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Precise detection of pH inside large unilamellar vesicles using membrane-impermeable dendritic porphyrin-based nanoprobe

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Abstract

Accurate real-time measurements of proton concentration gradients are pivotal to mechanistic studies of proton translocation by membrane-bound enzymes. Here we report a detailed characterization of the pH-sensitive fluorescent nanoprobe Glu³, which is well suited for pH measurements in microcompartmentalized biological systems. The probe is a polyglutamic porphyrin dendrimer in which multiple carboxylate termini ensure its high water solubility and prevent its diffusion across phospholipid membranes. The probe's pK is in the physiological pH range, and its protonation can be followed ratiometrically by absorbance or fluorescence in the ultraviolet-visible spectral region. The usefulness of the probe was enhanced by using a semiautomatic titration system coupled to a charge-coupled device (CCD) spectrometer, enabling fast and accurate titrations and full spectral coverage of the system at millisecond time resolution. The probe's pK was measured in bulk solutions as well as inside large unilamellar vesicles in the presence of physiologically relevant ions. Glu³ was found to be completely membrane impermeable, and its distinct spectroscopic features permit pH measurements inside closed membrane vesicles, enabling quantitative mechanistic studies of membrane-spanning proteins. Performance of the probe was demonstrated by monitoring the rate of proton leakage through the phospholipid bilayer in large vesicles with and without the uncoupler gramicidin present. Overall, as a probe for biological proton translocation measurements, Glu³ was found to be superior to the commercially available pH indicators.

Keywords

Porphyrin; Dendrimer; Polyglutamic; Fluorescence; pH; Proton pumping; Molecular probe; Liposome; LUV; pH gradient; pH sensor

Advanced molecular sensors is a rapidly developing area of research [1], and proton nanosensors attract special interest because of their applicability in monitoring proton concentrations in biological microcompartments. Proton concentration gradients play a pivotal role in the processes of respiratory and photosynthetic energy conversion and regulation. These gradients are created and maintained by bioenergetic proton-translocating membrane-bound protein complexes, the reactions of which involve highly reactive intermediates that can produce damaging radicals. These bioenergetic enzymes are implicated in natural aging [2] and serious degenerative conditions such as Parkinson's disease [3–6]. Furthermore,

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misbalanced pH in cellular compartments is a key miscreant in cancer drug resistance, and the proton pumps and channels involved in tumor biology are under intense scrutiny [7–10].

Much structural information on bioenergetic proteins has emerged over the past decade. However, to understand the molecular mechanisms of their action, it is crucial to accurately measure electron transfer and proton translocation stoichiometries, and for this, quantitative methods for monitoring proton translocation are needed.

In proton translocation studies, the enzyme of interest must reside in the membrane of a closed vesicle, and the membrane itself must be proton impermeable on the time scale of the translocation process. The closed vesicle may be the native membrane of the protein (see, e.g., Ref. [11]) or one artificially prepared from a known phospholipid composition. The latter offers a well-defined experimental system where no unknown or unwanted proteins are present. To monitor proton concentrations in this system, noninvasively and dynamically, pH-sensitive molecular probes are used. Ideally, the proton concentration inside the vesicles and that of the bulk solution should be monitored simultaneously. Unfortunately, the characteristics of the available pH probes do not accommodate such measurements; many of them are membrane permeable, and most of them have too weak spectroscopic signals to report pH from the interior of vesicles dispersed in a bulk solution. These difficulties have been cleverly sidestepped in proton translocation studies on several important protein complexes (see, e.g., Refs. [12–14]). However, experiments with available probes have reached their full potential; further progress requires better molecular pH sensors.

Several common commercial colorimetric pH indicators are presented in Table 1. Membrane-permeable pH-sensitive dyes, such as neutral red, have been used to monitor intravesicular pH changes using a solution of low buffer strength inside the vesicle and high buffer strength in the bulk phase. Although neutral red could diffuse freely in and out of the vesicle, the different buffer concentrations ensured that the detected spectral changes arose exclusively from the probe present inside the vesicles. However, a probe that diffuses through and associates with the membrane is likely to alter the membrane properties, affecting the measurements in an unpredictable manner. Neutral red was reported to show significant protonophore activity, facilitating proton transfer across the membrane [15]. A permeable probe may also accumulate on one side of the membrane as the pH changes. In addition, the difference in the buffering capacities required for these experiments excludes simultaneous pH measurements on both sides of the membrane. Phenol red [16] is less likely to diffuse across the membrane but displays relatively weak spectroscopic signals, making it less suitable for intravesicular pH determination. A combination of experiments using membrane-permeable neutral red and membrane-impermeable phenol red resulted in pumping stoichiometries of 2 H⁺/e[−] for the respiratory complex I [17,18], and 3.6 H⁺/e[−] for complex I reconstituted in proteoliposomes were obtained [14,19]. The seminaphthofluorescein chromophore SNARF^{1,2} also monitors pH and does not diffuse significantly through lipid bilayers. It was, however, shown to bind to the membrane surface of large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs), resulting in a sensor subpopulation with characteristics different from those of the dissolved probe [20]. The ratiometric fluorescence probe pyranine is more suitable for monitoring intravesicular pH and has been combined with the membrane potential probe oxonol for mechanistic studies of H⁺-ATPase [21]. However, the modest brightness of pyranine

¹Abbreviations used: SNARF, seminaphthorhodafluor; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; Glu³, generation 3 polyglutamic porphyrin dendrimer; Glu⁴, generation 4 polyglutamic porphyrin dendrimer; CCD, chargecoupled device; UV, ultraviolet; PMT, photomultiplier tube; H₂TPP, tetraphenylporphyrin; SNR, signal-to-noise ratio; fb, free base; dc, dication; EDTA, ethylenediaminetetraacetic acid; cryo-TEM, transmission electron microscopy at cryogenic temperatures; DMSO, dimethyl sulfoxide; H₂P, tetraarylporphyrin chromophore.

²SNARF pH indicators (Invitrogen).

fluorescence limits the sensitivity of these measurements. These problems may be avoided by using improved membrane-impermeable pH probes.

Ideal characteristics of a pH probe for proton translocation measurements include membrane impermeability, high molar extinction coefficients in both protonated and nonprotonated forms, high water solubility, and no tendency to aggregate or form complexes with biological macrostructures. If measurements involve fluorescence, the emission quantum yields of both protonated and nonprotonated forms should be high enough to allow ratiometric pH detection. The only commercially available probes of this kind are SNARF–dextrans, which have been used in several proton-pumping studies [22,23]. The ligation of a dextran moiety to the SNARF chromophore effectively eliminates probe diffusion through membranes, but the SNARF group in these conjugates can still bind to membrane lipids. Furthermore, the molecular compositions of SNARF–dextrans are not uniquely defined, and although they permit ratiometric measurements, the spectral changes accompanying their protonation are not large.

Dendritic pH probes offer a number of advantages over SNARF–dextran complexes. Porphyrin-based probes, such as Glu³ (see Fig. 1) [24], possess distinctly different absorption and fluorescence features in protonated and nonprotonated forms (see below). Their absorption (Soret) bands are sharper and stronger than those of SNARF, facilitating detection of small pH changes. Hydrophilic dendritic cages render probes like Glu³ very water soluble, whereas multiple peripheral carboxylates prevent interactions with biological membranes. Furthermore, we found that the SNARF chromophore is easily lost during the preparation of proteoliposomes (see “Probe adhesion to hydrophobic beads” section in Materials and methods), whereas Glu³ remains stable and in solution throughout the procedure. This combination of properties makes Glu³ an attractive alternative to SNARF–dextrans for biological proton translocation measurements.

In this work, we characterize the pH-sensitive, membrane-impermeable probe Glu³ (generation 3 polyglutamic porphyrin dendrimer [25]) and validate its potential for biological pH measurements. Glu³ consists of a tetraarylporphyrin core with four covalently attached polyglutamic dendrons, terminated by carboxylic acid groups. The optical spectra of the porphyrin are distinctly different in the nonprotonated (free-base) and doubly protonated (dication) forms (see Fig. 2A). The negative charges of the ionized carboxylates shift the *pK* of the intrinsically weakly basic porphyrin to higher pH values, placing it in the physiological pH range [25]. The peripheral charges also render Glu³ highly water soluble and membrane impermeable [24]. These qualities allow monitoring of proton translocation across membranes by nonintrusive detection of pH inside closed phospholipid vesicles.

Glu⁴ (generation 4 polyglutamic porphyrin dendrimer), a dendritic probe similar to Glu³, was used in our previous experiments involving SUVs (20–30 nm in diameter). Glu⁴ was demonstrated to be retained inside the vesicles and to report on their internal pH [24]. Later, this probe was used to demonstrate formation of synthetic membrane-spanning channels made of dendritic dipeptides [26,27]. The current probe Glu³ exhibits a *pK* similar to that of Glu⁴ but can be synthesized in fewer steps. Neither probe has yet been calibrated with the precision required for mechanistic studies of proton-translocating enzymes. For such measurements, the ability to monitor small pH changes at low dye concentrations is required; many proton-pumping studies have been carried out in the absence of buffer, with exact control of the buffering capacity being sacrificed so as to obtain detectable signals [12,13,28]. Furthermore, the behavior of the probe in the presence of metal cations, biological macromolecules, and phospholipid membranes needs to be evaluated accurately.

Rather than using poorly defined SUVs with small internal volumes (on the order of 10^{−21} L/vesicle), we have adapted methods for probe incorporation into well-characterized LUVs of

uniform size (on the order of 10^{-18} L/vesicle). The LUVs have an approximately 10 times larger diameter than the SUVs, accommodating larger amounts of the probe and, thus, permitting accurate determination of pH not only by fluorescence but also by absorption.

In this work, Glu³ was investigated using a newly developed semiautomatic titration system.³ The system is based on a charge-coupled device (CCD) spectrometer from which output is fed automatically into an analysis program. This setup ensures precision, eliminates user-to-user variability, and significantly reduces the time required for titration experiments. CCD detection enables the full absorption spectrum to be collected in 1.1 ms, providing the user with continuous control of all chromophores in the sample. The evaluation presented in this article provides the basis for a robust, quantitative pH detection method, optimized specifically for pH measurements in the interior of liposomes. This method can be applied directly to determination of proton-pumping stoichiometries of bioenergetic enzymes. By adding a different pH probe to the bulk solution or monitoring the bulk pH with an electrode, the pH change on each side of the vesicle membrane can be monitored simultaneously [24]. This excludes proton concentration changes incurred by reactions other than proton translocation and enhances the sensitivity of the measurements. If the protein contains optically detectable cofactors, these will be monitored as well, giving a uniquely comprehensive picture of the proton translocation process.

Materials and methods

Optical spectroscopy and titrations

Optical absorption spectra were recorded either on a PerkinElmer Lambda 35 ultraviolet-visible spectrophotometer or by using an in-house-constructed CCD-based spectrometer (see below). Steady-state fluorescence measurements were performed on an SPEX Fluorolog-2 spectrofluorometer (HORIBA Jobin Yvon) equipped with Hamamatsu R2658P photomultiplier tube (PMT). Emission spectra were obtained using aqueous solutions (50 mM phosphate buffer, total $[K^+] = 50$ mM) of Glu³ (4–5 μ M) with absorbance of approximately 0.05 OD at the excitation maxima. The emission quantum yields of Glu³ as the free base (pH 8.0) and dication (pH 4.0) were determined relative to the fluorescence of freebase tetraphenylporphyrin (H_2 TPP) in deoxygenated benzene ($\phi_f = 0.11$) [29]. For pH titration by fluorescence, the pH of a buffered solution of the probe (~30 ml total volume) was adjusted by adding small portions of concentrated solutions of HCl and KOH (5 M). The pH step was 0.5 to 1.0 units. At each pH, two emission spectra, excited at 515 nm (free base) and 600 nm (dication), were recorded. The spectra were integrated, and the ratio of the integrals (plotted against pH) was analyzed (see below) to yield the value of the apparent pK . Three independent titrations were averaged to obtain the calibration curve.

Titrations by absorption were performed using an in-house-constructed system. The spectra were recorded using a fiber-optic absorption spectrometer (AvaSpec-2048, Avantes) with a tungsten/halogen light source. The spectrometer was coupled to a sample holder featuring stirring, a controlled atmosphere, an electrode duct, and two capillary inlets. Two Hamilton syringes (100 μ l total volume), attached to computer-controlled syringe pumps (NE-500, New Era Pump Systems), were connected to the capillary inlets of the sample holder. The syringe pumps administered precise volumes of 0.5 M HCl and KOH solutions, calculated by the controlling software to achieve the desired pH. The actual pH of the bulk solution was measured by a combination pH microelectrode (Orion ROSS, Thermo Scientific) fitted to the electrode duct of the sample holder. The software was configured to set a series of pH points and acquire the absorption spectrum at each pH.

³Autonomous Science Machines.

To investigate the effects of metal cations, Glu³ was added to solutions of potassium or magnesium salts to a final probe concentration of approximately 2 μ M. The acquisition time for each spectrum was 1.1 ms, and 1024 spectra were averaged for each pH point. This configuration results in an extremely high signal-to-noise ratio (SNR) throughout the entire spectral range, with a temporal resolution of 1.2 s. If a lower SNR is acceptable, the system achieves millisecond time resolution.

Data analysis

The fraction of nonprotonated Glu³ at a given pH (R_{fb}) was derived from the absorbances at the Soret peaks of the free base (fb, where A_{415} is absorbance at 415 nm) and the dication (dc, where A_{439} is absorbance at 439 nm):

$$R_{fb} = \frac{1.04 \times A_{415} - 0.22 \times A_{439}}{A_{415} + A_{439}} \quad (1)$$

In Eq. (1), the absorbances were corrected for the slightly differing extinction coefficients of the free-base and cation Soret maxima ($\epsilon_{fb}/\epsilon_{dc} = 1.17$) and for the partial overlap of the absorption bands. The correction values were determined experimentally using the spectra acquired at pH 4.5 for the dication and at pH 7.2 for the free base. (Complete protonation of the porphyrin requires very acidic conditions at which polyglutamic branches might undergo partial hydrolysis. At pH 4.5, less than 5% of the probe is deprotonated, introducing less than 1% error in Eq. 1.) Measurements involving liposome-encapsulated Glu³ required subtraction of the scattering baseline prior to determination of A_{415} and A_{439} .

The equation for the analysis of the fluorescence data was

$$R_{fb} = \frac{I_{E515} - 0.18 \times I_{E600}}{0.77 \times I_{E515} + 2.12 \times I_{E600}}, \quad (2)$$

where I_{E515} is the integrated fluorescence spectrum when exciting at 515 nm, corresponding to the free base, and I_{E600} is the integrated fluorescence spectrum when exciting at 600 nm, corresponding to the dication. As in Eq. (1), corrections for different band intensities and spectral overlap were made. The protonation state of the dye, determined by absorbance (Eq. 1) or fluorescence (Eq. 2), was fitted with a sigmoidal curve according to the Hill equation [30]:

$$R_{fb}(pH) = \frac{10^{n(pH-pK)}}{1 + 10^{n(pH-pK)}}, \quad (3)$$

where pK refers to the apparent protonation constant (see below) and corresponds to the pH at which $R_{fb} = 0.5$. The Hill equation with $n = 1$ is valid for a protonation reaction involving a single proton. However, it can be applied to protonations involving more than one proton by using the floating parameter n if the protonations have close equilibrium constants. The resulting apparent pK represents the average of the protonation constants. Notably, constant K in Eq. (3) corresponds to the equilibrium where the forward reaction is the dissociation of protons from the dication (see Scheme 1) rather than the protonation of the free base.

Glu³ can also be analyzed ratiometrically by monitoring the absorbance ratio $R = A_{415}/A_{439}$ or the ratio of fluorescence intensities or integrals (vide infra). Plotting R versus pH results in a

sigmoidal curve, displaying a midpoint that is significantly higher than the apparent pK and a larger dynamic range along the y axis.

Liposome preparation and dye inclusion

LUVs were prepared following the method described by Rigaud and Levy [31]. High-purity phosphatidyl choline from egg yolk (Sigma–Aldrich) was used to prepare multilamellar vesicles by reverse-phase evaporation. In each preparation, 0.5 ml of lipid in chloroform at 100 mg/ml was used. The chloroform was evaporated under vacuum, and the resulting lipid film was dissolved in 3 ml of diethyl ether and sonicated with 1 ml of 20 mM potassium phosphate buffer with 0.5 mM ethylenediaminetetraacetic acid (EDTA, pH 7.2). This resulted in a water-in-oil emulsion. Sonication was performed on ice during four periods of 30 s separated by periods of 15 s. The organic phase was removed by evaporation under 0.5 atm of nitrogen for 2 h. Also, 2 ml of the buffer was added to the resulting material, and the suspension was dried for 1 h to remove the remaining traces of diethyl ether. The suspension was extruded in series through polycarbonate filters with pore sizes of 400 and 200 nm using an extruder (LiposoFast, Avestin) [32]. A total of nine extrusions were performed. The preformed liposomes were diluted in a buffer to a final lipid concentration of 5 mg/ml, solubilized by the addition of 80 mg of the detergent Triton X-100 to achieve a 2:1 (w/w) detergent/lipid ratio, and stirred for 20 min or until the solution became optically transparent. Glu³ was added to a final concentration of approximately 0.2 mM. Detergent was removed by adding moist beads (SM-2 Bio-Beads, Bio-Rad) in four portions of 800 mg, with 1 h of gentle stirring between the additions. The removal of the detergent was monitored optically by the OD at 700 nm, which represents light scattering caused by the assembling liposomes. The vesicles were harvested 15 min after the OD became constant. Prior to use, the beads were washed with methanol and deionized water alternatively (three cycles) and then with deionized water (three times) to remove traces of methanol. To remove Glu³ from the bulk solution, the liposomes were purified by size exclusion chromatography [24] on a Sephadex G200 column (GE Healthcare) containing a layer of Sephadex A50 anion exchange resin (GE Healthcare) at the bottom [24]. The resin was degassed under vacuum before use. The resulting vesicles were stored at 4 °C.

Probe adhesion to hydrophobic beads

Five commercially available pH-sensitive dyes and Glu³ (see Table 1) were each added to a separate 2-ml sample of 20 mM potassium phosphate buffer solution at pH 7.2 to give the final dye concentration of 200 μ M. Each probe solution was divided into two aliquots of 1 ml, one of which was put aside for control. Prewashed SM-2 beads (100 mg) were added to each sample, followed by 60 min of stirring. Then a second portion of 50 mg of beads was added, followed by 60 min of stirring. The dye solutions were separated from the beads, and the absorbance spectra of these solutions were compared with those of the reference samples.

Cryo-TEM

The morphology of the liposomes was investigated by transmission electron microscopy at cryogenic temperatures (cryo-TEM) [33]. For the preparation of the unstained, frozen, hydrated liposome specimen, a 5- μ l droplet of the liposome suspension (~4 mg of lipid/ml) was deposited on an electron microscopy grid. After removing the excess solution with a filter paper, the thin layer of aqueous solution was rapidly plunged into liquid ethane at -178 °C. The grid, kept under liquid nitrogen, was then transferred into a cryoholder maintained at -170 °C, which in turn was introduced into the electron microscope for observation at the same temperature. Liposomes from several cryo-TEM images were measured to estimate the size distribution.

Monitoring the probe inside liposomes

Titration measurements were performed using LUV suspensions in potassium phosphate buffer (20 mM) at 20 °C in the absence and presence of gramicidin. Gramicidin was added as a solution in dimethyl sulfoxide (DMSO) to a final concentration of approximately 1 µg/ml. In the pH jump experiment (see Fig. 8), pH was changed from 7.2 to 5.6 by a single addition of 0.5 M solution of HCl. Following the addition, the spectrum of the probe was monitored for 30 min, after which gramicidin was added and the final spectrum was recorded. The net flux of protons and hydroxyl ions, J_{net} , was calculated as

$$J_{\text{net}} = \frac{\Delta \text{pH}_i}{\Delta t} \times \frac{B_i \times V_i}{A}, \quad (4)$$

where ΔpH_i is the change of internal pH during the time interval Δt , V_i is the internal volume, B_i is the buffer capacity of the internal volume, and A is the total membrane area [34].

Results and discussion

Probe Glu³

The molecular structure of the probe Glu³ is shown in Fig. 1. The probe was synthesized by the mixed convergent/divergent method described previously [24,25,35]. The probe's molecular weight as a polyglutamic acid is 4402 Da. The porphyrin macrocycle in Glu³ (tetraarylporphyrin chromophore [H₂P], shown inside the circle in Fig. 1) undergoes two sequential protonations, resulting in the formation of the dication. It is more common to consider dissociation of the protons from the dication as the forward reaction rather than the protonation of the free base. The dication H₄P²⁺ can then be viewed as a Brønsted acid characterized by two ionization constants K_1 and K_2 (Scheme 1).

Protonation of porphyrins is accompanied by distinct changes in the optical spectra, for example by red shifts of the Soret bands (see Fig. 2A). The protonations can be followed spectroscopically by plotting a characteristic absorbance intensity of either of the forms, or their ratio, versus pH (Fig. 2B). For the majority of porphyrins, constants K_1 and K_2 are very close, and the monocation H₃P⁺ is an elusive species whose concentration is extremely low at all proton concentrations [36]. Accordingly, instead of fitting the data to a function describing two sequential protonations, it is more practical to use the analytical equation describing a single protonation (Eq. 3), allowing variation of the parameter n . The derived apparent constant K is suitable for the characterization of the overall equilibrium [37].

The use of a floating parameter n in Eq. (3) is also justified by the fact that the p*K* values of the multiple glutamic carboxylates at the periphery of Glu³ span a broad range. Although the p*K* values of an isolated glutamic acid are well defined, 2.19 and 4.25, in Glu³, 16 closely positioned peripheral glutamic residues affect each other's protonation states and significantly broaden the protonation range of the core porphyrin. The net effect of the ionized glutamates is stabilization of the porphyrin dication and a shift of the p*K* to higher values compared with the “naked” porphyrin [24,25].

Automated pH titration spectroscopy

We have constructed an automated titration system coupled to a fiber-optic CCD spectrometer, as detailed in Materials and methods. CCD detection sidesteps baseline drift problems associated with monochromators and provides full spectral coverage suitable for monitoring the state of the sample continuously. Automated titration eliminates user-to-user variability, minimizes titration errors, and facilitates fast and reproducible titrations and analyses of large

numbers of samples. Once the experimental parameters have been set and the titration has been initiated, this system performs the complete titration without user attendance. A typical pH titration experiment consisting of 20 spectral measurements at pH values separated by $\Delta\text{pH} = 0.1$ is completed within 25 min. The resulting spectra are automatically analyzed to reveal the protonation state of the sample at each pH (Eq. 1), after which the data are fitted to Eq. (3).

Porphyrin dendrimers as pH sensors

The change of the Glu³ absorption spectrum during titration is shown in Fig. 2A. Overall, this titration features a stable baseline, a clean isosbestic point, and an accurate fit of the data points to Eq. (3) (Fig. 2B). The titration starts with the free-base form, exhibiting absorption maxima at 415 nm (Soret band) and 517 nm (Q band). These features decrease with a decrease in pH in favor of the peaks at 439 and 650 nm, originating from the doubly protonated (dicationic) form. The spectra of Glu³ are virtually identical to those reported previously for Glu⁴ [24].

The relative amount of the probe in its free-base form at any given pH can be estimated by comparing the intensity of the Soret peaks at 415 and 439 nm, as detailed in Materials and methods (Eq. 1). Plotting this fraction, R_{fb} , as a function of pH and fitting it with Eq. (3) yields the pK value of 5.8 for Glu³ in 20 mM potassium phosphate buffer with total $[K^+] = 34$ mM.

In the free-base form, Glu³ fluoresces with quantum yield $\phi_f(\text{fb}) = 0.16$. In spite of the strong nonplanar deformation of the porphyrin macrocycle induced by protonation [38], the Glu³ dication fluoresces with nearly the same quantum yield, $\phi_f(\text{dc}) = 0.15$, as the free base. On protonation, the emission spectra undergo a red shift (Fig. 3A and B) mirroring that of the absorption. The probe was excited at wavelengths corresponding to the free base and the dication: 515 and 600 nm, respectively. Using the integrated fluorescence spectra (Fig. 3A and B) as indicators of the dication and free-base concentrations, the fraction of freebase Glu³ was calculated using Eq. (2), plotted versus pH, and fitted with Eq. (3) (Fig. 3C). The fit yields $pK = 5.8$, in good agreement with that derived from the titration by absorption (Fig. 2B).

Fig. 3 clearly demonstrates that Glu³ can be used as a ratiometric fluorescent pH sensor, as it exhibits practically equal brightness (molar extinction times quantum yield) in its nonprotonated and protonated forms. This property is rare for fluorescent pH indicators, most of which fluoresce only in one form and do not permit ratiometric measurements. The accuracy of R_{fb} (Eqs. 1 and 2) is enhanced considerably when both states are followed spectroscopically. When the ratio of the integrated fluorescence spectra, I_{E515}/I_{E600} is, plotted against pH, as is shown in the inset of Fig. 3C, the midpoint of the plot occurs at a pH higher than the pK (6.45 compared to 5.8).

The following experiments were based on absorption measurements conducted using the system described above.

Ion effects on the dye

The absorption spectrum of Glu³ changes gradually and irreversibly in the presence of Zn^{2+} ions, probably as a result of the insertion of Zn^{2+} into the porphyrin. Subsequently, all zinc-containing materials were excluded from the experimental system and 0.5 mM EDTA was added to all solutions studied. Cu^{2+} effects similar spectroscopic changes (data not shown), and other metal ions with high affinity to the tetrapyrrole macrocycle can potentially influence the signal. Fortunately, common two-valent cations, such as Ca^{2+} or Mg^{2+} , do not form stable complexes with porphyrins in aqueous solution and do not affect the measurements.

The pK values of dendritic polyglutamic probes such as Glu³ can be influenced by the complexation between the peripheral carboxylate groups and metal cations. Such complexation can reduce the effective negative charge around the porphyrin, decreasing its apparent pK

[24,25]. To quantify this effect, we performed a series of pH titrations in the presence of K^+ and Mg^{2+} ions. $[K^+]$ was varied between 2.5 and 200 mM, and $[Mg^{2+}]$ was varied between 0 and 8 mM. The results are displayed in Fig. 4. For both K^+ and Mg^{2+} , the pK of the probe decreases with an increase in the cation concentration. This effect is much more pronounced in the case of Mg^{2+} . The salt effect is reproducible and stable, and it provides a means by which to tune Glu^3 toward a desired pK .

At the concentrations $[K^+] = 50$ mM and $[Mg^{2+}] = 1$ mM, typical for many protein assays, the pK of Glu^3 at 2 μ M is close to 5.6. At a lower ion concentration, $[K^+] = 10$ mM and no Mg^{2+} , pK is approximately 6.3. This pK enables pH measurements at physiological pH values, and the pK is increased further when monitoring intravesicular pH (see below). The effect of Na^+ was similar to that of K^+ (data not shown).

Dye adsorption to hydrophobic beads

Most protein reconstitution methods include a step of detergent removal conducted by dilution, dialysis, or the use of detergent-adsorbing hydrophobic polystyrene beads [31,39]. Hydrophobic beads are preferable because they guarantee virtually complete detergent removal, a narrow distribution of vesicle sizes, and no sample dilution. For the dye to be included in the liposomes, it needs to be added prior to detergent removal. Therefore, it is crucial that the probe remains dissolved throughout this process. We have tested several commercially available pH probes with respect to their adsorption to beads (see Table 1). All of the membrane-impermeable colorimetric probes were found to adsorb to hydrophobic beads to some extent. Glu^3 stands out, showing a very low propensity to adsorb to the beads. This makes this probe particularly suitable for inclusion into proteoliposomes.

Inclusion of the probe into liposomes

To calibrate Glu^3 for intravesicular pH measurements, it was characterized while encapsulated in LUVs. Glu^3 was enclosed in phospholipid LUVs, and the excess probe was rigorously removed from the bulk solution outside the vesicles as described in Materials and methods. The probe concentration was 200 μ M (i.e., ~100 times higher than that in the bulk experiments, Figs. 2–4). A cryo-TEM image of the Glu^3 -containing vesicles is shown in Fig. 5A. The vesicles are predominantly unilamellar. The darkening of the vesicle centers reveal that they protrude out of the surface of the isomorphous ice, suggesting that they have not been flattened during the TEM sample preparation procedure. The excellent mechanical stability of the vesicles is also confirmed by the fact that close-lying vesicles do not coalesce but rather show slight signs of deformation. The LUVs have a mean diameter of 150 nm and exhibit a relatively homogeneous size distribution; the diameters of 80% of the vesicles are distributed within ± 50 nm of the mean value (Fig. 5B).

The spectra and pK of Glu^3 in liposomes were measured in the presence of gramicidin (Fig. 6A). Gramicidin is a pore-forming peptide [40] in which oligomers form membrane-spanning channels, allowing protons, but not large charged molecules such as Glu^3 , to pass through the lipid bilayer. Thus, in the presence of gramicidin, the bulk pH and the pH inside the vesicles are rapidly equilibrated, whereas Glu^3 remains inside. The spectra of protonated and nonprotonated Glu^3 captured inside the vesicles are shown in Fig. 6A. The slope of the baseline is caused by the scattering of the vesicles; however, the spectra themselves are identical to those in Fig. 2A. Since the scattering does not vary with pH, the SNR of the titration curve is practically unaffected by the vesicle encapsulation. The strength of the measured signal suggests that the probe concentration and/or the spectral averaging time can be decreased considerably without compromising measurement accuracy.

Notably, when titrated inside the gramicidin-containing vesicles, the probe showed significantly higher pK than in the solution experiments (6.3 vs. 5.8) (Fig. 6B). This increase may be explained by the cation effect discussed above. Although the ion concentration is the same within the vesicles as in the solution ($[K^+] = 34 \text{ mM}$), the probe concentration inside the vesicles is 100 times higher than it was in the solution experiments. Thus, the salt concentration per probe molecule is lower, and the pK attenuation effect (Fig. 4) is diminished. To test this hypothesis, we determined the pK of Glu^3 in $4 \text{ mM } K^+$ solution at $[\text{Glu}^3] = 20$ and $2 \text{ }\mu\text{M}$. This 10-fold increase of Glu^3 concentration led to a 1% increase in pK ($pK = 6.55$ and 6.48 , respectively, $n = 1.1$ in both cases [data not shown]), consistent with a minor effect of the Glu^3 concentration on the pK value.

Fig. 7 displays the results of an experiment identical to that in Fig. 6 but performed in the absence of gramicidin. While pH in the medium was titrated from 7.5 to 4.6, the probe inside the liposomes remained virtually unaffected (solid spectra). When gramicidin was added at the end of the titration, rendering the lipid bilayer proton permeable, the probe was immediately protonated (dashed spectrum, lowest data point in Fig. 7B). The titration curve of the encapsulated probe in Fig. 6B is superimposed on Fig. 7B (inversed with the inversed x axis), demonstrating nearly perfect reproducibility at the end points of the titration. The slight increase in the pH after the addition of gramicidin is due to the accessibility of the encapsulated buffer ions and dye molecules (which also have buffering capacity) to the bulk protons. The buffer is near its pK inside the vesicles and so only 50% protonated, whereas the bulk buffer ions have nearly no buffering capacity at the end of the titration (pH 4.6). When gramicidin is added, the encapsulated buffer and dye molecules bind protons from the bulk, causing the observed increase in pH. The total volume of intravesicular solution is approximately 2% of the total sample volume.

Vesicle stability and leakage

A sample of the liposomes with Glu^3 inside was stored for 6 days in the dark at 4°C , and the spectra from day 1 and day 7 were virtually indistinguishable (data not shown). This demonstrates that the liposomes are extremely stable and that the probe does not change characteristics inside the LUVs over time. This experiment shows that the observed changes in Figs. 7 and 8 are not caused by dye variation over time but rather are a true reflection of the intravesicular pH.

Proton leakage through the phospholipid bilayer was investigated by exposing the liposomes, containing a probe solution equilibrated with the bulk at pH 7.2, to a sharp decrease of external pH to 5.6. Fig. 8 shows the temporal evolution of R_{fp} after this pH jump administered at time $t = 0$. There was no baseline drift over time; such a drift would be immediately evident from the spectra that were recorded throughout the measurement. Thus, the slope in Fig. 8 represents the change in pH inside the vesicles. It is clear that the decrease in external pH affected the internal pH only very slowly.

After 35 min, gramicidin was added, effecting an immediate pH equilibration across the membrane. At this point, the intravesicular pH had drifted from its original value by only 0.2 pH units. From these data, the half-time of proton leakage is estimated to be 130 min. This corresponds to an approximate leak rate of one proton per second and vesicle or 10 protons per second and μm^2 . This very low leakage rate shows that our vesicles are virtually proton impermeable in the absence of proteins and under the current experimental conditions, providing a good system for studying proton translocation.

Sensitivity of Glu³ as an intravesicular pH indicator

The previously reported experiment with Glu⁴ in SUVs [24] had one dye molecule per 1000 to 10,000 vesicles, leaving the majority of vesicles spectroscopically silent. Nonetheless, distinct fluorescence changes of Glu⁴, encapsulated in gramicidin-containing SUVs, were observed on a pH jump of 1 unit. In the current study, we used 200 μ M Glu³ in the dye incorporation step, resulting in an average of 170 probe molecules per LUV. Our vesicle concentration is on the order of 10 times lower than that in the SUV experiment [24]. Thus, the current setup should yield an approximately 100-fold higher intrinsic signal level, with all LUVs acting as detection units and each one reporting an average readout from 170 dye molecules. As is evident from the data in Figs. 2–4 and Fig. 6, at such a high signal level, the detection of Δ pH = 0.01 in the linear regime of the indicator is entirely feasible. If necessary, even smaller effects can be monitored by using fluorescence rather than absorbance and/or by following a single wavelength with a PMT detector that is more sensitive than the CCD system.

Conclusions

The probe/liposome system presented is well suited for measurements of biological proton transfer across phospholipid bilayers, and the titration/spectroscopy system enhances efficiency and accuracy. The probe Glu³ has a pK appropriate for biological applications. The probe was successfully included into liposomes and did not traverse the membrane, displaying excellent localizability. The absorbance signal from Glu³ enclosed in liposomes is intense and dominates the spectrum over the scattering caused by the vesicles. LUVs loaded with the probe show good stability over time and can be stored for several days. The proton permeability of the LUV phospholipid bilayer is very low under our experimental conditions, approximately 10 protons per second and μ m².

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Fig. 1. Molecular structure of probe Glu³. The tetraarylporphyrin chromophore (H₂P) is shown inside the dashed circle, and the nitrogen atoms undergoing protonation are shown in bold. The molecular weight of the probe is 4406 Da as the polycarboxylic acid and 5110 Da as the poly-Na⁺ salt.

Fig. 2.

(A) Absorption spectra of Glu³ in 5 mM potassium phosphate buffer with total $[K^+] = 9$ mM and $[Mg^{2+}] = 1$ mM, recorded with pH varying from 7.2 to 4.4. (B) Fraction of free-base Glu³ derived from the data in panel A using Eq. (1). The data were fitted by Eq. (3) (solid line), giving $pK = 5.8$ ($n = 1.3$).

Fig. 3. (A, B) Fluorescence spectra of Glu³ in 50 mM potassium phosphate buffer: (A) $\lambda_{\text{ex}} = 515$ nm; (B) $\lambda_{\text{ex}} = 600$ nm. (C) Fraction of free-base Glu³ derived from the integrated fluorescence spectra from panels A and B using Eq. (2). The data were fitted by Eq. (3) (solid line), giving $pK = 5.8$ ($n = 1.2$). The inset shows the ratio of the integrated fluorescence spectra, $I_{\text{E515}}/I_{\text{E600}}$, plotted against pH and fitted to a sigmoidal curve. The midpoint pH derived from the inset is 6.45. $[\text{Glu}^3] = 4\text{--}5$ μM ; total $[\text{K}^+] = 50$ mM.

Fig. 4. pK values of Glu³ as a function of [K⁺] (A) and [Mg²⁺] (B). For clarity, the data were fitted with these functions: $7.04 \times \text{pH}^{-0.00531}$ (K⁺) and $5.82 \times \text{pH}^{-0.00417}$ (Mg²⁺).

Fig. 5.
(A) Transmission electron micrograph of LUVs made from egg yolk phosphatidyl choline containing Glu³. (B) Size distribution of 216 vesicles.

Fig. 6.

(A) Absorption spectra of Glu³ enclosed in LUVs with membranes percolated by gramicidin. (B) Fraction of free-base Glu³ derived from the data in panel A using Eq. (1) as a function of pH. The experimental data (solid line) were fitted by Eq. (3), giving $pK = 6.31$ at $[K^+] = 34$ mM ($n = 1.1$).

Fig. 7. (A) Absorption spectra of Glu³ enclosed in LUVs in the absence of gramicidin. (B) Fraction of free-base Glu³ derived from the data in panel A using Eq. (1) as a function of pH. The lowest point was recorded last, after the addition of gramicidin. The fit from Fig. 6B is indicated.

Fig. 8. Fraction of free-base Glu³ enclosed in LUVs without gramicidin as a function of time. The initial pH was 7.2. At time 0, the bulk pH was changed to 5.6. After 35 min, gramicidin was added.

Scheme 1.
Protonation of the core porphyrin (H_2P) in Glu^3 .

Table 1

Comparison of pH and membrane-potential probes.

Indicator	Membrane permeable?	Detection ^a	Indicates ^b	Residual dye after bead incubation	Reference
Neutral red	Yes	A	[H ⁺]	n.a.	[41]
Phenol red	No	A	[H ⁺]	<1%	[16]
Pyranine (HPTS)	No	A, F	[H ⁺]	44%	[42]
BCECF	No	A, F	[H ⁺]	67%	[43]
SNARF	No	A, F	[H ⁺]	< 1%	[22]
SNARF-dextran (10 kDa)	No	A, F	[H ⁺]	72%	[23]
Glu ³	No	A, F	[H ⁺]	> 99%	
Oxanol	Yes	A	Δψ	n.a.	[44]
ACMA	Yes	F	Δ[H ⁺]	n.a.	[45]

Note. n.a., not applicable; HPTS, *hydroxypyrenetrisodium*; BCECF, bis(carboxyethyl) carboxyfluorescein; ACMA, aminochloromethoxy acridine.

^a Absorbance (A) and/or fluorescence (F).

^b Proton concentration ([H⁺]), membrane potential (ψ), or proton concentration difference (Δ[H⁺]).