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Precise Proton Mapping near Ionic Micellar Membranes with Fluorescent Photoinduced-Electron-Transfer Sensors

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Abstract: One of the challenges for fluorescent sensors is to reduce their target environment size from a micrometer scale, such as biological cells, to a nanometer scale. Proton maps near membranes are of importance in bioenergetics and are the first goal in nanometer-scale analysis with fluorescent sensors. Thirty-three fluorescent photoinduced-electron-transfer pH sensors bearing an environment-sensitive benzofurazan fluorophore and having different hydrophobicity/hydrophilicity and hydrogen-bonding abilities are prepared. These sensors are scattered in nanospaces associated with anionic and cationic micelles as model membranes to indicate proton availability and polarity in local spaces. Gathering the data from the sensors allows the successful drawing of proton maps near anionic and cationic micelles, in which electrostatic attraction/repulsion of protons by the charged head groups of micelles and dielectric suppression of protons are clearly observed.

Introduction

The proton is the simplest and most influential ion in science and our daily life. In aqueous media, protons always exist under the dissociating equilibrium of water molecules. Clearly, measuring proton concentration in aqueous media (defined as 'pH' by Sørensen in 1909^[1]) is quite important. On a macroscopic scale, such as a solution in a glass flask, the proton concentration can be measured by a glass electrode.^[2] Colorimetric pH indicators (e.g., bromothymol blue and phenolphthalein) are alternatives.^[3] When the size of the subject is reduced to the micrometer scale, e.g., a biological cell or a microfluidic circuit, fluorescent pH sensors can monitor the local proton concentration^[4] and even map it with the assistance of a microscope.^[5] Until now, a variety of methods for accurately measuring proton concentration in spaces larger than the microscale have been established.

Similarly, the proton concentration in smaller spaces, i.e., on a nanometer scale, have attracted much attention from scientists. In the nanospaces associated with highly organized three-dimensional molecules such as proteins and membranes, proton concentration is influenced by neighboring structures and is rather dependent on the local position. For instance, a distinct proton gradient is created across the lipid membranes of mitochondria and is responsible for the entire field of bioenergetics.^[6] To measure a proton gradient in a nanospace, a new method using a series of multiple molecular sensors that are located in a narrow space should be established; notably, a sensor is not scattered widely near three-dimensional nanostructures, and its position is determined mainly by its hydrophobicity/hydrophilicity.^[7]

Fluorescent sensors are advantageous for functioning in nanospaces due to their small size relative to those spaces. In most previous studies on monitoring position-dependent environmental factors (e.g., dipoles,^[8] hydrophobicity,^[9] water content,^[10] and concentrations of protons^[11] and sodium ions^[12]), a series of fluorescent sensors with different hydrophobicities were adopted to collect environmental information at different positions. Thus, nanometer-scale gradients of these environmental factors have been successfully measured, although the positional difference between these fluorescent sensors was deduced only from the chemical structures (but was not experimentally confirmed).

In our previous communication, we reported the first proton mapping near nanospaces defined by micellar membranes by using a series of fluorescent photoinduced-electron-transfer (PET) sensors (**1–18**, Figure 1).^[13] Micelles were adopted as a simple model of membrane systems;^[14] moreover, nanoenvironments created by micelles have already been associated with some unique fluorescent sensing systems.^[15] Sensors **1–18** consist of position tuners (red parts), a polarity-sensitive fluorophore (blue), a proton receptor (orange), and a spacer (green). Due to the position tuners, **1–18** can be located at different positions near micellar membranes. The fluorophore-spacer-receptor system is essential to PET-type fluorescent off-on sensors^[16] and proton concentration can be evaluated by the switching behavior. The position of each sensor in the micellar membranes could be evaluated from the maximum emission wavelength, which is affected by environmental polarity. Thus, proton mapping could be performed by collecting information concerning proton concentration and polarity near all of **1–18**. Overall, we could show proof-of-concept proton mapping near micellar membranes, but we also found an issue that subsequently needed to be worked out: While sensors **1–18** allowed us to draw smooth proton gradient curves for neutral micellar membranes created by Triton X-100 (radius: < 4.8 nm) and octyl β -glucopyranoside (~ 2.3 nm), some sensors were pinned in a narrow area near anionic sodium dodecyl sulfate (SDS) micelles (< 3.6 nm)^[17] and cationic cetyltrimethylammonium chloride (CTAC) micelles (< 3.5 nm)^[17], resulting in proton mapping with low resolution.

In this study, we improved the resolution of our proton mapping method for anionic SDS and cationic CTAC micelles by adding new fluorescent pH sensors **19–33** (Figure 1) to the series of original sensors (**1–18**). Because the pinning of some of the sensors near SDS and CTAC micelles likely originated from undesired attractive interaction between the fluorescent sensors and the ionic head group of the micelles (e.g., ion-dipole interaction and hydrogen bonding^[18]), we designed a new series of sensors that are suitably free of these undesired interactions:

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Sensors **19–27** are more hydrophobic than **1–18** due to the additional *n*-octyl group, and sensors **28–33** lack the hydrogen bonding site of the amino group at the 4-position in the corresponding sensors **6, 9, 15, 18, 24, and 27**. In addition, we also perform proton mapping for another type of micelle, anionic sodium laurate (NaL) micelles,^[19] to demonstrate the applicability of our method even to pH-sensitive micellar membranes and the generality of a proton gradient pattern for anionic micelles.

<div> <div>position tuner</div> <div>H⁺ receptor</div> <div>spacer</div> <div>polarity-sensitive fluorophore</div> <div>position tuner</div> </div>		<div> </div>		
R ¹	R ²	R ³ , R ⁴		
		Me, Me	Me, ⁿ Oc	ⁿ Oc, ⁿ Oc
CH ₂ COO ⁻	CH ₂ COO ⁻	1	10	19
CH ₂ COO ⁻	Et	2	11	20
H	H	3	12	21
CH ₂ CH ₂ OH	CH ₂ CH ₂ OH	4	13	22
CH ₂ CH ₂ OCH ₂ CH ₂		5	14	23
Me	Me	6	15	24
H	Et	7	16	25
CH ₂ COOMe	Et	8	17	26
Et	Et	9	18	27
Me	Me	28	30	32
Et	Et	29	31	33

Figure 1. Fluorescent sensors **1–33** for proton mapping near micellar membranes. The orders of **1–9, 10–18, and 19–27** are determined by the log *P* (*n*-octanol/water partition coefficient) value of a corresponding amine (R¹R²NH).^[13] Sensors **1–18** were originally reported in ref [13], and **19–33** were newly synthesized in the present study to improve the resolution of proton mapping.

Results and Discussion

1. Synthesis of fluorescent pH sensors **19–33**.

In the present study, we newly obtained fluorescent pH sensors **19–33**. Sensors **19–27** and **30–33** were synthesized from 4-chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole (CBD-Cl).^[20] First, the chlorosulfonyl group of CBD-Cl was reacted with di-*n*-octylamine or *N*-methyl-*n*-octylamine, followed by replacement of the remaining chloro group at the 4-position by the amino group of ethylenediamines. Further derivatization of a tail amino group was performed for **19, 20** and **26**. Sensors **28** and **29** were synthesized from 7-chloro-4-*N,N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-Cl)^[20] and ethylenediamines. Detailed synthetic procedures and full characterization (mp, NMR, HR-MS and/or elemental analysis) are described in the Supporting Information.

Figure 2. Fluorescence behavior of a) **28** in water and b) **28** in SDS solution with varying pH. Left: spectra, right: intensities, where *FI* is the fluorescence intensity. Vertical axes for the insets: $\log[(F_{\max}-F)/(F-F_{\min})]$. The excitation wavelength was 440 nm for a) and 410 nm for b), and the concentrations were 10 μ M for **28** and 0.20 M for SDS.

2. General procedure of proton mapping near membranes and proton mapping for anionic SDS micelles.

The procedure for proton mapping of micellar membranes was established in our previous paper and widely spread throughout the scientific community.^[13]

1) As explained in the seminal work of Fernández and Fromherz in 1977,^[21] the ΔpK_a value arises from a difference of chemical potentials in bulk and membrane-bounded water with respect to all species involved in the prototropic equilibrium of the probe. This is directly due to the local electric field and due to the local polarity, both of which differ from the corresponding values in bulk water. However, it is well-appreciated that a low local polarity repels protons, as does a local electric field originating in cationic head groups of the membrane. A corresponding electric field arising from anionic membraneous head groups would attract protons. Therefore the ΔpK_a value indicates an effective local proton concentration indirectly.

More specifically, the local proton concentration can be examined by the ΔpK_a value (pK_a in micellar solution- pK_a in water) of the conjugate acid of the receptor amine, which was originally established by Fernández and Fromherz.^[21] It should be noted that the pK_a value in micellar solution is an apparent value because only the bulk pH is accessible instead of the local pH value (see the Experimental Section). The ΔpK_a value is affected by the electrostatic potential and dielectric constant at the sensor location but is independent of the intrinsic acidity/basicity of the sensor.^[21,22] If the local effective proton concentration is higher than that in bulk water, a positive ΔpK_a value is obtained.^[11,23] As our sensors possess a fluorescence “off-on” switching system by controlling PET processes with a fluorophore-spacer-receptor format,^[16] the ΔpK_a value can be determined from the fluorescence intensity, along with pH profiles arising from titrations. Figure 2 shows representative fluorescence titrations of **28** in water and in SDS aqueous solution, in which the ΔpK_a value

of **28** in the SDS solution was determined from the two pK_a values to be +0.83 (9.02 minus 8.19).^[24]

2) The local polarity is estimated from the maximum emission wavelength (λ_{em}) of the polarity-sensitive fluorophore 4-sulfamoyl-7-aminobenzofurazan. The λ_{em} value is strongly redshifted with increasing environmental polarity and is well related to the dielectric constant (ϵ) of the solvent.^[7] Thus, the relationship between the λ_{em} and ϵ values is obtained beforehand for each sensor from the fluorescence spectra in water, methanol, ethanol, and a mixture of water and methanol (see Table S3 in the Supporting Information). Obtaining the λ_{em} - ϵ relationship for the sensors under both acidic and basic conditions is important because the emission of **1–33** is blueshifted by protonation of the amine receptor in a homogeneous medium. For example, the λ_{em} of the protonated form of **28** in water is 585 nm (at pH = 5.49), whereas that of free **28** is 598 nm (i.e., at pH = 10.32) (Figure 2a). This blueshift is due to an interaction between the protonated receptor and the internal charge-transfer excited state of the fluorophore across the dimethylene spacer.^[25] In the representative case of **28** in SDS solution shown in Figure 2b, the local ϵ values were estimated to be 32.1 and 34.1 under acidic and basic conditions, respectively, from the emission wavelengths (562 and 566 nm, respectively). Generally, the ϵ value obtained under acidic conditions was larger than that obtained under basic conditions (see Table S6 in the Supporting Information) because the protonation of the receptor increases the hydrophilicity of the sensors, causing a change in its position to a more polar location. In addition, in anionic micelles, electrostatic attraction is expected between only the protonated sensor and head groups of the surfactant, which can also vary the sensor position between acidic and basic conditions. Considering these characteristics, the average of the ϵ values obtained under acidic and basic conditions (represented as $\bar{\epsilon}$) is adopted as a parameter of the polarity near a sensor.

3) The position of a sensor near the micellar membrane is altered by changing its substituents R^1 – R^4 and introducing a methyl group to the amino group at the 4-position of the fluorophore. A sensor bearing more hydrophilic substituents is expected to stay in a more hydrophilic region in the nanospace. Finally, proton concentration maps near micellar membranes can be drawn by collecting the environmental data from **1–33** in the form of ΔpK_a - $\bar{\epsilon}$ diagrams.

Figure 3. ΔpK_a -polarity ($\bar{\epsilon}$) diagrams of SDS micelles (0.20 M) obtained with **1–33** (10 μ M). The number in italics represents the sensor corresponding to each point. The data points obtained with **1–18** (pink) were originally reported in ref [13], and those obtained with **19–33** (red) are newly added. A dotted line indicates a trend in points.

Figure 3 shows a ΔpK_a - $\bar{\epsilon}$ diagram for anionic SDS micelles. The new sensors (**19–33**, red) were more scattered in the ΔpK_a - $\bar{\epsilon}$ diagram than the original sensors (**1–18**, pink). This result demonstrated that position tuning by introducing two octyl groups and by eliminating the hydrogen-bonding site of the amino group at the 4-position was effective.^[26] In the ΔpK_a - $\bar{\epsilon}$ diagram for SDS micelles, the most hydrophilic sensor (**1**) gave the largest $\bar{\epsilon}$ value (53.5), where the proton concentration (in other words, proton availability) was similar to that in bulk water (i.e., $\Delta pK_a \sim 0$). Most sensors located in areas with moderate polarity ($30 < \bar{\epsilon} < 50$) gave positive ΔpK_a values. This result is observed because protons are concentrated by the electrostatic effect near the anionic head sulfonate groups of the SDS micelles. The maximum proton concentration near SDS micelles was experimentally estimated to be 7,760% of that in bulk water ($\Delta pK_a = +1.89$ from **23**). Sensors **29**, **31**, and **33** were located in more hydrophobic areas ($28 < \bar{\epsilon} < 30$) in SDS micelles, where the positive electrostatic effects attracting protons were cancelled by the additional repulsive dielectric effects in these apolar locations. As a result, the ΔpK_a value is ~ 0 . Thus, we could successfully perform proton mapping for SDS micelles by using a series of fluorescent pH sensors (**1–33**) and confirm the local nanoscale proton abundance near the head group of the surfactant.

3. Proton mapping for cationic CTAC micelles.

Figure 4 is a proton map for cationic CTAC micelles. The new sensors (**19–33**) could increase the number of scattered data points to improve the resolution of the map. In particular, sensors **31** and **33**, which are highly hydrophobic and lack the hydrogen-bonding site, were located deep inside the micelles ($\bar{\epsilon} \sim 37$), where the available proton concentration is only approximately 0.01% of that in bulk water (i.e., $\Delta pK_a \sim -4$). Considering that the most hydrophobic sensor (**18**) in the original series indicated that $\Delta pK_a = -2.83$ at the position where $\bar{\epsilon} = 41.5$, the extension of the series of sensors to **1–33** in the present study successfully recorded the proton availability at this extreme location. The ΔpK_a - $\bar{\epsilon}$ diagram in Figure 4 clearly indicates that there are much fewer protons inside cationic CTAC micelles than outside the micelles due to the double repelling effects (i.e., dielectric effect and electrostatic effect between protons and the cationic head groups of CTAC).

Figure 4. ΔpK_a -polarity ($\bar{\epsilon}$) diagrams of CTAC micelles (5.0 mM) obtained with **1–33** (10 μ M). The number in italics represents the sensor corresponding to each point. The data points obtained with **1–18** (pale orange) were originally reported in ref [13], and those obtained with **19–33** (orange) are newly added. A dotted line indicates a trend in points.

4. Proton mapping for anionic NaL micelles.

In the present study, we also tried to map proton concentration near another type of anionic micelle, NaL micelles, to confirm the applicability of our established method to a wide range of micelles and to confirm the generality in the pattern of proton availability for anionic micelles. Nevertheless, attempts to map the proton availability for NaL micelles faced unavoidable restrictions because of the weak acidity of NaL ($pK_a = \sim 7.5$ [27]): Fluorescence titrations with varying pH could be performed at a range of pH values greater than 8.8, whereas in a more acidic solution, NaL precipitated and could not be kept in a micellar form. This restriction of the pH range prevented the use of sensors **4**, **5**, **8**, **13**, **14**, **17**, **22**, **23**, and **26** with functional ranges in acidic regions. Even some sensors with functional ranges in more basic regions enabled only partial fluorescence titration in limited pH ranges. For these cases, $F_{\max}$ values were estimated from multiple fluorescence intensity data points in basic pH regions by fitting a sigmoidal curve to the data (see the Experimental Section), from which the pK_a values were obtained. For the same reason, the λ_{em} values of protonated sensors could not be determined. Thus, for proton mapping of NaL micelles, the ϵ value corresponding to the λ_{em} value of the free sensor form under basic pH conditions (denoted ϵ_{base}) was used as a parameter of local polarity instead of the $\bar{\epsilon}$ values.

Figure 5 is the ΔpK_a -polarity (ϵ_{base}) diagram for NaL micelles, which was drawn with data obtained from 24 sensors (excluding sensors **4**, **5**, **8**, **13**, **14**, **17**, **22**, **23**, and **26** from the full set). Although the number of sensors utilized for proton mapping of the anionic NaL micelles was less than that used for the another anionic SDS micelles, the 24 sensors were well scattered and thus provided ΔpK_a values at different locations, enabling us to accomplish proton mapping. The proton map for NaL micelles (Figure 5) shows considerable similarity to that for SDS micelles (Figure 3), supporting that the ϵ_{base} value could replace the $\bar{\epsilon}$ value in the current case. Similar to SDS micelles, NaL micelles created a nanospace with a high proton concentration by electrostatic attraction between protons and the carboxylic head groups (the highest proton concentration was 4,270% of that in bulk water, as determined from $\Delta pK_a = +1.63$ with **12**), but this effect was reversed with dielectric repulsion in the deep interior of the NaL micelles ($\epsilon_{base} \sim 30$). It was also concluded that the pattern of a proton map is independent of the chemical structure of micelles unless the charge of the head group is different.

Figure 5. ΔpK_a -polarity (ϵ_{base}) diagrams of NaL micelles (54.4 mM) obtained with **1–33** (excluding **4**, **5**, **8**, **13**, **14**, **17**, **22**, **23**, and **26**) (10 μM). The number in italics represents the sensor corresponding to each point. A dotted line indicates a trend in points.

Conclusions

In this study, a series of fluorescent proton sensors having different hydrophilicity/hydrophobicity enabled proton mapping for anionic SDS and NaL micelles and cationic CTAC micelles with nanoscale spatial resolution. This advancement is considered a rational step toward fluorescent sensors that function in nanospaces. Our research direction will be divided into two important lines: The first is straightforward proton mapping of more complex membrane systems such as vesicles and liposomes. DNA is also an interesting subject. The second is the expansion of target ion species. For instance, both sodium and potassium ions are abundant near biological membranes and are involved in significant functions. For the latter purpose, the synthesis of fluorescent ion sensors with an environment-sensitive fluorophore and a strong binding moiety (receptor) is an apparent hurdle and should be addressed for further progression of fluorescent sensors.

Experimental Section

Materials and detailed synthetic procedures for new fluorescent pH sensors **19–33** are described in the Supporting Information.

Fluorescence measurements with sensors 1–33. For the determination of ΔpK_a ($= pK_a$ in micelle solution $- pK_a$ in water) values and local dielectric constants (ϵ values), the following procedures^[13] were adopted:

1. pK_a value in water. To a solution (30 ml) containing hydrochloric acid (10 mmol/l) and the fluorescent sensor (**1–9**, **28** and **29**, 10 $\mu mol/l$), an appropriate amount of solution containing sodium hydroxide (100 mmol/l) and the fluorescent sensor (**1–9**, **28** and **29**, 10 $\mu mol/l$) was added. The pH value of the solutions was measured with a Horiba D-21 or F-73 pH meter. Then, the fluorescence spectrum under each pH condition was obtained with excitation at 410 nm, except for **28** in water (which was excited at 440 nm). For the fluorescence titrations using **1** and **2**, ethylenediamine tetraacetic acid (EDTA, 0.2 mmol/l) was also added to the solutions to avoid the effects of trace metal ions on the pK_a value. The pK_a value was determined with least-squares analysis by fitting the obtained fluorescence intensity (F) at a fixed wavelength (maximum emission wavelength of the protonated form) and the pH value using eq. (1)

$$\log[(F_{\max} - F)/(F - F_{\min})] = a(pH - pK_a) \quad (1)$$

where $F_{\max}$, $F_{\min}$, and a are the maximum fluorescence intensity, the minimum fluorescence intensity, and the gradient, respectively. The obtained pK_a values are listed in Table S1.

2. pK_a value in micelle solution. To a solution (30 ml) containing hydrochloric acid (10 mmol/l), the fluorescent sensor (**1–33**, 10 $\mu mol/l$) and the surface active agent (SDS (200 mmol/l), CTAC (5 mmol/l), or NaL (54.4 mmol/l)), an appropriate amount of solution containing sodium hydroxide (100 mmol/l), the fluorescent sensor (10 $\mu mol/l$) and the surface active agent (same concentration as above) was added. For the fluorescence titrations using **1**, **2**, **10**, **11**, **19**, and **20**, EDTA (0.2 mmol/l) was also added to the solutions. The pK_a value was determined with least-squares analysis by fitting the obtained F at the maximum emission wavelength of the protonated form and the pH value indicated by the pH meter using eq. (1). For the fluorescence titration in the NaL solution, the pH of the solution should be kept above 8.8; otherwise, the protonated form of NaL precipitates, and the solution becomes turbid. Thus, the fluorescence titrations in NaL micelles could not be performed for **4**, **5**, **8**, **13**, **14**, **17**, **22**, **23**, and **26** and could not be completed for **1**, **2**, **6**, **9–11**, **15**, **18–20**, **24**, and **27–33**. For the latter cases, the pK_a value was obtained by eq. (1) with the estimated $F_{\max}$ value that afforded the maximum correlation coefficient

(*r*) in the fitting of eq. (1) to the experimental data. The determined pK_a values are listed in Table S1.

3. ΔpK_a values. The ΔpK_a value was obtained by subtracting the pK_a value in water from the pK_a value in micelle solution and is listed in Table S2. The determination of the ΔpK_a values for **10–18** (and **19–27**) uses the pK_a values for **1–9** in water instead of those for **10–18** (and **19–27**) in water because of the insolubility of **10–18** (and **19–27**). Similarly, the pK_a values for **28** and **29** in water were used for the determination of the ΔpK_a values for **30–33**. The error in the determination of the ΔpK_a values was estimated to be within 0.1.

4. ϵ values. The fluorescence spectra of **1–9**, **28** and **29** (1–30 mmol/l) were measured in water ($\epsilon = 78.48$), water-methanol (3:1 (v/v) ($\epsilon = 69.43$), 1:1 (v/v) ($\epsilon = 58.77$) and 1:3 (v/v) ($\epsilon = 46.79$)),^[28] methanol ($\epsilon = 32.66$) and ethanol ($\epsilon = 24.55$) under acidic (+ 0.5% sulfuric acid) and basic (+ saturated sodium carbonate) conditions. Then, the observed maximum emission wavelengths (λ_{em} , listed in Table S3) and ϵ values of the solvents were found to be best fit with the following linear relationships (eq. (2)) by least-squares analyses, even though the theoretical expectation is a double-reciprocal relationship.^[29]

$$\lambda_{em} [\text{nm}] = m \times \epsilon + n \quad (2)$$

The optimized parameters (*m* and *n*) and correlation coefficient (*r*) in the linear approximation are summarized in Table S4. In the linear relationships, **1–9** were treated as model compounds of **10–18** and **19–27** with the assumption that the difference in the R^3 and R^4 groups has no effect on the emission wavelength. In a similar manner, **28** and **29** were model compounds of **30–33**. The local ϵ value at each sensor position in micelle solution was calculated by using eq. (2) with the maximum emission wavelength of the sensor in micelle solution (Tables S5 and S6). The error in the determination of the ϵ values was estimated to be within 4.

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Conflict of interest

The authors declare no conflict of interest.

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Entry for the Table of Contents

FULL PAPER

By using 33 fluorescent pH sensors with different hydrophobicity/hydrophilicity and hydrogen-bonding abilities, we successfully drew proton maps near anionic and cationic micelles with high spatial resolution. In the obtained proton maps, electrostatic attraction/repulsion of protons by the charged head groups of micelles and dielectric suppression of protons were observed.

Seiichi Uchiyama, Kayo Yano, Eiko Fukatsu, A. Prasanna de Silva**

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Precise Proton Mapping near Ionic Micellar Membranes with Fluorescent Photoinduced-Electron-Transfer Sensors