A facile method to build a proton nanosensor with neutral to basic pH sensitive range Post-print

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Abstract

Bio-nanosensors (Bio-NSs) have attracted much attention recently due to their unique properties. Among all of the bio-NSs, the intracellular proton sensor is significant for biomedicine studies and clinic diagnosis. Proton nanosensors (PNSs) with different pH sensitive ranges could satisfy different research requirements. Here we report a facile method to build a PNS with a neutral to basic pH sensitive range, in which the commercial pH indicator, fluoresceinamine (FA), was covalently coupled to the carboxylic-rich amphiphilic polymer (AP) coated gold nanoparticles (AuNPs).

Full Text

Preamble

A Facile Method to Build a Proton Nanosensor with Neutral to Basic pH Sensitive Range

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Bio-nanosensors (Bio-NSs) have attracted considerable attention recently due to their unique properties. Among various bio-NSs, intracellular proton sensors are particularly significant for biomedical research and clinical diagnosis. Proton nanosensors (PNSs) with different pH-sensitive ranges can satisfy diverse research requirements. Here we report a facile method to construct a PNS

with a neutral to basic pH-sensitive range, in which the commercial pH indicator fluoresceinamine (FA) was covalently coupled to carboxylic-rich amphiphilic polymer (AP)-coated gold nanoparticles (AuNPs).

Keywords: Fluoresceinamine, Proton nanosensor, Fluorescence, Spectrometer, Amphiphilic polymer
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Introduction

Nowadays, bio-nanosensors (bio-NSs) are opening new possibilities for intracellular measurements at submicron dimensions. Building bio-NSs is believed to provide great advances in our understanding of cellular function, thereby thoroughly revolutionizing cell biology. Ion channels embedded in cellular membranes control transmembrane ion gradients and intercellular signaling, making ionic homeostasis paramount for living systems, with any ionic perturbation potentially serving as a hallmark of disease. For example, tumor cells thrive in lower pH environments, while multidrug-resistant cells prefer higher pH surroundings. Consequently, engineering proton nanosensors to monitor intracellular pH will be valuable for both scientific research and clinical diagnosis.

Numerous commercial H^+ probes are available, such as BCECF, SNARF, and Oregon Green, which are widely applied in biomedical research and commonly called pH indicators. In principle, biologists could directly use these commercial indicators for intracellular pH sensing. However, several inherent disadvantages exist with these organic molecules. First, these indicators are small molecules, so cells cannot efficiently uptake them even when chemically engineered as ester derivatives. Second, upon entering cells, they are susceptible to digestion by intracellular enzymes. Third, the local spatial intensity of free indicators typically yields poor signal-to-noise ratios that cannot meet increasing demands for both scientific research and clinical diagnostics.

To address these three issues, a new generation of proton nanosensors (PNS) has been developed based on nanotechnology over the past decade. First, CdTe-based quantum dots (QDs) can directly sense pH as fluorescent nanocrystals due to additional passivation of the CdTe nanoparticle surface by a shell of cadmium thiolate complexes. In addition, lifetime-based pH sensing of CdSe/ZnS QDs has been reported. Second, fluorescence resonance energy transfer (FRET)-based NSs have been constructed by covalently coupling organic pH-sensitive dyes to QDs. Third, two or more dyes can be loaded into a single carrier for pH sensing, which also shows promise for developing multiplexed ion sensing.

Based on these advances in PNSs, several advantages emerge from linking organic proton indicators to NPs. First, the cellular uptake rate of NP-based PNSs is more efficient than that of free organic indicators. Second, when coupled to NPs, organic indicators can successfully evade intracellular enzymatic digestion due to surface-inhibition effects. Third, compared with free organic indicators, PNSs can provide much higher signal-to-noise ratios at subcellular

spatial resolution and have the potential to enable multi-ionic detection. In this paper, we demonstrate a facile approach to construct a PNS with an extended pH-sensitive range simply by conjugating a commercially available indicator to carboxylic-rich amphiphilic polymer (AP)-coated gold NPs (AuNPs).

Experimental Section

A. Hydrophobic AuNPs Synthesis

Dodecanethiol-protected AuNPs were synthesized according to previously published protocols with minor modifications. Briefly, 2.17 g of tetraoctylammonium bromide was dissolved in 80 mL toluene in a round flask. Meanwhile, 300 mg of tetrachloroauric acid was dissolved in 25 mL Milli-Q water under sonication. These two solutions were mixed in a 500 mL separation funnel and gently shaken for approximately 5 minutes. The aqueous solution was discarded, and the remaining solution was transferred to a round flask stirred with a magnetic bar. Next, 334 mg of freshly dissolved sodium borohydride in 25 mL Milli-Q water was added dropwise to the round flask within 1 minute. The reaction solution was stirred continuously for at least 60 minutes, then transferred back to a clean separation funnel and washed sequentially with 25 mL HCl (10 mM), NaOH (10 mM), and Milli-Q water. This washing procedure was repeated at least 4 times until the aqueous phase became colorless. The final solution was transferred to a new round flask and stirred overnight to achieve good size distribution via Ostwald ripening. The resulting solution was then poured into 10 mL 1-dodecanethiol and heated to 65 °C in a water bath for 2 hours with stirring. The solution was precipitated several times by either centrifugation or methanol addition to remove large aggregates and small impurities. Finally, all AuNPs were collected and redissolved in chloroform at a storage concentration of 6 µM.

B. AP Synthesis

The AP was synthesized according to published protocols. In practice, 2.70 g (15 mmol) of dodecylamine powder was dissolved in 100 mL THF. Then 3.084 g (20 mmol monomer) of poly(isobutylene-alt-maleicanhydride) powder was dissolved in the dodecylamine solution in a round flask under sonication. The solution was heated at 55–60 °C for 1 hour under stirring, during which the volume was reduced under vacuum to enhance the reaction, then left to react overnight under stirring. Finally, the THF was completely evaporated and the AP was redissolved in 40 mL chloroform to a final monomer concentration of 0.5 M.

C. Polymer Coating

The polymer coating procedure was completed according to previous reports. In this work, we mixed the AP solution with hydrophobic NPs at a molar ratio of 100–200 polymer monomers per nm^2 of the NP' s effective surface area (the core diameter of the hydrophobic NPs was determined by TEM analysis; the effective

diameter (d_{eff}) includes organic molecules considered to contribute a layer thickness of 1 nm around the inorganic core; the surface area per NP (A_{eff}) was estimated using the equation A_{eff} = 4π(d_{eff}/2)^2). After mixing, the solvent was slowly evaporated under reduced pressure until the sample was completely dried. The remaining solid film in the flask was redissolved in SB12 buffer (sodium borate, 50 mM, pH 12) under vigorous stirring until the solution turned clear.

D. Purification

In this work, we used ultrafiltration with ultrafilters (membrane: 100 kDa MW cutoff PES, Sartorius Stedim) and size exclusion chromatography (SEC) with a self-packed sephacryl S-300 HR column (GE Healthcare) connected to a high-performance liquid chromatography (HPLC) system (Agilent 1260) equipped with an autosampler, fraction collector, UV-vis absorption detector, fluorescence detector, and additional refractive index detector (RID). The mobile phase was SB9 buffer (sodium borate, 50 mM, pH 9), and the flow rate was maintained at 0.5 mL/min.

E. Size and Zeta-potential Measurement

Dynamic light scattering (DLS) equipment (Malvern, ZS90) was used to measure both size and zeta-potential of nanoparticles before and after linking with FA.

Results and Discussion

A. Chemical Engineering of PNS

To construct a PNS, we designed a facile chemical synthesis route as shown in Fig. 1. The main concept is to use AP-coated NPs (AP-NPs) coupled with commercial pH indicators, based on several considerations: AP coating is a general approach applicable to all hydrophobic NPs with different material properties because it employs hydrophobic interactions between AP and NPs. In detail, NPs synthesized in nonpolar solvents exhibit much more robust and higher quality than those synthesized in aqueous solution due to higher temperature annealing that eliminates most surface defects. Considering synthesis simplicity, we selected dodecanethiol-protected AuNPs as a typical NP example. Using previously published coating methods, the hydrophobic AuNPs were first coated with AP, followed by EDC chemistry to covalently conjugate FA to AP. Due to the substantial size difference, the PNS could be readily purified by SEC to remove excess FA molecules, which could also be verified by gel electrophoresis (GE, data not shown).

B. Characterization of PNS

From the design and synthesis strategy, the only impurities requiring removal are excess AP, AP-AuNP, AP-FA, and FA itself. Normally, amphiphilic molecules

tend to self-assemble into micelles in buffers, and AP is no exception. However, because AP micelles and AP-AuNPs share the same surface chemistry, neither of these impurities will influence the pH sensitivity of the PNS; in fact, AP micelles can be easily removed by ultracentrifugation or GE rather than SEC due to their similar size. Therefore, the only concern is FA, which can be readily removed by SEC because of the substantial size difference (Fig. 2Figure 2: see original paper). Using DLS, we measured the size of purified AP-AuNPs before and after linking with FA via EDC chemistry. The results show that after conjugation with FA, AP-AuNPs exhibit no significant size change (Fig. 2(b) and 2(c), both 12 nm in diameter), indicating that the small FA molecules do not substantially contribute to the PNS size. Zeta potentials were measured at -30 mV and -25 mV for AP-AuNPs before and after linking with FA, respectively. This confirms successful covalent conjugation via EDC chemistry, as each FA molecule consumes one carboxylic group of AP to form a peptide bond, and the increased zeta potential reflects the reduction of carboxylic groups on the surface.

C. pH Sensitivity Test

Both free FA and the PNS were tested for pH sensitivity using a static fluorescence spectrometer. As shown in Fig. 3[Figure 3: see original paper], free FA molecules exhibit pH-dependent fluorescence with maximum intensity at 509 nm. However, we found that the pH sensitivity of FA is irregular below pH 5, particularly at pH 3 and pH 4 (Fig. 3(a) and 3(b)), which is attributed to interference from the primary amino group (pKa = 9.4) on FA (carboxylic fluorescein does not show the same spectra, data not shown). In contrast, the newly constructed PNS shows improved pH-dependent fluorescence spectra (Fig. 3(c)), and from pH 2 to pH 10 all fluorescence intensities (maximum slightly shifted to 512 nm, Fig. 3(d)) increase regularly as pH rises, further indicating that the primary amino group disturbs the pH sensitivity of the FA indicator. From Fig. 3, both the free indicator and the newly built NS show a positive correlation between pH and fluorescence intensity. However, when plotting maximum fluorescence intensities against pH, a distinct optimization effect is observed after conjugating the free indicator to AP-AuNPs. For the free indicator, the positive correlation exists only from pH 5 to pH 8, whereas for the PNS, the positive correlation extends from pH 5 to pH 10, which is attributed to the Debye-screening effect as discussed in our previous work. More importantly, the pH sensitivity of the newly built PNS is significantly better than that of the free indicator itself, as evidenced by the gradients of the plotted sigmoid curves.

Conclusion

By conjugating a commercial pH indicator FA to AP-coated AuNPs, we have demonstrated a method to construct a new PNS with not only improved pH sensitivity (a lower gradient) but also an extended pH-sensitive range (from pH 5 to pH 10). To our knowledge, this represents the first PNS capable of

sensing more than 5 pH units. We attribute this altered pH sensitivity to a combination of the conjugated amino group of the free indicator and the carboxylic-rich AP-coated AuNPs, an approach that promises applicability to other existing molecular probes. We also hope this work provides inspiration for novel engineering of NP-based NS.

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Note: Figure translations are in progress. See original paper for figures.