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Expanding the dynamic measurement range for polymeric nanoparticle pH sensors†

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Conventional optical nanoparticle pH sensors that are designed for ratiometric measurements in cells have been based on utilizing one sensor fluorophore and one reference fluorophore in each nanoparticle, which results in a relatively narrow dynamic measurement range. This results in substantial challenges when conducting live cell measurements, which often leads to misleading results. In the present work we provide a simple solution to this problem.

A range of nanoparticle based sensors (nanosensors), based on optical detection using fluorophores, have been developed in recent years,¹ and have valuable properties in comparison to methods using free dyes such as higher signal, a potential for controlled cellular uptake and low non-specific permeability through cellular membranes, possibility for ratiometric measurements and often higher dye photostability.¹ Quantification of H⁺ concentrations in intracellular compartments is highly important for understanding a number of cellular processes, e.g. the cell internalization pathways (the endosome–lysosome system) and transmembrane H⁺ gradients in mitochondria, and furthermore has potential applications in tumor diagnostics.²,³ as well as a tool in development of new drug delivery systems.⁴,⁵

Nanoparticle pH sensors are particularly suited for measuring the pH in the endosome–lysosome pathway, where the pH ranges from ~4.0 to ~7.4, as nanoparticles are efficiently internalized by cells through endocytosis if they are designed correctly. As most commercially available fluorophores only have a sensitive range of up to 2 pH units (pKₐ ±1, as a rule of thumb) they are not very suited for kinetic measurements in the endosome–lysosome pathway. A few pH sensitive fluorophores cover a broader pH range, such as pHrodo, but the fluorescence intensity of pHrodo does not change linearly with pH in the sensitivity range, which is problematic for microscopy based measurements.

We and others have earlier synthesized nanoparticle pH sensors that have the advantage of reducing fluorophore cytotoxicity and protein binding and interference, by coupling a pH sensitive fluorophore (e.g. fluorescein) and a pH insensitive dye (e.g. rhodamine B) to a polymeric nanoparticle allowing ratiometric measurement of pH in living mammalian cells.⁸,⁹ When plotting the fluorescence intensity ratio (R = fluorescein/rhodamine) against pH, we obtain a standard pH titration curve from which we can quantify the pH in an intracellular measurement without knowing the sensor concentration inside the cell. A sensor based on fluorescein can measure pH from ~5.5 to ~7.3 (fluorescein pKₐ = 6.4) and by changing the sensor fluorophore to Oregon Green 488 (Oregon) having a pKₐ = 4.8, we can change the measurement range to 3.9–5.7.⁸,⁹ However, when utilizing these sensors in cellular measurements in the endosome–lysosome pathway we have recently learned that the rather limited measurement range creates fundamental problems. The sensors will often encounter a pH that is at the limit of the detection range, which results in erroneous quantification of pH leaving such sensors practically unusable for measurements in living cells.

Fluorescein and Oregon have different pH sensitivity ranges, but have the same emission maximum (520 nm), which was envisaged to provide a possibility for expanding the measurement range of nanosensors by combining two or more pH sensitive fluorophores in the right ratio into a nanoparticle. The necessary ratio will depend on the quantum yield of the fluorophores at a specific pH. We have investigated this possibility with the aim of developing a nanoparticle sensor that can measure pH in the entire range of the endosome–lysosome pathway and have utilized a versatile synthetic strategy where multiple fluorophores are easily conjugated to the nanoparticles (NPs).

We aimed to synthesize polymeric NPs in the form of a hydrogel with free amines distributed in the network that act as functional groups for fluorophore attachment and in addition render the overall ζ-potential of the particle positive. Positively charged particles are effectively endocytosed by cells. To achieve this, we first prepared the NP hydrogels as previously described.⁸ However, when preparing the nanoparticles by reverse microemulsion polymerization using hexane and water, in the presence of dioctyl sodium sulfosuccinate (AOT) and with Brij30 as a co-surfactant, we found that even after 5 days of dialysis, the nanoparticles still had a negative ζ-potential (~15 mV), which was due to AOT adhesion to the surface of the NPs.¹⁰ The negative surface charge results in poor cellular uptake of the nanoparticles and the AOT...
influences further synthetic manipulation. To prevent this, we used the non-ionic surfactant TX-100 for preparing the NPs.\textsuperscript{11} The nanoparticles with free amines (NP–NH\textsubscript{2}) were prepared by copolymerization of acrylamide and bismethylacrylamide with 3-amino-propyl-acrylamide in a microemulsion (for experimental details see ESI\textsuperscript{w}).

To synthesize a nanosensor with three different fluorophores (see Scheme 1), to broaden the measurement range, it is crucial to adjust the ratio of two pH sensitive fluorophores, in this case Oregon and fluorescein. The accumulated fluorescence intensity of Oregon and fluorescein should preferably increase linearly in the pH range 3.9 to 7.3. As Oregon and fluorescein have approximately the same quantum yield,\textsuperscript{1} it is expected that the optimal molar ratio should be approximately 1 : 1.

The conjugation of the fluorescence dyes to the nanoparticles was easily achieved by a simple one-pot procedure using the commercially available isothiocyanates (ITC), \textit{i.e.} FITC, Oregon Green ITC, RhB ITC that react readily with the free primary amine groups of the nanoparticles (Scheme 1). The reaction was carried out in a carbonate buffer solution at pH 9.2 for 1 hour at ambient temperature in the dark. The nanosensors were purified by dialysis against MilliQ water for 2 days. The success of the conjugation was verified by conducting a fluorescence emission scan using an excitation of 488 nm (Oregon and fluorescein) and 545 nm (RhB) giving an emission maximum of 520 nm for fluorescein/Oregon and 580 nm for RhB (Fig. 1). The calibration curves of NP–Oregon–fluorescein–RhB show an extended linear range at pH 3.9 to 7.3 in comparison to the free dyes Oregon–RhB and fluorescein–RhB with a linear pH range of 3.9 to 5.7 and 5.8 to 7.3, respectively (Fig. 2 and 3). This illustrates the simplicity of the method for expanding the measurement range of nanoparticle sensors.

We have employed a post-conjugation method to bind the fluorophores. This method is much more efficient than our earlier reported method,\textsuperscript{8} where we synthesized acrylamide-fluorophores that could be co-polymerized during the nanoparticle synthesis. We easily obtain 10 times higher concentrations of fluorophores in the nanoparticles, giving strong fluorescence intensity, which is important when conducting cellular measurements using microscopy.

The synthesized nanosensor NP–Oregon–fluorescein–RhB with a pH measurement range of pH 3.9 to pH 7.3 successfully serves the purpose of providing a sensor that can measure the pH in the endosome–lysosome pathway, with a minor limitation in the upper pH if one wants to measure e.g. the pH in the cytosol where the pH is expected to be 7.2–7.4. We have investigated how to enhance the pH measurement range further. One possibility is to add an additional sensor fluorophore with a high pK\textsubscript{a}, but it would be more ideal if we could get away with only using two sensor fluorophores (and one reference).

2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) with pK\textsubscript{a} 7.0 has been used to measure neutral pH in cells and
RhB with a fluorophore in the far red, Alexa 633. To avoid any further complications with FRET, we exchanged energy transfer (FRET) between the sensor dyes and RhB. To curve which could be attributed to weak Förster resonance energy transfer (FRET) between the sensor dyes and RhB. The titration curve showed a linear range from 3.9 to 7.9 due to insufficient overlap in buffering capacity of the fluorophores in the region of pH 5.9 (pKₐ too far apart, Fig. 3). We found that a mole ratio of Oregon/BCECF 1 : 1, by titrating in the pH interval 3.9 to 7.9, did not provide a sufficient linearity even though the quantum yields of the fluorophores are similar. We further found that the RhB concentration also affected the titration curve which could be attributed to weak Förster resonance energy transfer (FRET) between the sensor dyes and RhB. To avoid any further complications with FRET, we exchanged RhB with a fluorophore in the far red, Alexa 633, with a maximum absorption at ~633 nm and a emission maximum at ~644 nm, which was easily synthesized using succinimidyl esters to give a new nanosensor NP–Oregon–fluorescein–RhB, and NP–Oregon–BCECF–Alexa 633.

The pH measurement range of individual free pH sensitive fluorophores Oregon, fluorescein, and BCECF and the polymeric nanosensor NP–Oregon–fluorescein–RhB, and NP–Oregon–BCECF–Alexa 633 was measured in MilliQ water. The ε-potential of these two new nanosensors NP–Oregon–fluorescein–RhB and NP–Oregon–BCECF–Alexa 633 was 13.4 mV, 14.5 mV, respectively. Dynamic light scattering indicated that the hydrodynamic diameter of both nanosensors was 77 nm. Atomic force microscopy indicated that the sensors have a spherical morphology and did not show any sign of aggregation (Fig. 4).

In conclusion, by labelling polyacrylamide nanoparticles with two pH sensitive fluorophores Oregon (pKₐ = 4.8) and fluorescein (pKₐ = 6.4), and the pH inert fluorophore RhB, the nanosensor is capable of measuring a pH range from 3.9 to 7.3. To further expand the pH measurement range of the pH nanosensor, we developed another nanosensor containing two pH sensitive fluorophores Oregon and BCECF and the pH inert fluorophore Alexa 633. The new sensor with pH measurement range 3.9 to 7.9 could have great potential for measuring pH in almost all mammalian cellular environments. This method of labelling nanoparticles with two pH sensitive fluorophores can successfully expand the pH measurement range, to a range that is impossible to reach by using single fluorophores. This strategy offers a simple procedure for labeling nanoparticles with dual or multiple pH sensitive fluorophores to expand the pH measurement range and the method can be used in development of sensor technology for other metabolites.

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Notes and references