Affinity diversification of a polymer probe for pattern-recognition-based biosensing using chemical additives

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Abstract

Pattern-recognition-based sensing has attracted attention as a promising alternative to conventional sensing methods that rely on selective recognition. Here, we report a novel strategy using chemical additives with the ability to modulate probe/analyte interactions to more easily construct pattern-recognition-based sensing systems for proteins and cells. The fluorescence of dansyl-modified cationic poly-L-lysine (PLL-Dnc) is enhanced upon binding to proteins in aqueous solution, while the addition of salts, inert polymers, or alcohols modulates the protein/PLL-Dnc interactions via a variety of mechanisms. Subsequent readout of the fluorescence changes produces response patterns that reflect the characteristics of the analytes. Multivariate analysis of the response patterns allowed accurate identification of not only eight structurally similar albumin homologues, but also four mammalian cells. This strategy, which uses inexpensive and common additives, significantly improves the accessibility of pattern-recognition-based sensing, which will offer new opportunities for the detection of various bioanalytes.

Keywords: biosensors, human cells, pattern recognition, polymers, proteins, chemical additives
Introduction

Protein sensing is of great importance in areas ranging from basic biological research to medical diagnosis. Most conventional sensing techniques are based on the ‘lock-and-key’ approach, in which proteins are detected using highly selective probe molecules such as antibodies and aptamers. However, in such methods, achieving high selectivity to prevent false recognition is costly and laborious due to the complex design and synthesis/production of suitable probe molecules. In contrast, sensing techniques that exploit characteristic patterns generated using non-selective interactions with proteins, i.e., ‘pattern-recognition-based sensing’, represent a promising alternative to selective strategies. Pattern-recognition-based sensing systems are generally constructed using an array of probe molecules that are capable of non-selectively interacting with the target proteins. The addition of the target proteins to the array and subsequent detection of optical signals from each probe molecule generate the characteristic optical response patterns. Accurate identification of proteins is possible via processing these response patterns using multivariate analysis.

In the pattern-recognition-based sensing of proteins, polymers and nanoparticles are often used as probe materials, as recognition units can be introduced onto these materials in high density, endowing them with the potential to interact strongly with the analytes at multiple points. These materials are covalently or non-covalently combined with dyes that exhibit color or fluorescence changes in response to their local environment, enabling information regarding the interactions to be converted into readable optical signals. Probe materials that meet these requirements are expected to be easier to produce than highly selective probes such as antibodies. Although such sensing systems could present potential advantages in terms of cost and labor, current mainstream approaches involve laborious syntheses of sets of probe materials into which different functional groups are introduced in order to generate response patterns unique to the
target proteins.

To reduce the synthetic effort required for pattern-recognition-based systems, methods to generate response patterns by modifying the aqueous environment wherein the probe molecules interact with the analytes have been proposed. For example, the pH value of an aqueous solution strongly affects the charge states and thus the electrostatic interactions of analytes and probe molecules. This allows the generation of response patterns with sufficient information to identify various analytes, ranging from metal ions\textsuperscript{10} to peptides\textsuperscript{11} and even complex beverages such as tea,\textsuperscript{12} with only a small number of probe molecules. We have recently reported a strategy for pattern-recognition-based sensing that combines modulation of the ionic strength and the pH value, in which a cationic poly-L-lysine modified with environmentally responsive dansyl groups (PLL-Dnc) was used as a probe molecule.\textsuperscript{13,14} The addition of salts weakens the electrostatic interactions between the target analytes and PLL-Dnc due to the electrostatic shielding on account of the increased ionic strength, which in turn increases the relative contributions of the other interaction factors. Thus, the system can successfully generate response patterns for identifying proteins and human-derived cell lines.

In the field of protein science, particular chemical compounds have long been known to modulate ‘soft-interfaces’ related to proteins,\textsuperscript{15} such as protein structures and interactions, via a variety of mechanisms.\textsuperscript{16-18} For example, alcohols can reduce the solubility of proteins, causing them to precipitate,\textsuperscript{19} or lower the dielectric constant of the solution, causing the proteins to denature.\textsuperscript{20} Inert hydrophilic polymers can enhance the thermal stability of proteins through crowding effects, which increase their resistance to denaturation, or favor the assembly of macromolecules, which promotes the formation of complexes, amorphous aggregates, and amyloid fibrils.\textsuperscript{21} The effect of salts is not limited to simply increasing the ionic strength. The Hofmeister series classifies ions based on their effect on the interfacial tension of the protein/water interface and preferential binding to proteins, e.g., thiocyanate ions destabilize protein
conformations and increase their solubility, whereas sulfate ions exhibit the opposite effect.\textsuperscript{22} In addition, we have shown that different additives exhibit various effects on the protein structures\textsuperscript{24} and on the complex formation between polyelectrolytes and proteins.\textsuperscript{16} Considering these findings, we hypothesized that the addition of such compounds to modulate protein structures and interactions could provide a new means to efficiently generate diverse protein-response patterns.

Here, we report the effects of various chemical additives on a pattern-recognition-based sensing system (Fig. 1). The addition of five different additives modulated the protein/PLL-Dnc interactions in various ways, altering the fluorescence response of PLL-Dnc to the proteins. When this strategy was applied to the identification of eight albumin homologues with very similar sequences and tertiary structures, multivariate analysis of the resulting response patterns provided highly accurate identification. In addition, this strategy was also capable of identifying human cell lines, which would potentially not only provide useful information about biological processes, but also enable cancer diagnosis through, e.g., tissue and liquid biopsy techniques. Our strategy, which does not rely on complex syntheses, but instead involves the simple addition of inexpensive compounds to generate response patterns, will provide new opportunities for pattern-recognition-based sensing for the identification of various biological analytes.
Fig. 1 Schematic representation of the additive-mediated pattern-recognition-based sensor. (a) Chemical structure of PLL-Dnc and the additives. (b) Modulation of protein/PLL-Dnc interactions by the additives to alter the fluorescence responses. (c) Resulting fluorescence response patterns and (d) identification of proteins by multivariate analysis.
Experimental

Materials

Poly-L-lysine hydrobromide (PLL) (degree of polymerization: 39; estimated by multiangle laser light scattering (MALLS)), 3-(N-morpholino)propanesulfonic acid (MOPS), 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (EPPS), 2-(N-morpholino)ethanesulfonic acid (MES), albumin from human serum (HSA), albumin from bovine serum (BSA), ovalbumin from chicken egg white (OVA), α-lactalbumin from bovine milk (BLA), lactosylated BSA (BSA-Lac; number of modifications: 15), and glucosylated HSA (HSA-Glu; number of modifications: 2) were obtained from Sigma Chemical Co. Sodium sulfate (Na₂SO₄), sodium thiocyanate (NaSCN), and 2,2,2-trifluoroethanol (TFE) were obtained from Fujifilm Wako Pure Chemical Ind. Poly(ethylene glycol) with an average molecular weight of 10000 (PEG) was obtained from Fluka. Dansyl chloride (Dnc-Cl) and 1,6-hexanediol (1,6-hex) were obtained from Tokyo Chemical Industry Co., Ltd. Cells from human breast carcinoma cell lines (MCF-7 and MDA-MB-453), the human cervix carcinoma cell line (HeLa), and the human Burkitt’s lymphoma cell line (HL-60) were obtained from RIKEN BioResource Center (Ibaraki, Japan). The PLL-Dnc polymer (4 out of 39 lysine units per side chain were modified with Dnc groups) was synthesized according to a previous report. The concentrations of the proteins were determined from the absorbance at 280 nm using a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) with extinction coefficients determined based on a previous report for 0.646 (mg/mL)⁻¹ cm⁻¹ (BSA and BSA-Lac), 0.518 (mg/mL)⁻¹ cm⁻¹ (HSA and HSA-Glu), 0.657 (mg/mL)⁻¹ cm⁻¹ (RSA), 0.687 (mg/mL)⁻¹ cm⁻¹ (PSA), 0.712 (mg/mL)⁻¹ cm⁻¹ (OVA), and 2.006 (mg/mL)⁻¹ cm⁻¹ (BLA).
**Preparation of cell suspensions**

Adherent cell lines (HeLa, MCF-7 and MDA-MB-453) were grown on a 10 cm-diameter cell culture dish (AGC Techno Glass Co.) in Dulbecco's modified eagle medium (DMEM; Wako Pure Chemical Ind.) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Science) and 1% of a penicillin-streptomycin-neomycin (PSN) antibiotic mixture (Thermo Fisher Scientific Inc.) at 37 °C in humidified air with 5% CO₂. The adherent cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Wako Pure Chemical Ind.), and subsequently treated with trypsin (Life Technologies). The harvested cells were suspended with chemically defined serum-free medium (CDCHO medium; Thermo Fisher Scientific Inc.) supplemented with 8 mM L-glutamine (Sigma Chemical Co.). A non-adherent cell line (HL-60) was grown in a T-25 Flask (Thermo Fisher Scientific Inc.) in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% FBS and 1% PSN at 37 °C in humidified air with 5% CO₂. The cells were washed with DPBS, and subsequently suspended with CDCHO supplemented with 8 mM L-glutamine.

**Fluorescence measurements**

Fluorescence measurements of PLL-Dnc/protein mixtures in the presence of various concentrations of the additives were performed using a Cytation5 Imaging Reader (BioTek Instruments, Inc.). To each well of a 96-well microplate (96 Well Black Flat-Bottom Polystyrene NBS Microplates; Corning Inc.), 200 μL of a mixture of 4 μg/mL PLL-Dnc and 20 μg/mL HSA in 25 mM MOPS (pH = 7.5) with or without one of the additives (0-40 v/v% TFE; 0-25 w/v% 1,6-hex; 0-10 w/v% PEG; 0-0.5 M Na₂SO₄; 0-0.5 M NaSCN) was added. After incubation at 35 °C for 10 min, the fluorescence intensity was recorded at 35 °C for 10 min using excitation/emission wavelengths (λ_ex/λ_em) of 340 nm/520 nm.
Pattern-recognition-based sensing

Aliquots of 160 μL of (i) buffer solutions [25 mM MOPS (pH = 7.5), 25 mM EPPS (pH = 8.5), and 25 mM MES (pH = 5.5)] or (ii) 25 mM MOPS (pH = 7.5) with different additives (the final concentrations of additives were as follows: 10 or 20 v/v% TFE; 25 w/v% 1,6-hex; 5 w/v% PEG; 0.1 M Na₂SO₄; 0.1 M NaSCN) were loaded into each well of the 96-well plates using a PIPETMAX system (Gilson Inc.), followed by the addition of 20 μL of 40 μg/mL PLL-Dnc in deionized water. After incubation at 35 °C for 10 minutes, the fluorescence intensities were measured (λ_{ex}/λ_{em}: 340 nm/520 nm). Subsequently, 20 μL aliquots of 200 μg/mL solutions of the proteins were added to each well, and the fluorescence intensities were recorded again after 10 minutes of incubation at 35 °C. For the human cell lines, the final concentrations of PLL-Dnc and the cell line were 0.2 μg/mL and 2.0×10⁴ cells/mL, respectively. The measurement process was repeated six times to generate a training data matrix, which was then processed using linear discriminant analysis (LDA) in SYSTAT 13 (Systat Inc.). For a blind test, the same measurement process was repeated six times to generate a test data matrix. The test data were classified into the groups generated by the training matrix according to their shortest Mahalanobis distances.

Results and Discussion

PLL-Dnc was chosen as a model probe material to examine whether unique protein response patterns could be effectively generated using additives that modulate the interactions between the proteins and probe materials. As PLL-Dnc contains positively charged amino groups (pKa ≈10.5), hydrophobic n-butyl and dansyl groups, as well as amide bonds with hydrogen-bonding ability, it can bind to proteins at multiple points through a variety of interactions. The dansyl group exhibits only weak fluorescence in aqueous solution, while its fluorescence increases with decreasing polarity of the surrounding environment, e.g., when bound to
Due to these properties, the interaction mode of PLL-Dnc was expected to vary in the presence of the additives, resulting in changes in fluorescence intensity.

To modulate the interactions of PLL-Dnc with proteins, we selected three classes of additives (Fig. 1): salts, an inert hydrophilic polymer, and alcohols. The addition of the chosen salts (NaSCN and Na\(_2\)SO\(_4\)) should weaken the electrostatic interactions between PLL-Dnc and proteins through electrostatic shielding. In addition, these two salts have different effects on the dissolution state of proteins. Namely, the chaotropic salt NaSCN reduces the interfacial tension between water and the protein and consequently inhibits protein association, whereas the cosmotropic salt Na\(_2\)SO\(_4\) does the opposite.\(^{22}\) The hydrophilic polymer PEG, which is generally inert toward proteins, stabilizes more compact states, such as complexes and aggregates, via a crowding effect.\(^{21}\) Increasing alcohol concentrations strengthen the electrostatic interactions due to the decrease in the dielectric constant of the aqueous solution. Furthermore, TFE promotes the formation of α-helix structures in proteins,\(^{27}\) while 1,6-hex inhibits hydrophobic interactions.\(^{28}\) Using these five additives, as well as the buffer solutions with different pH values reported previously for comparison,\(^{13}\) we investigated the effects of the additives on pattern-recognition-based protein sensing.

First, the fluorescence intensity of PLL-Dnc was measured after the addition of different concentrations of the additives to mixed solutions of PLL-Dnc and HSA (Fig. 2). Similarly to in the previous report,\(^{13}\) the fluorescence intensity increased 3.6-fold when 25 \(\mu\)g/mL HSA was mixed with 4 \(\mu\)g/mL PLL-Dnc in 20 mM MOPS (pH = 7.5). The addition of additives modulated the fluorescence response of PLL-Dnc depending on the type of compound and its concentration. For example, the fluorescence response decreased gradually with increasing concentrations of Na\(_2\)SO\(_4\), NaSCN, and 1,6-hex (Figs. 2a-c). In the presence of 0.25 M Na\(_2\)SO\(_4\), the increase in the fluorescence intensity of PLL-Dnc due to binding with HSA was almost negligible (Fig. 2a), while the addition of more than 0.25 M NaSCN reduced the fluorescence intensity to a level lower than
that observed for PLL-Dnc alone in aqueous solution (Fig. 2b). The decreased fluorescence in the presence of the salts is presumably due to weakening of the electrostatic interactions between PLL-Dnc and HSA. NaSCN probably suppresses even the interactions between PLL-Dnc molecules or affects the polarity of the surrounding environment of the dansyl groups, resulting in the decreased fluorescence intensity compared to that of PLL-Dnc alone. 1,6-Hex reduced the fluorescence intensity by 70% (Fig. 2c), which suggests that not only electrostatic but also hydrophobic interactions contribute substantially to the binding between HSA and PLL-Dnc. In contrast, PEG increased the fluorescence response at concentrations above 2 w/v% (Fig. 2d). This was attributed to the crowding effect, which promotes HSA/PLL-Dnc binding. Interestingly, in the case of TFE, the fluorescence response decreased at concentrations of up to 8 v/v%, increased at 20 v/v%, and then decreased again at 40 v/v% (Fig. 2e). Although these complex changes are difficult to fully understand, they might be partially explained by considering the effects of both a decreased polarity of the solution and changes in the protein conformation on the interactions; i.e., the interactions may have been weakened due to structural changes in HSA at concentrations of up to 8 v/v% TFE, while at higher concentrations the electrostatic interactions between PLL-Dnc and HSA may have increased due to the decreased dielectric constant of the solution. Overall, these results show that the fluorescence responses, which reflect the binding of PLL-Dnc and HSA, can be easily modulated to different extents using additives that alter their interaction modes.
Fig. 2 Effect of additives on the enhancement of the PLL-Dnc fluorescence in the presence of HSA. Changes in the fluorescence intensity of 4 µg/mL PLL-Dnc and 20 µg/mL HSA upon addition of different concentrations of (a) Na$_2$SO$_4$, (b) NaSCN, (c) 1,6-hex, (d) PEG, and (e) TFE in 25 mM MOPS (pH = 7.5); λ$_{ex}$/λ$_{em}$: 340 nm/520 nm. Values shown represent mean values ± standard deviation ($n = 3$).

Subsequently, we attempted to use the additive-induced changes in the protein/PLL-Dnc interactions for pattern-recognition-based protein sensing. In this study, six albumins from various sources were selected as target proteins (Table 1) in order to ensure comparability with previous reports. Given that the four serum albumins are nearly 70% identical in sequence and that the false detection of these albumins by the immune system can cause allergic reactions, their discrimination is suitable for evaluating the performance of the developed sensing system. In addition, we used two serum albumins with non-enzymatic glycation (HSA-Glu and BSA-Lac), which is regarded as a biomarker for diabetes and potentially for neurodegenerative diseases.

For the sensing procedure, each albumin (20 µg/mL) was mixed with PLL-Dnc (4 µg/mL) in 25 mM MOPS (pH = 7.5) with or without one of the five additives, 25 mM EPPS (pH = 8.5), or 25 mM MES (pH = 5.5) in a 96-well plate. The concentration of each compound was chosen so that the change in fluorescence intensity would be sufficiently high (Fig. 2). For the two salts, concentrations where the fluorescence responses did not completely disappear were chosen. The fluorescence signals were recorded as $I/I_0$ values with λ$_{ex}$/λ$_{em}$ = 340 nm/520 nm, generating a
dataset of 9 solution conditions × 8 albumins × 6 replicates.

| protein                  | abbr. | pI  | Mw  |
|--------------------------|-------|-----|-----|
| bovine milk albumin      | BLA   | 4.8 | 14,000 |
| egg white albumin        | OVA   | 5.2 | 43,000 |
| bovine serum albumin     | BSA   | 5.6 | 68,000 |
| human serum albumin      | HSA   | 5.7 | 67,000 |
| rabbit serum albumin     | RSA   | 5.7 | 66,000 |
| porcine serum albumin    | PSA   | 5.8 | 67,000 |
| lactosylated BSA         | BSA-Lac | -  | -  |
| glucosylated HSA         | HSA-Glu | -  | -  |

In the absence of the additives at pH = 7.5, all albumins increased the fluorescence intensity of PLL-Dnc (Fig. 3; for raw data, see Table S1). As expected, the additives had different effects on the responses. For BLA and OVA, both tested concentration of TFE (10 v/v% and 20 v/v%) increased the fluorescence intensity, while other albumins, including HSA, showed decreased fluorescence in the presence of 10 v/v% TFE and increased fluorescence at 20 v/v%, suggesting that BLA and OVA are different from the other albumins in terms of the effect of TFE on their interaction with PLL-Dnc or on their tertiary structures. However, the effects of other compounds differed even between BLA and OVA; the response of BLA was almost negligible in the presence of both salts, whereas OVA retained some fluorescence intensity. Overall, the effects of the additives varied depending on the type of protein, and could not simply be predicted. This property should be advantageous for obtaining information related to various aspects of the proteins from their response patterns.
Fig. 3 Patterns of changes in the fluorescence intensity upon addition of various albumins (20 µg/mL) to an array consisting of 4 µg/mL PLL-Dnc in 25 mM MOPS (pH = 7.5) without additives or one of the five additives, 25 mM EPPS (pH = 8.5), or 25 mM MES (pH = 5.5). Values shown represent mean values ± standard deviation (n = 6).

To visualize the statistical differences between the albumin responses, the fluorescence patterns were subjected to linear discriminant analysis (LDA), which is a representative pattern recognition algorithm that provides a graphical output that offers insight into the clustering of the data, i.e., the classification ability. The percentages shown on each axis indicate the extent to which each discriminant score explains the features (Fig. 4a), i.e., the first discriminant score [score (1)], which provided the best discrimination among the classes, accounted for 75.2% of the total variance, while score (2) comprised the second highest degree of variance at 18.9%. In the plot of these two scores, the individual points represent the response patterns of a single analyte. The clusters corresponding to each albumin were well separated, suggesting that distinct response patterns were obtained for structurally similar serum albumins as well as the glycated albumins via the modulation of the probe/protein interactions by the additives. In particular, the differentiation of non-enzymatic glycation of albumin associated with diseases is medically significant.
Discriminant score plots for various albumins (20 µg/mL) under (a) all conditions and (b) two selected conditions [MOPS (pH = 7.5) with Na\textsubscript{2}SO\textsubscript{4} or without any additives], obtained from the array consisting of 4 µg/mL PLL-Dnc. Ellipsoids represent the confidence interval (±1 standard deviation) for each analyte.

The fluorescence patterns were then subjected to a principal component analysis (PCA). PCA is a so-called unsupervised pattern recognition algorithm that unbiasedly concentrates pattern information within a dataset in a lower dimensional space in a way that the most significant characteristics of multidimensional data are preserved. A PCA score plot showed that most clusters were separated, while a slight overlap between clusters of HSA/BSA and PSA/HSA-Glu were observed (Fig. S1). Thus, we have demonstrated the ability of our system to recognize the differences between these albumins.

Two cross-validation methods were then used to determine the identification accuracy of the array: A leave-one-out cross-validation (LOOCV) test (the so-called ‘jackknife test’) and a holdout test. In both methods, the dataset is divided into ‘training data’ to create a model for identification and ‘test data’ for evaluating the constructed model. In the LOOCV test, one response pattern is removed from the dataset (n = 12) and treated as test data, while the remaining patterns are used as training data. This procedure is repeated for all the data. In the holdout test,
the training data \((n = 6)\) and test data \((n = 6)\) are fixed. In both methods, the test data are assigned to clusters based on the nearest Mahalanobis distances, which are calculated using the training data. The accuracy is determined based on whether the analyte in the test data is assigned to the correct albumin. Analysis of the nine-dimensional response patterns provided 100% classification accuracy in both tests, demonstrating that the developed system can discriminate the eight albumins with high accuracy. In the case of a previously reported system in which only the pH value was changed\(^{13}\), the holdout test afforded 94% accuracy (Table S2 and Fig. S2). Therefore, the identification accuracy can easily be improved by combining the pH-focused method with the new strategy using various additives.

For practical use, it is preferable to construct a small sensor system that can achieve sufficient accuracy. Therefore, the different solution conditions were analyzed to determine which could be effectively paired with the additive-free data (Table S3). High accuracy was found to be achieved when the \(\text{Na}_2\text{SO}_4\) data was combined with the additive-free results (100% in the LOOCV test and 98% in the holdout test), despite using only two-dimensional response patterns (Fig. 4b). Thus, our present strategy will also serve to expand the pool of response patterns that are candidates for constructing small sensor systems.

To examine the versatility of pattern-recognition-based sensing using additives, we attempted to apply our system to the identification of human cells. As with protein sensing, cell identification techniques are crucial in research areas ranging from basic biological research to medical diagnosis. Conventionally, methods using antibodies as cellular biomarkers have been used, but biomarker expression is often challenging as it is regulated by various cellular processes, and detection of a limited number of biomarkers is inconclusive. As the molecular composition of the cell surface reflects a variety of cellular conditions, such as cancer\(^{34,35}\) and metabolic diseases,\(^{36}\) pattern-recognition-based cell sensing that can recognize complex characteristics of the cell surface has also attracted attention in such cell identification.\(^{37}\) We investigated four
human cancer cell lines from different sources, namely, HeLa (cervix), HL-60 (circulatory system), MDA-MB-435 (breast, more-aggressive subtype), MCF-7 (breast, less-aggressive subtype). Interestingly, the effects of the additives on the fluorescence response of PLL-Dnc for each cell line were different from those for albumins. For example, while NaSCN decreased the fluorescence intensity of all the cell lines and all albumins, Na$_2$SO$_4$ did not have a marked effect on the cells (Fig. 5a; for the raw data, see Table S5). In addition, PEG decreased the fluorescence intensity of the cells, despite causing only slight changes to that of the albumins. These results suggest that the effects of the additives on the cell/PLL-Dnc interactions or cellular states are different from those on the albumins. Cell-surface components include phospholipids, membrane proteins, and sulfonated glycosaminoglycans. As many of these components are negatively charged and/or hydrophobic, PLL-Dnc can interact with them at multiple points through electrostatic and hydrophobic interactions. Thus, the generated responses reflect overall information on the entire cell surface, and the addition of additives may have modulated these individual interactions.

Fig. 5 Discrimination of human cancer cell lines using pattern-recognition-based sensing mediated by additives. (a) Patterns of changes in the fluorescence intensity upon addition of human cancer cell solutions ($2.0\times10^4$ cells/mL) to an array consisting of 0.2 µg/mL PLL-Dnc in
25 mM MOPS (pH = 7.5) without additives or one of the five additives, 25 mM EPPS (pH = 8.5), or 25 mM MES (pH = 5.5). Values shown represent mean values ± standard deviation (n = 6). (b) Discriminant score plot for various human cancer cell lines using the four selected conditions [MOPS (pH = 7.5) with 20 v/v% TFE, 5 w/v% PEG, and 0.1 M Na₂SO₄, and MES (pH = 5.5)] from the PLL-Dnc array. Ellipsoids represent the confidence interval (±1 standard deviation) for each analyte.

Analysis of response patterns using LDA provided 92% accuracy in both the LOOCV and the holdout tests when the full nine-dimensional dataset was used (Table S5 and Fig. S3), while the accuracy of the test improved to 96% using selected conditions (Table S6). Using these selected conditions, distinct clusters of cells were observed in a three-dimensional LDA plot (Fig. 5b).

In pattern-recognition-based sensing, the control of probe/analyte interactions via changes in pH and ionic strength has been used to simplify the construction of sensing systems for proteins\textsuperscript{13} and cells.\textsuperscript{14} In this study, we have successfully demonstrated that this method can be further extended for the sensing of proteins and human cells by adding various classes of additives. Even though the effects of additives on protein structure are significant,\textsuperscript{16-18} the effects of additives on pattern changes appear to be limited. In the case of cells in particular, the fluorescence responses did not vary dramatically with the type of additives, although the cell membrane is composed of various components. The efficacy can possibly be improved by careful selection of the additives and polymers. As for additives, for example, the present results showed that Na₂SO₄, a salt with a salting-out effect,\textsuperscript{22} TFE, an alcohol that causes a change of the dielectric constant\textsuperscript{19} and transition in secondary structure,\textsuperscript{27} and PEG, an inert hydrophilic polymer with a crowding effect,\textsuperscript{21} have characteristic effects. In addition, the use of different classes of additives, such as denaturants and surfactants, may also be useful. Additives may also exhibit more pronounced
effects by choosing polymers with different structures that have enhanced hydrophobicity or additional hydrogen bonding ability.

Conclusions

In summary, we have shown that the use of chemical additives with the ability to modulate probe/analyte interactions allows obtaining increased response information in pattern-recognition-based sensing without increased synthetic effort. The addition of chaotropic/cosmotropic salts, an inert hydrophilic polymer, or alcohols to mixed solutions of PLL-Dnc and the target analytes induced significant changes in the fluorescence response of PLL-Dnc in an analyte-specific manner. This strategy produced characteristic response patterns for the analytes and enabled accurate discrimination of eight structurally similar albumins and four human cell lines via multivariate analysis. In addition to the additives used in this study, other compounds with different functionalities are commercially available at low cost, and their use can be expected to easily facilitate a further expansion of the candidate response pool. Furthermore, we have shown that minimal sensing systems can be constructed for target proteins and cells by selecting appropriate additive compounds. Therefore, we are convinced that our additive-based strategy will lead to the construction of small but effective sensing systems for a variety of biological analytes, which will expand the potential of pattern-recognition-based sensing.

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References

1. Y. Y. Broza, X. Zhou, M. Yuan, D. Qu, Y. Zheng, R. Vishinkin, M. Khatib, W. Wu, and H. Haick, *Chem. Rev.*, **2019**, *119*, 11761.

2. C. Solier and H. Langen, *Proteomics*, **2014**, *14*, 774.

3. H. Xiong, J. Yan, S. Cai, Q. He, D. Peng, Z. Liu, and Y. Liu, *Int. J. Biol. Macromol.*, **2019**, *132*, 190.

4. Z. Li, J. R. Askim, and K. S. Suslick, *Chem. Rev.*, **2019**, *119*, 231.

5. S. Tomita, S. Ishihara, and R. Kurita, *ACS Appl. Mater. Interfaces*, **2019**, *11*, 6751.

6. N. D. B. Le, A. K. Singla, Y. Geng, J. Han, K. Seehafer, G. Prakash, D. F. Moyano, C. M. Downey, M. J. Monument, D. Itani, U. H. F. Bunz, F. R. Jirik, and V. M. Rotello, *Chem. Commun.*, **2019**, *55*, 11458.

7. A. Bigdeli, F. Ghasemi, H. Golmohammadi, S. Abbasi-Moayed, M. A. F. Nejad, N. Fahimi-Kashani, S. Jafarinejad, M. Shahrajabian, and M. R. Hormozi-Nezhad, *Nanoscale*, **2017**, *9*, 16546.

8. S. Xu, T. Gao, X. Feng, X. Fan, G. Liu, Y. Mao, X. Yu, J. Lin, and X. Luo, *Biosens. Bioelectron.*, **2017**, *97*, 203.

9. S. Pandit, T. Banerjee, I. Srivastava, S. Nie, and D. Pan, *ACS Sens.*, **2019**, *4*, 2730.

10. X. Zhou, J. Nie, and B. Du, *ACS Appl. Mater. Interfaces*, **2017**, *9*, 20913.

11. S. A. Minaker, K. D. Daze, M. C. F. Ma, and F. Hof, *J. Am. Chem. Soc.*, **2012**, *134*, 11674.

12. B. Wang, J. Han, M. Bender, S. Hahn, K. Seehafer, and U. H. F. Bunz, *ACS Sens.*, **2018**, *3*, 504.

13. S. Tomita, S. Ishihara, and R. Kurita, *ACS Appl. Mater. Interfaces*, **2017**, *9*,
22970.

14. H. Sugai, S. Tomita, S. Ishihara, and R. Kurita, *ACS Sens.*, **2019**, 4, 827.

15. M. Maeda, A. Takahara, H. Kitano, T. Yamaoka, and Y. Miura, “*Molecular Soft-Interface Science*”, **2019**, 1st ed., Springer, Tokyo.

16. M. Mimura, K. Tsumura, A. Matsuda, N. Akatsuka, and K. Shiraki, *J. Chem. Phys.*, **2019**, 150.

17. H. Hamada, T. Arakawa, and K. Shiraki, *Curr. Pharm. Biotechnol.*, **2009**, 10, 400.

18. T. Arakawa, S. J. Prestrelski, W. C. Kenney, and J. F. Carpenter, *Adv. Drug Deliv. Rev.*, **2001**, 46, 307.

19. H. Yoshikawa, A. Hirano, T. Arakawa, and K. Shiraki, *Int. J. Biol. Macromol.*, **2012**, 50, 865.

20. N. Hirota, K. Mizuno, and Y. Goto, *J. Mol. Biol.*, **1998**, 275, 365.

21. H. X. Zhou, G. Rivas, and A. P. Minton, *Annu. Rev. Biophys.*, **2008**, 37, 375.

22. Y. Zhang and P. S. Cremer, *Curr. Opin. Chem. Biol.*, **2006**, 10, 658.

23. C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, and T. Gray, *Protein Sci.*, **1995**, 4, 2411.

24. K. Shiraki, S. Tomita, and N. Inoue, *Curr. Pharm. Biotechnol.*, **2015**, 17, 116.

25. O. Hayashida, N. Ogawa, and M. Uchiyama, *J. Am. Chem. Soc.*, **2007**, 129, 13698.

26. Y. De Zhuang, P. Y. Chiang, C. W. Wang, and K. T. Tan, *Angew. Chem. Int. Ed.*, **2013**, 52, 8124.

27. K. Shiraki, K. Nishikawa, and Y. Goto, *J. Mol. Biol.*, **1995**, 245, 180.

28. D. L. Updike, S. J. Hachey, J. Kreher, and S. Strome, *J. Cell Biol.*, **2011**, 192, 939.
29. M. Chruszcz, K. Mikolajczak, N. Mank, K. A. Majorek, P. J. Porebski, and W. Minor, *Biochim. Biophys. Acta*, 2013, 1830, 5375.

30. J. Vicente-Serrano, M. L. Caballero, R. Rodríguez-Pérez, P. Carretero, R. Pérez, J. G. Blanco, S. Juste, and I. Moneo, *Pediatr. Allergy Immunol.*, 2007, 18, 503.

31. C. E. Guthrow, M. A. Morris, J. F. Day, S. R. Thorpe, and J. W. Baynes, *Proc. Natl. Acad. Sci. U. S. A.*, 1979, 76, 4258.

32. Y. Wei, L. Chen, J. Chen, L. Ge, and R. Q. He, *BMC Cell Biol.*, 2009, 10, 1.

33. P. Anzenbacher, P. Lubal, P. Bucek, M. A. Palacios, and M. E. Kozelkova, *Chem. Soc. Rev.*, 2010, 39, 3954.

34. D. W. Kufe, *Nat. Rev. Cancer*, 2009, 9, 874.

35. M. N. Christiansen, J. Chik, L. Lee, M. Anugraham, J. L. Abrahams, and N. H. Packer, *Proteomics*, 2014, 14, 525.

36. J. W. Dennis, I. R. Nabi, and M. Demetriou, *Cell*, 2009, 139, 1229.

37. H. Sugai, S. Tomita, and R. Kurita, *Anal. Sci.*, 2020, 36, 923.
Graphical Index

Additive-mediated generation of characteristic patterns