Enzyme-responsive Fluorescent Ionic Liquid

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Herein, we describe the development of a novel material, “enzyme-responsive fluorescent ionic liquid”, which enabled a highly sensitive detection of alkaline phosphatase (ALP). We prepared a plasticized poly(vinyl chloride) (PVC) membrane using this new material as a plasticizer and quantified ALP in aqueous solutions. Preliminary results suggested that the PVC membrane responded to ALP at an interface between the membrane and the sample solution with anion extraction to maintain electroneutrality in the membrane phase. The developed PVC membrane showed an approximately six-times higher sensitivity than the conventional membrane, thereby demonstrating highly sensitive ALP detection. These results suggested the potential applicability of the proposed membrane for highly sensitive protein detection by using ALP-labeled antibodies.

Keywords Alkaline phosphatase, enzyme activity assay, ionic liquid, optical sensor, plasticized PVC membrane

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Plasticized poly(vinyl chloride) (PVC) membranes have been applied for the preparation of optical ion sensors (ion-selective optode). The response mechanism of an optode is based on the ion extraction equilibrium of an analyte between the organic phase (membrane) and the aqueous phase (sample solutions).¹⁻³ We have investigated the application of the functional PVC membrane to capillary-based micro-analytical devices that include ion sensing,^{4,5} enzyme biosensing,⁶ and enzyme immunoassay (EIA).⁷ Among these, a plasticized PVC membrane containing a fluorescent alkaline phosphatase (ALP) substrate facilitated single-step EIA by using a water-soluble membrane containing enzyme-labeled antibodies and a hydrogel membrane possessing the molecular sieving function.⁷ However, because the solubility of the dye in the plasticizer was low (40 mmol kg⁻¹), the sensitivity enhancement was limited.

On the other hand, functional ionic liquids (ILs) have gained attention as being smart materials because of their various characteristics such as photochromic,^{8,9} pH-sensitive,¹⁰⁻¹² temperature-sensitive,¹³⁻¹⁷ and electrochromic properties¹⁸⁻²⁰ and many other functions. Thus, ILs have been utilized as functional sensor materials in the field of analytical chemistry.²¹⁻²⁴ Recently, our group developed a plasticizer-free PVC membrane, which was composed of an ionic liquid-based dye (IL-Dye) in order to overcome a limitation of the dye solubility in the PVC membrane.²⁵⁻²⁷ The IL-Dye serves dual functions of a lipophilic pH indicator dye (chromophore) and a plasticizer. In the IL-Dye-containing membrane, an extremely high concentration of the dye was realized, making the absorbance sensitivity very high, although the membrane thickness was very thin (< 1 μm). In our previous studies, we achieved a highly sensitive absorbance detection of ions. However, therein, the detection target was limited to ions; thus, it was impossible to expand the

applicability of the IL-Dye to the detection of proteins or enzymes.

Herein, we describe how we developed an enzyme-responsive IL-Dye ([P₆₆₆₁₄]₂[12-PC]). This is a novel IL, which can serve as a fluorescent enzyme substrate and a plasticizer. [P₆₆₆₁₄]₂[12-PC] is composed of bulky lipophilic phosphonium cations and lipophilic fluorescent anions possessing an ALP-responsive phosphate group (Fig. 1(a)). By utilizing [P₆₆₆₁₄]₂[12-PC] as a

Fig. 1 (a) “Enzyme-responsive fluorescent ionic liquid” ([P₆₆₆₁₄]₂[12-PC]). (b) General concept of increasing the dye concentration in the PVC membrane based on “IL-Dye”. Highly-sensitive detection can be achieved using a much thinner membrane.

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Fig. 2 Comparison of the fluorescence response curves of $[P_{6614}]_2[12-PC]$ -based PVC membranes for Cl^- -containing samples and Cl^- -free samples.

plasticizer, the fluorescence sensitivity can be improved by considerably increasing the amount of dye in the PVC membrane, as shown in Fig. 1(b). Figure S1 (Supporting Information) shows the fluorescence response mechanism of a PVC membrane composed of $[P_{6614}]_2[12-PC]$, which involves the cleavage of phosphate groups from the substrate by an enzymatic reaction at the interface, and a subsequent extraction of anions to maintain the electroneutrality in the PVC membrane phase.

First, we synthesized $[P_{6614}]_2[12-PC]$ from the bulky lipophilic phosphonium IL ($[P_{6614}][Cl]$) and the lipophilic fluorescent coumarin derivative (12-PC) by ion exchange. We then obtained a light-yellowish liquid product. The detailed method of the synthesis and the 1H NMR data are included in Supporting Information. The response of $[P_{6614}]_2[12-PC]$ to ALP was confirmed by fluorescence spectroscopy. Figure S2 (Supporting Information) shows the typical solution-phase fluorescence spectra, indicating the response to ALP.

Next, a 340-nm-thick PVC membrane plasticized by $[P_{6614}]_2[12-PC]$ was prepared by spin-coating (Fig. S3(a), Supporting Information). For a comparison, the conventional 340-nm-thick PVC membrane composed of bis(2-ethylhexyl) sebacate (DOS) and 12-PC was prepared. The detailed preparation methods of these membranes are described in Supporting Information.

Furthermore, a PDMS microchannel array ($0.5\text{ cm} \times 1\text{ cm}$) was placed on the PVC membrane (Fig. S3(b)). The combination of the PDMS channel array with the PVC membrane allowed multiplex detections with the same membranes. After that, $0.2 - 2000\text{ U L}^{-1}$ ALP in the HEPES buffer (50 mmol L^{-1} , pH 9.0 (the optimum pH of ALP)), which contained 0 or 0.1 mol L^{-1} NaCl, were introduced into a PDMS microchannel array in parallel. NaCl was chosen as a model ionic species included in serum. Next, we acquired fluorescence images (Figs. S4(a) and S4(b), Supporting Information) and plotted the differences in the fluorescence intensities of the channels between $0.2 - 2000\text{ U L}^{-1}$ ALP and 0 U L^{-1} ALP at an 80 min reaction time (Figs. S5(a) and S5(b), Supporting Information). It should be noted that the 12PC was not leached out from the PVC membrane after 80 min, because of the high lipophilicity of the dye. This was more clearly confirmed by photographs

Fig. 3 Comparison of the fluorescence response curves of a $[P_{6614}]_2[12-PC]$ -based PVC membrane and the conventional PVC membrane containing 2 wt% of 12-PC.

shown in the graphical index where the photo was taken after soaking in an ALP sample for 80 min. Figure 2 shows the fluorescence response results, which indicate a higher fluorescence intensity for the sample solutions including NaCl than those without NaCl. These results suggested that $[P_{6614}]_2[12-PC]$ responded to ALP at the interface based on a mechanism involving the extraction of anions in order to maintain electroneutrality in the PVC membrane phase. These results also suggested that a swelling of the membrane did not strongly occur since the membrane components were very hydrophobic. Thus, ALP sample solutions containing 0.1 mol L^{-1} NaCl were used in following measurements in order to obtain a higher fluorescence intensity.

Finally, the fluorescence response of a PVC membrane consisting of $[P_{6614}]_2[12-PC]$ was quantitatively compared with that of the conventional membrane. The same ALP sample solutions containing 0.1 mol L^{-1} NaCl were introduced into PDMS microchannel arrays combined with the conventional PVC membrane. Fluorescence microscopy images (Fig. S4(c)) were analyzed and the differences in the fluorescence intensities were plotted in a manner identical to the former experiment (Fig. S5(c)). The limit of detection (LOD, defined by 3σ of the blank signal) of a $[P_{6614}]_2[12-PC]$ -based PVC membrane was 41.8 U L^{-1} , which was lower than that of the conventional PVC membrane (516 U L^{-1}). Further optimization of the experimental conditions would allow the $[P_{6614}]_2[12-PC]$ -based devices to show a lower LOD. By comparing the slope of the response curves shown in Fig. 3, the fluorescence intensities of the $[P_{6614}]_2[12-PC]$ -based PVC membrane exhibited an approximately six-times higher sensitivity than that of the conventional one in the concentration range of more than 20 U L^{-1} . The $[P_{6614}]_2[12-PC]$ -based PVC membrane contained *ca.* 600 mmol/kg dye, which was 15-times higher than the dye concentration in the conventional membrane (*ca.* 40 mmol/kg). Therefore, since the dye concentration per membrane-thickness extremely increased, a sensitivity enhancement was achieved. In this study, a 15-fold sensitivity enhancement was not achieved, possibly because of fluorescence quenching in the membrane due to a high concentration. However, these preliminary results indicated the potential applicability of the present approach for highly sensitive enzyme activity detection.

In conclusion, we developed a novel material, ALP-responsive fluorescent IL, which serves multiple functions of the enzyme-responsive fluorophore and the plasticizer. This IL can considerably increase the dye concentration in the PVC membrane, thereby facilitating a highly sensitive response. The application of this material to functional devices (such as array-type devices,²⁸ capillary-type devices,⁷ and nanoparticle-type devices^{29,30}) would lead to the development of sensitive and simple methods or devices for protein detection by using ALP-labeled antibodies.

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Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

References

1. E. Bakker, P. Bühlmann, and E. Pretsch, *Chem. Rev.*, **1997**, 97, 3083.
2. P. Bühlmann, E. Pretsch, and E. Bakker, *Chem. Rev.*, **1998**, 98, 1593.
3. G. Mistlberger, G. A. Crespo, and E. Bakker, *Annu. Rev. Anal. Chem.*, **2014**, 7, 483.
4. H. Hisamoto, Y. Nakashima, C. Kitamura, S. Funano, M. Yasuoka, K. Morishima, Y. Kikutani, T. Kitamori, and S. Terabe, *Anal. Chem.*, **2004**, 76, 3222.
5. H. Hisamoto, M. Yasuoka, and S. Terabe, *Anal. Chim. Acta*, **2006**, 556, 164.
6. E. Tsutsumi, T. G. Henares, K. Kawamura, T. Yao, and H. Hisamoto, *Chem. Lett.*, **2010**, 39, 436.
7. S. Funano, M. Sugahara, T. G. Henares, K. Sueyoshi, T. Endo, and H. Hisamoto, *Analyst*, **2015**, 140, 1459.
8. L. C. Branco and F. Pina, *Chem. Commun.*, **2009**, 6204.
9. J. Avó, L. Cunha-Silva, J. C. Lima, and A. J. Parola, *Org. Lett.*, **2014**, 16, 2582.
10. S. Das, P. K. S. Magut, S. L. de Rooy, F. Hasan, and I. M. Warner, *RSC Adv.*, **2013**, 3, 21054.
11. H. Enozawa, S. Ukai, H. Ito, T. Murata, and Y. Morita, *Org. Lett.*, **2019**, 21, 2161.
12. K. Y. Yung, A. J. Schadock-Hewitt, N. P. Hunter, F. V. Bright, and G. A. Baker, *Chem. Commun.*, **2011**, 47, 4775.
13. H. Rodríguez, M. Williams, J. S. Wilkes, and R. D. Rogers, *Green Chem.*, **2008**, 10, 501.
14. N. Aoyagi, K. Shimojo, N. R. Brooks, R. Nagaishi, H. Naganawa, K. V. Hecke, L. V. Meervelt, K. Binnemans, and T. Kimura, *Chem. Commun.*, **2011**, 47, 4490.
15. Y. Qiao, W. Ma, N. Theysen, C. Chen, and Z. Hou, *Chem. Rev.*, **2011**, 117, 6881.
16. Y. Kohno, S. Saita, Y. Men, J. Yuan, and H. Ohno, *Polym. Chem.*, **2015**, 6, 2163.
17. B. Monteiro, M. Outis, H. Cruz, J. P. Leal, A. T. Laia, and C. C. L. Pereira, *Chem. Commun.*, **2017**, 53, 850.
18. A. Branco, L. C. Branco, and F. Pina, *Chem. Commun.*, **2011**, 47, 2300.
19. A. Branco, J. Belchior, L. C. Branco, and F. Pina, *RSC Adv.*, **2013**, 3, 25627.
20. A. Kavanagh, K. J. Fraser, R. Byrne, and D. Diamond, *ACS Appl. Mater. Interfaces*, **2013**, 5, 55.
21. S. V. Muginova, D. A. Myasnikova, S. G. Kazarian, and T. N. Shekhovtsova, *Anal. Sci.*, **2017**, 33, 261.
22. K. Shimojo, *Anal. Sci.*, **2018**, 34, 1345.
23. M. Atanassova, H. Okamura, A. Eguchi, Y. Ueda, T. Sugita, and K. Shimojo, *Anal. Sci.*, **2018**, 34, 937.
24. W. Yoshida, F. Kubota, R. Kono, and M. Goto, *Anal. Sci.*, **2019**, 35, 343.
25. T. Mizuta, K. Sueyoshi, T. Endo, and H. Hisamoto, *Sens. Actuators, B*, **2018**, 258, 1125.
26. Y. Niwa, T. Mizuta, K. Sueyoshi, T. Endo, and H. Hisamoto, *Analyst*, **2019**, 144, 6858.
27. T. Mizuta, K. Sueyoshi, T. Endo, and H. Hisamoto, *Bunseki Kagaku*, **2019**, 68, 945.
28. T. Mizuta, K. Maeno, K. Sueyoshi, T. Endo, and H. Hisamoto, *Anal. Sci.*, **2018**, 34, 517.
29. X. Xie and E. Bakker, *Anal. Bioanal. Chem.*, **2015**, 407, 3899.
30. A. Shirai, K. Sueyoshi, T. Endo, and H. Hisamoto, *FlatChem*, **2019**, 13, 34.