Supplementary information

A hydrogel based nanosensor with an unprecedented broad sensitivity range for pH measurements in cellular compartments

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Supplementary methods

Materials

The acrylamide and N,N’-methylenebis(acrylamide) monomers were purchased from Sigma-Aldrich. The reactive amino group containing monomer N-(3-aminopropyl)methacrylamide hydrochloride was obtained from Polyscience Inc. N,N,N’,N’-Tetramethylethlenediamine (TMEDA), ammonium persulfate, Triton X-100, 1-hexanol, cyclohexane, fluorescein isothiocyanate (FITC), EDC.HCl, N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and dialysis tubing (MWCO = 12 kDa) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (anhydrous), oregon green 488 isothiocyanate (F2FITC) mixed isomer and alexa fluor NHS ester were purchased from life technologies. The fluorophore difluoro-Oregon Green (DFOG) was synthesized as described in the main text. All other reagents and solvents were obtained from Sigma-Aldrich and used without further purification.

Preparation of poly(acrylamide) nanoparticle having primary amino groups

The poly(acrylamide) nanoparticle was synthesized as follows. Typically, 1.02 g of acrylamide, 0.255 g of N,N’-methylenebis(acrylamide) and 0.20 g of N-(3-aminopropyl)methacrylamide hydrochloride were dissolved in 3.075 mL of milliQ water. This monomer solution (2.85 mL) was added drop wise to 120 mL of oil phase, which was prepared by dissolving 62.5 g TX-100 and 153.27 g 1-hexanol in 1000 ml cyclohexane. A reverse microemulsion was formed under stirring for 10 min, after which the reaction mixture was degassed through five freeze-vacuum-thaw cycles and kept under argon atmosphere. Thirty microlitter of 25% (w/w) ammonium persulfate solution (free radical generator) and 20 µL of TMEDA (activator for free radical formation) were added to initiate the polymerization. The reaction mixture was stirred at room temperature for 3 h and the progress of the reaction was monitored by 1H NMR to ensure full conversion. The nanoparticles were precipitated by addition of ethanol and filtered using an Amicon ultrafiltration cell (Millipore Corp., Bedford, MA). The NPs was redispersed in MilliQ water with ultrasonic treatment and was dialyzed (MWCO = 12 kDa) against MilliQ water for three days (Dh = 55 nm and zeta potential (ζ = 29 mV) and lyophilized.

Preparation of pH nanosensor

Fluorophore stock solutions (1 mg/mL) were prepared in anhydrous DMSO.

Poly(acrylamide) nanoparticle (25 mg) was redispersed in MilliQ water with ultrasonic treatment and dialyzed (MWCO = 12 kDa) against PBS buffer (50 mM) for two days. To two milliliters of the nanoparticle dispersion in PBS buffer (12 mg/mL), was added F2FITC (50 µL), FITC (50 µL) Alexa 568 NHS (25 µL) and activated DFOG (150 µL) (the carboxylate group of DFOG was activated by treatment with EDC.HCl and Sulfo-NHS in MilliQ water at room temperature). The reaction mixture was stirred at room temperature for 12 h in the absence of light. The poly(acrylamide) pH nanosensor was transferred into dialysis tube (MWCO = 12 kDa) and
dialyzed against PBS buffer (50 mM) for three days and then against MilliQ water for another three days. The final concentration of the sensor was 6.25 mg/mL.

**Size and zeta potential measurements**

Dynamic light scatting measurements (DLS) and zeta potential measurements were carried out in MilliQ water using a ZetaPALS analyzer (Brookhaven Instruments Corporation) at room temperature. DLS measurements were carried out at fixed scattering angle (90°) and the measured number-averaged hydrodynamic diameter is an average of six measurement (duration of each measurement was 30 second). The given zeta potential value was an average of 3 measurement, each measurement consist of 10 runs and each runs having 10 cycles. Fluorescence measurements were carried out on an Olis upgraded SLM based spectrofluorometer. The obtained values for the nanosenser are: Dh = 53 ± 3 nm and ζ = 26 ± 4 mV.
Supplementary information

Emission spectra

Emission spectra were constructed by fluorescence measurements of the nanosensor at 0.152 mg/mL in 60 mM buffers (20 mM BIS-TRIS propane (pKa: 6.8; 9.0), 20 mM citric acid (pKa 3.1; 4.8; 6.4), 20 mM maleic acid (pKa: 2.0; 6.2) and 140 mM KCl) from pH 1.4 to 8.1 at 25°C. Absorbance spectra were obtained using an Olis upgraded SLM based spectrofluorometer to determine the optimal excitation wavelengths. The emission spectra were thus obtained by excitation at 500 (pH sensitive fluorophores) and 568 nm (reference fluorophore). The emission spectra in response to pH are presented in Figure S1. The three pH-sensitive fluorophores have identical excitation and emission spectra with respect to wavelength, and can therefore not be separated as such. But they do have different intensity profiles with respect to pH. From Figure S1 left it can be observed that the combined spectra are sensitive to pH in a range from approx. pH 1.4 to 8.1 with a 50 fold increase in intensity. This indicates that all three pH-sensitive fluorophores contributes to the sensitivity. Figure S1 right shows the pH sensitivity of the reference fluorophore Alexa 568 and it reveals that this fluorophore also has slight pH sensitivity between pH values 1.4 and 4.2. However, the change in intensity is only 2.2 fold, which will not have any influence on the overall pH-sensitivity of the nanosensor as the sensitivity of the green pH-sensitive fluorophores is much larger.

Figure S1. Emission spectra of the quadruple-labelled nanosensor by spectro-fluorometry in different pH buffers. Left: Combined spectra of the three pH-sensitive fluorophores DFOG, OG and FA. Right: Spectra of the reference fluorophore Alexa 568.
Distributions of intensity ratios

Images of nanosensor in buffers can be converted into histograms of ratios. In this case we have normalized the ratios according to the corresponding calibration curve which is fitted to equation 2 (presented in the main text); the normalization was performed by subtraction of $R_{\text{min}}$ and thus division by $(R_{\text{max}} - R_{\text{min}})$. The mean and the standard deviation (SD) of each of the normalized ratio distributions obtained for each tested pH value were thus calculated and the relative SD was obtained by division of the absolute SD by the normalized intensity ratio. Presented in figure S2 are thus the normally distributed curves with the respective mean and relative SD for each pH value. Due to the normalization of the ratios, the lower limit of the nanosensor corresponds to 0 and the upper limit corresponds to 1. Measurements that fall outside this limit cannot be used for conversion to pH via the calibration curve, and it is thus preferable to have as little measurements that fall outside these limits. It can be observed that even though the mean of the distributions of normalized intensity ratios are within this limit, many measurements within some of the distributions fall below or above these limits. It is therefore preferable to have as narrow distributions as possible. The width of a distribution is (among other parameters) related to the variation in numbers of the different fluorophores that are incorporated per particle. As more different fluorophores are introduced into a particle the number of each fluorophore will decrease. This means that a variation in the number of each fluorophore will be relatively larger the more different fluorophores are incorporated in the particle. As the distribution of normalized intensity ratios are dependent upon this variation in number of fluorophores, the distributions becomes wider the more fluorophores are incorporated. Therefore, there is a limit to the number of different fluorophores that can be incorporated into a nanoparticle in order to widen the sensitivity range.

![Figure S2](image)

**Figure S2.** Ratio histograms obtained from images of calibrations curves of the quadruple-labeled nanosensor in buffers with different pH values. Presented are normal distributions with
mean and relative SD obtained from the normalized intensity ratios of measurements of the nanosensor in buffers with different pH values.

**Experimental control measurements**

pH distributions are obtained from the images presented in figure 5 in the main text. These distributions of the control experiments are presented in figure S3 together with the distributions presented in figure 4b for ease of comparison. HeLa cells treated with Na$_2$SO$_4$ shows a comparable distribution with the untreated cells. This indicates that the lower pH observed after treatment with ATP is due to the direct action of ATP on the V-ATPase pump, and not due to an increase in the sodium concentration. Furthermore, the cells treated first with bafilomycin A$_1$ and then with ATP show a comparable pH distribution as for the bafilomycin A$_1$ treated cells. This indicates that the bafilomycin A$_1$ is indeed directly inhibiting the V-ATPase pump causing the observed increase in pH compared to untreated cells.

![Figure S3](image)

**Figure S3.** HeLa cells with internalized nanosensor after 24 h were either left untreated or treated with either ATP, Na$_2$SO$_4$, bafilomycin A$_1$ or first bafilomycin A$_1$ and then ATP where after they were imaged by confocal microscopy. The intensity ratios of each pixels in the obtained images were this converted to pH via the respective calibration curve, and histograms of pH distributions obtained. Mean ± SEM (n > 15 images). No measurements are below the lower limit of the nanosensor of pH 1.4. The following percentages of measurements are above the upper limit of the nanosensor of pH 7.0: 0.3%, 0.3%, 0.8%, 15% and 13% for the ATP, Na$_2$SO$_4$, untreated, Baf and Baf-ATP, respectively. Representative of four independent experiments.

The pH measurements presented in figure 4b in the main text, covering a pH interval from 3.0 to 7.0, cannot be performed with one single triple-labelled nanosensor, as its pH range is too narrow. To illustrate this we have performed some measurements with a triple-labelled nanosensor having a pH range of 3.6 to 6.9. Figure S4a shows measurements of untreated and bafilomycin A$_1$ treated cells as well as measurements performed on cells treated with the
ionophores nigericin and monensin in a buffer with pH 3.0. This treatment enables protons to cross the cellular membranes and the intracellular pH will equalize with the outside pH. This triple-labelled nanosensor measures nicely the pH of the untreated and bafilomycin A₁ treated cells, but the low pH in the ionophore treated cells cannot be measured. As the lower limit of the nanosensor is 3.6, all measurements obtained in these cells fall below this limit, and are thus presented as < 3.6. It can be observed that no measurements of the untreated and bafilomycin A₁ treated cells falls below this limit. This shows that it would not be possible to perform all the measurements presented in Figure 4 and Figure S3 with this triple-labelled nanosensor.

**Figure S4.** (a) HeLa cells with internalized nanosensor after 24 h were either left untreated or treated with either bafilomycin A₁ or the ionophores nigericin and monensin in a K⁺ rich buffer with pH 3.0 where after they were imaged by confocal microscopy. The intensity ratios of each pixel in the obtained images were then converted to pH via the respective calibration curve (b), and histograms of pH distributions obtained. Mean ± SEM (n > 10 images). 100% of the measurements obtained from the ionophore treated cells are below the lower limit of the nanosensor, whereas no measurements obtained from the untreated and Baf treated cells fall below this limit.