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

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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 Glu$^3$, 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 p*K* 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 p*K* was measured in bulk solutions as well as inside large unilamellar vesicles in the presence of physiologically relevant ions. Glu$^3$ 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, Glu$^3$ was found to be superior to the commercially available pH indicators.



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, 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

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**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 | $\Delta\psi$ | n.a. | [44] |
| ACMA | Yes | F | $\Delta[H^+]$ | 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 ($\psi$), or proton concentration difference ($\Delta[H^+]$).

(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 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 $Glu^3$ (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 $Glu^3$ 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 $Glu^3$ remains stable and in solution throughout the procedure. This combination of properties makes $Glu^3$ an attractive alternative to SNARF–dextrans for biological proton translocation measurements.

In this work, we characterize the pH-sensitive, membrane-impermeable probe $Glu^3$ (generation 3 polyglutamic porphyrin dendrimer [25]) and validate its potential for biological pH measurements. $Glu^3$ 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 p*K* of the intrinsically weakly basic porphyrin to higher pH values, placing it in the physiological pH range [25]. The peripheral charges also render $Glu^3$ 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.

$Glu^4$ (generation 4 polyglutamic porphyrin dendrimer), a dendritic probe similar to $Glu^3$, was used in our previous experiments involving SUVs (20–30 nm in diameter). $Glu^4$ 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 $Glu^3$ exhibits a p*K* similar to that of $Glu^4$

[1] *Abbreviations used:* SNARF, seminaphthorhodafluor; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; $Glu^3$, generation 3 polyglutamic porphyrin dendrimer; $Glu^4$, generation 4 polyglutamic porphyrin dendrimer; CCD, charge-coupled device; UV, ultraviolet; PMT, photomultiplier tube; $H_2TPP$, 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; $H_2P$, tetraarylporphyrin chromophore.
[2] SNARF pH indicators (Invitrogen).