicates that the liposomes on the glass substrate maintain their structural integrity during the chip-preparation steps. As mentioned above, the terminal amino group of **1** is necessary to covalently attach the mixed liposomes onto the aldehyde-terminated glass surface. In addition to this role, the amino group, in the ammonium form, seems to act as an auxiliary ligand in recognizing phosphate ions, along with the Zn(DPA) ligand, or to stabilize the liposome structure through hydrogen bonding: when we used a hydroxyl-terminated pentacosadiynoic acid derivative for the formation of mixed liposome **5** instead of the amine-terminated **1**, there was little color change upon addition of phosphate or pyrophosphate anions to the liposome solution.

We evaluated the fluorescence-sensing behavior of the microarray system toward the anions examined in the buffer



Scheme 2. Fabrication of the liposome-microarray chip and molecular interaction with pyrophosphate ion.

solution (pyrophosphate $P_2O_7^{4-}$, azide, acetate, carbonate, bromide, chloride, nitrate, sulfate, perchlorate, and phosphate; all as their sodium salts). We also evaluated ATP and AMP, which, in the above solution study, showed blue to red color changes, but also caused precipitation. The microarray chip was immersed into each anion solution (10 nM, pH 7 HEPES buffer) and incubated at room temperature for 6 hours. The fluorescence images were then analyzed with appropriate filters for excitation and emission lights. The data clearly show red fluorescence only in the case of pyrophosphate ions among the anions examined (Figure 5). This is in contrast to the solution assay, in which the Zn-(DPA) liposome responds to phosphate, pyrophosphate, ATP, and AMP (even though precipitation occurs in the latter two cases). The reason for the specific response of the microarray chip toward pyrophosphate anions over other phosphate derivatives is not clear; however, the results indicate that the liposomes on the chip seem to provide conformationally more rigid binding sites relative to the liposomes in solution. In case of such rigid binding sites, the pyrophosphate ion seems to fit the space between the two zinc sites, whereas other phosphate derivatives are too small or too large to be accommodated in it. The binding of pyrophosphate ions between two nearby Zn(DPA) groups distorts the conjugated enyne backbone; hence, the conjugation length becomes shorter, which results in the red fluorescent phase. Note that known homogeneous sensing systems for pyrophosphate ions[2] use 1,3-bis[Zn(DPA)]phenyl deriva-

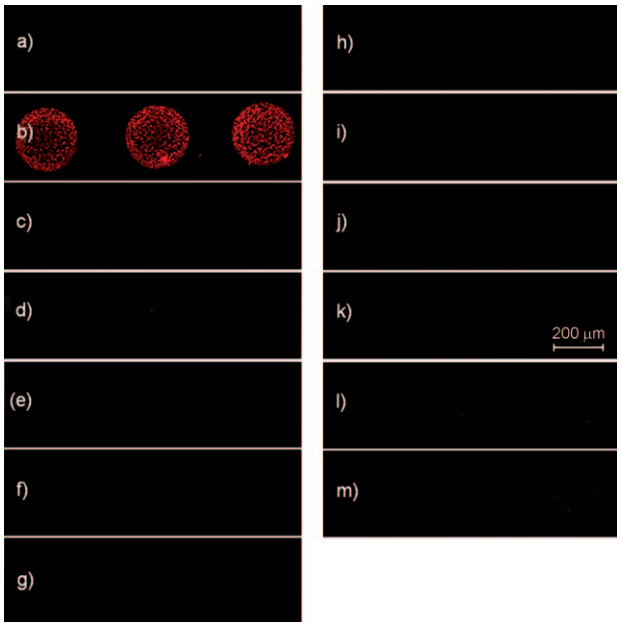


Figure 5. Fluorescence images of the liposome chip on interaction with various analytes: a) buffer only, b) $P_2O_7^{4-}$ (pyrophosphate), c) N_3^-, d) OAc^-, e) CO_3^{2-}, f) Br^-, g) Cl^-, h) NO_3^-, i) SO_4^{2-}, j) ClO_4^-, k) HPO_4^{2-}, l) ATP, and m) AMP. The images were taken after dipping the chip into each analyte solution (10 mM, pH 7 HEPES buffer) and incubating for 6 h at room temperature. Filters were used for excitation at 535±50 nm, and for emission at 610±75 nm. The diameter of each spot is about 200 nm.

tives as ligands to obtain selectivity over phosphate ions. In the present case, mono Zn(DPA) ligand is used; however, in its integrated form on the surface of the liposomes, specificity for pyrophosphate is achieved. Also, in our chip-based assay we can include analytes such as ATP and AMP, which precipitate in the solution assay, because such a solubility problem becomes irrelevant in the heterogeneous phase. This is an advantage of the chip-based assay over the solution-based assay.

Next, we evaluated the sensitivity of the microarray-chip system toward pyrophosphate ions by reducing the pyrophosphate concentration to 1 pM and analyzing the resultant fluorescence images. Red fluorescence spot images are clearly observable in the presence of pyrophosphate ions from the μM level to 1 nM range, and boundary spot images are observable even below that concentration (Figure 6). Even better sensitivity may be achieved by optimizing the spotting process; for example, simply by increasing the number of spotting. Such high sensitivity is not easily achieved with most known homogeneous sensing systems. A more significant advantage of the chip assay is its excellent selectivity toward pyrophosphate over other phosphate-containing molecules, which is rarely achieved with most known homogeneous sensing systems. The correlation curve between fluorescence intensity and pyrophosphate concentration is linear in the low nanomolar range (see the Supporting Information, Figure S2).

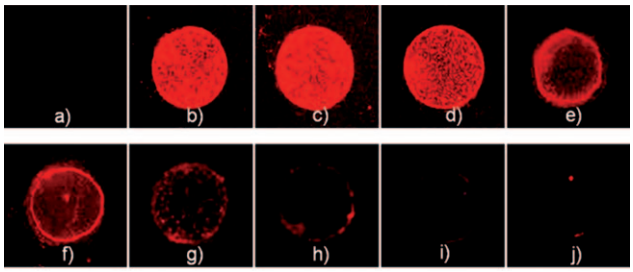


Figure 6. Fluorescence images of the liposome chip to pyrophosphate ($P_2O_7^{4-}$) ions at various concentrations: a) buffer only, b) 100 μM, c) 10 μM, d) 1 μM, e) 100 nM, f) 10 nM, g) 1 nM, h) 100 pM, i) 10 pM, and j) 1 pM. The images were taken after dipping the chip into each analyte solution (10 mM, pH 7 HEPES buffer) and incubating for 6 h at room temperature. Filters were used for the excitation (535±50 nm) and emission (610±75 nm). The diameter of each spot is about 200 nm.

Conclusions

We have fabricated the first microarray-chip system that detects pyrophosphate ions with unparalleled selectivity and sensitivity in aqueous media over competing phosphate derivatives and other anions. The chip system is constructed on a stimuli-responsive sensing platform of a PDA-based polymerized liposome that is structurally optimized from two components: a zinc(II)-dipicolylamine-functionalized component for binding phosphate ions; and an amine-terminated component, which acts as an auxiliary ligand, and also facilitates chemical ligation of the liposome onto a solid substrate. The microarray-chip system selectively responds to pyrophosphate ions among examined analytes; it shows apparent red fluorescence below 1 nM, in contrast to the solution assay with the liposome, which shows blue to red color changes toward phosphate, AMP, ATP, and pyrophosphate ions. The chip-based-assay system is promising for the detection of pyrophosphate ions, which play important roles in many biological processes; their in situ detection is currently under investigation.

Experimental Section

The synthesis of liposome components **1** and **2** is described in the Supporting Information.

Preparation of the Zn(DPA) Liposomes

A solution of **1** (2.1 mg, 5 μmol) and **2** (3.4 mg, 5 μmol) in chloroform (1 mL) was prepared in a vial from stock solutions of each component. The solvent was removed by a stream of Ar before the addition of deionized water (10 mL). The resulting suspension was sonicated for 20 min at 80 °C (with a Fisher Scientific sonic dismembrator, model 500) and then filtered through a cellulose acetate filter (0.8 μm) to remove dispersed lipid aggregates. The filtered solution was kept at 4 °C overnight to give a solution of the mixed liposome. An aqueous solution of $Zn(NO_3)_2$ (0.1 mL, 100 mM) was added to the liposome solution, and the resultant solution was exposed to 254 nm UV (1 mW cm^{-2}) for 2 min to give a solution of the polymerized Zn(DPA) liposome **4**, which was used for the solution assays below.

Detection of Anions Using the Liposome Solution

Solutions of azide, acetate, carbonate, bromide, chloride, nitrate, sulfate, perchlorate, phosphate, and pyrophosphate anions (as their sodium salts; 25 μL, 10 mM) were added at room temperature to a solution of **4** (1 mL, 250 μM), prepared as above. Photos showing the color change of each solution and UV/Vis spectra were taken within 30 min.

Preparation of the Zn(DPA) Liposome Microarray

The mixed-liposome solution prepared from a 1:1 mixture of components **1** and **2** (Scheme 1) was spotted onto an aldehyde-derivatized glass plate by using a manual microarrayer (Piezorray Perkin–Elmer LAS) with a pitch of 0.5 nm between spots. The printed slides were stored at room temperature for 6 h at 80% humidity. After being rinsed with deionized water and dried under a stream of N_2, the slides were dipped into an aqueous solution of $Zn(NO_3)_2$ (10 mM, 20 mL) for 30 min at room temperature. The slides were rinsed with deionized water and dried under a stream of N_2 before being exposed to 254 nm UV (1 mW cm^{-2}) for 2 min to afford the polymerized-liposome-microarray chips.

Detection of Pyrophosphate Ions with the Liposome Microarray Chip

A Zn(DPA) microarray chip prepared as described above was dipped into each analyte solution (pyrophosphate, azide, acetate, carbonate, bromide, chloride, nitrate, sulfate, perchlorate, phosphate, ATP, and AMP, as their sodium salts; 20 mL, 10 nM) and incubated for 6 h at room temperature. After being rinsed with deionized water, fluorescence images of the solutions were obtained by using a fluorescence microscope (Zeiss Axioplan 2). Similarly, the Zn(DPA)-microarray chip was incubated with a solution that contained varying concentrations of pyrophosphate ions (1 pM–100 μM: 2.0×10^{-11}–2.0×10^{-3} mol in 20 mL) for 6 h; images were obtained by using the fluorescence microscope. Quantitative fluorescence intensities from the dots were analyzed by using HCImage 1.0 software (Hamamatsu Corporation), and were repeated in four independent experiments.

Acknowledgements

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