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pH sensing in living cells using fluorescent microspheres

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Abstract—Intracellular pH in living cells is measured in real time at the single cell level using fluorescently covalently loaded microspheres as efficient carrier systems and stable sensors. The use of these sensors immobilized covalently onto polymeric particles allows analysis of intracellular pH flux over long period of time and eliminates the disadvantages such as dilution within the cell, elimination via leakage or compartmentalization.

The pH of the cytosol affects a wide range of cellular processes and functions and, as a consequence, is regulated within a narrow range by a variety of transport proteins that transfer ions across the cellular membrane.[1,2] Traditional methods to measure intracellular pH include the use of ^{31}P NMR spectroscopy,[3] microelectrodes[4] and fluorescent probes,[5,6] with recent approaches including the use of quantum dots (QDs)[7] and indicators based on green fluorescent protein (GFP).[8]

Fluorescent indicators in particular are valuable tools for measuring changes in intracellular proton concentration, providing the necessary sensitivity required for optical pH measurements inside living cells. Typical fluorescent probes used for pH_i measurement are based on fluorescein and its many derivatives, which exhibit multiple pH-dependent equilibria.[9,10] However, although fluorescein has been used to measure intracellular pH,[11] its use is restricted for two reasons. First because of its rapidity in leaking from cells and because it is very difficult to quantify intracellular pH (because the decrease in the cell fluorescence due to fluorescein leakage cannot be easily distinguished from that due to pH changes,[12] although some fluorescein-derivatives such as BCECF and SNARF have been developed to overcome this problem).[13,14] Second, because dyes of this type are typically loaded into the cell as their cell-permeable acetoxymethyl (AM) ester derivatives (the neutral molecule is able to pass across the membrane) and once inside the cell esterases cleave to reveal the acid groups; however, this method has disadvantages, such as compartmentalization and prolonged cellular leakage (as discussed above).[15] Another common problem that is encountered with all potential sensors or cellular tags is dilution and cellular degradation, which results in little or no signal. Commonly used dextran conjugates offer a solution to these problems, but the drawback of this approach is their compartmentalization within endosomes, which requires the addition of chloroquine to facilitate release and subsequent pH sensing.[7]

To be used for pH_i measurements the fluorescent probe should be non-toxic, have a pK_a within the physiological range (generally between ~6.8 and 7.4) to allow detection of small pH changes typical in a cell, while having excitation and emission wavelengths suitable for detection by flow cytometry, fluorescence microscopy or other techniques relying on cell fluorescence analysis. Also the fluorescence should be stable overtime.[16]

Previously we have demonstrated that amino functionalised polystyrene microspheres can be used successfully for calcium sensing while avoiding dilution and cellular

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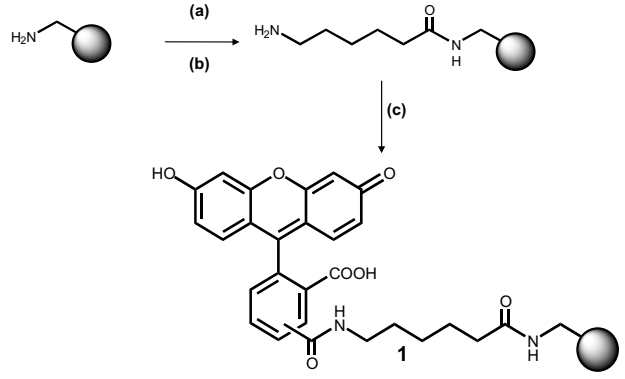
degradation within the cells.[17] In this paper, fluorescein-loaded microspheres are used for real-time pH sensing in living cells. Binding fluorescein covalently to microspheres eliminates leakage of the dye from cells and ensures a highly localised 'dye' that allows 'on-bead' analysis. Additionally, due to their micron size the labelled microspheres are easily visualised by microscopy.

The sensor-loaded microsphere **1** was prepared by coupling 5(6)-carboxyfluorescein onto 2 μm aminomethyl microspheres derivatized with an aminohexanoic acid spacer (Scheme 1).[18] This synthesis was achieved following Fmoc strategy for solid phase chemistry (see supporting information for experimental details).



Scheme 1. Preparation of fluorescein-labelled amino functionalised polystyrene microspheres **1**. Reagents and conditions: (a) *N*-Fmoc-aminohexanoic acid (5 equiv), HOBt (5 equiv), DIC (5 equiv), DMF, 12 h; (b) 20% piperidine in DMF; (c) 5(6)-carboxyfluorescein (5 equiv), HOBt (5 equiv), DIC (5 equiv), DMF, 12 h. HOBt = *N*-hydroxybenzotriazole; DIC = *N*,*N*′-diisopropylcarbodiimide, DMF = dimethylformamide.

Quantitative emission spectra were obtained using a custom-built system consisting of excitation and emission optical fibres mounted on a micro-capillary in which the sample was flown via a syringe pump. This setup allowed fluorescence measurement of a sample free from artefacts such as aggregation, scattering, photo-bleaching and sedimentation. In this manner spectrofluorimetric studies were carried out to evaluate how immobilization of the sensor affected its sensitivity to $[H^+]$ by examining the microspheres at different buffered pH values. All measurements of pH were made using the ratio of the fluorescence emitted at 525 and 610 nm. This ratio corrects for changes in cell volume and fluorochrome uptake.[19] Importantly, the sensor had very similar fluorescence profile when bound to the microspheres as in solution (Fig. 1a) (for the 'in solution' details, see supporting information). Flow cytometry studies were also carried out to analyze the fluorescence properties as a function of pH and showed an identical profile (see Fig. 1b).

Several cell lines (B16F10, HEK-293T and L929) were treated in triplicate with 2 μm fluorescein-loaded microspheres **1** at a range of concentrations (0.1–0.8 mg/mL) over four different time periods (3, 6, 12 and 24 h) (for detailed protocol, see the supporting information). Following incubation, the excess microspheres were removed, and analysis by flow cytometry and fluorescence microscopy showed that cellular uptake of the microspheres was effective in all cases. The microspheres were delivered into the cells, with varying

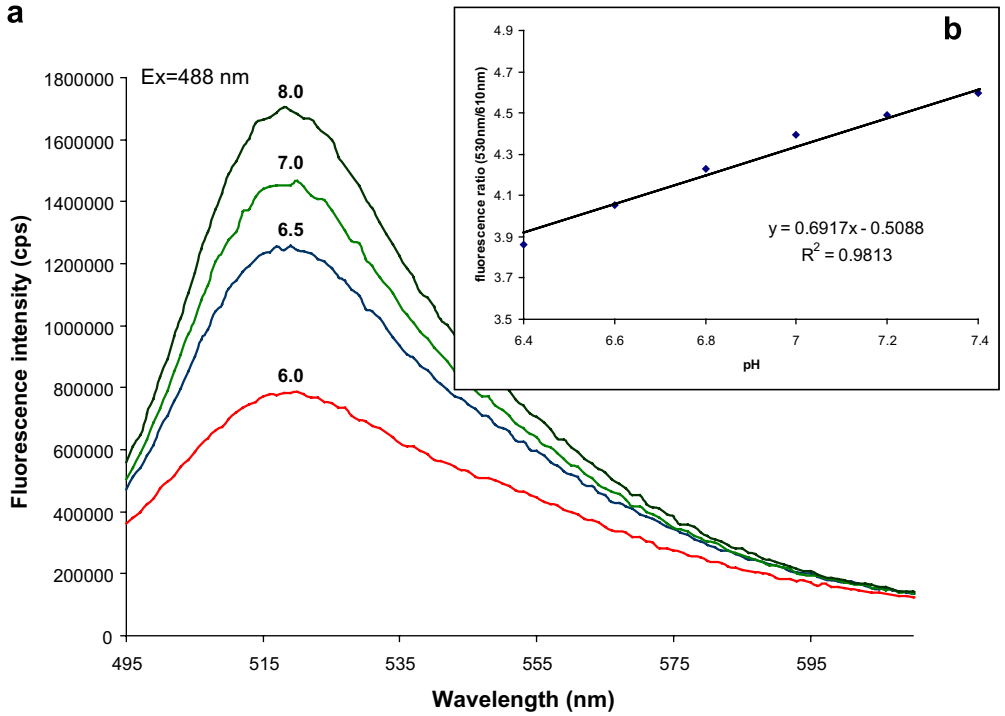


Figure 1. (a) Fluorescence emission spectra (λ_{ex} = 488 nm) and (b) calibration curve of pH versus fluorescence intensity ratio (525 nm/610 nm) (obtained by flow cytometry analysis) for fluorescein-loaded microspheres **1** as a function of pH.[20]

degrees of success depending on the cell type investigated. Figure 2a shows the results of HEK-293T cells (as a representative example) after incubation with 2 μm fluorescein-loaded microspheres (0.1 mg mL^{-1}) for 24 h. Figure 2b shows the confocal image obtained when B16F10 cells were incubated with 2 μm fluorescein-loaded microspheres (0.1 mg mL^{-1}) for 6 h. Cells containing the beads (ca. 30%) were isolated by fluorescence-activated cell sorting and were shown by confocal microscopy after 72 h of incubation at 37 °C to be healthy with the sensor-microspheres inside the cells. The cell membrane was stained with a red fluorescent dye (PKH26, Sigma–Aldrich) which allows long-term in vitro analysis of live cells. Figure 2c shows the flow cytometric analysis of the rate of uptake of fluorescein-labelled microspheres (0.1 mg mL^{-1}) over a range of incubation times. A total of 10,000 events per sample were analyzed. FITC (530/30 nm) band pass filters were used for fluorescence analysis of the cell suspensions. Cellular uptake was found to be concentration and time dependent.

To analyze the stability of the microspheres some experiments were performed. The fluorescence intensity of the microspheres inside cells was analyzed at different time points. We found that fluorescence was stable after these incubation times (see supporting information for details). This result shows that the microspheres are stable and they are not metabolized by the intracellular enzymes.

To determine if the microspheres internalized by the cells are in lysosomes, an acidic organelle-selective fluorescent probe was used (Lysotracker Red DND-99, Invitrogen). The cells were incubated with fluorescein-loaded microspheres and the red dye at 37 °C. This experiment shows that the microspheres lie outside of the lysosomes, since the green fluorescence is anticorrelated with the red fluorescence, which marks the periphery of the lysosomes (see supporting information for details). This observed behaviour of the particles not being in the lysosomes greatly increases the potential applicability of these materials for cytosolic analysis.

Microsphere-induced cytotoxicity was investigated by a MTT assay to evaluate the impact of uptake on cell viability.[21] The fluorescein-loaded microspheres were found to be non-toxic at all of the concentrations tested (see supporting information for details).

To evaluate the microspheres as intracellular pH sensors, the beads were examined in triplicate by flow cytometry with cells plated at various values of pH. A total of 10,000 events per sample were analyzed. The green fluorescence was recorded using the 515–545 nm band pass filter and the red fluorescence with the 600–620 nm band pass filter.

A calibration curve (fluorescence ratio against pH) was constructed from labelled cells resuspended in a high concentration of potassium buffer of appropriate known pH by treatment with nigericin before flow cytometry analysis.[19] (In the presence of nigericin, an antiporter of H^+ and K^+, the intracellular [H^+] becomes equilibrated to the extracellular [H^+].) Figure 3 shows the response obtained for HeLa and HEK-293T cells loaded with 2.0 μm fluorescein-labelled microspheres, showing

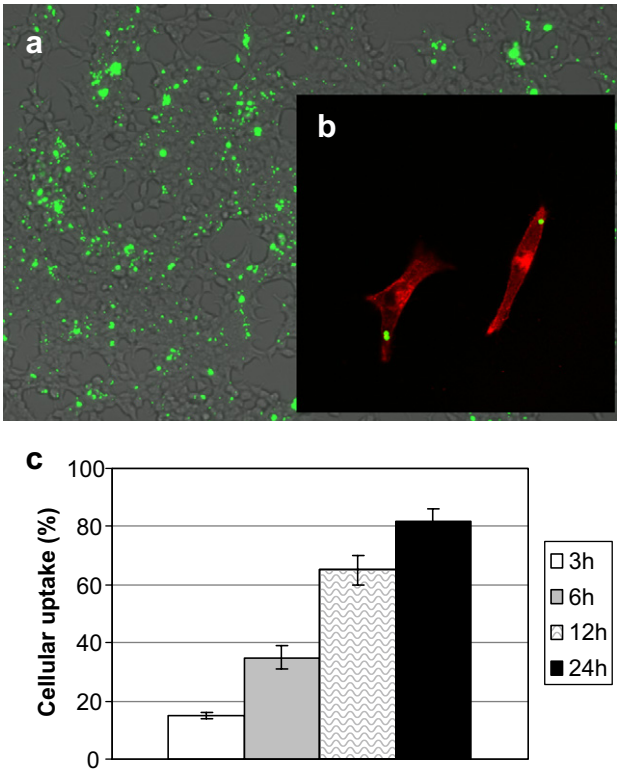


Figure 2. (a) Microscopy image for living HEK-293T cells after incubation at 37 °C for 24 h with 2 μm fluorescein microspheres. (superimposed image of bright phase and fluorescein filter images). (b) Confocal microscopy image of melanoma cells (B16F10) loaded with fluorescein microspheres **1** (green circles) after 6 h of incubation at 37 °C. (c) Flow cytometry results for cellular uptake of 2 μm fluorescently labelled microspheres into B16F10 cells overtime (0.1 mg/mL), at 37 °C with 5% CO_2.

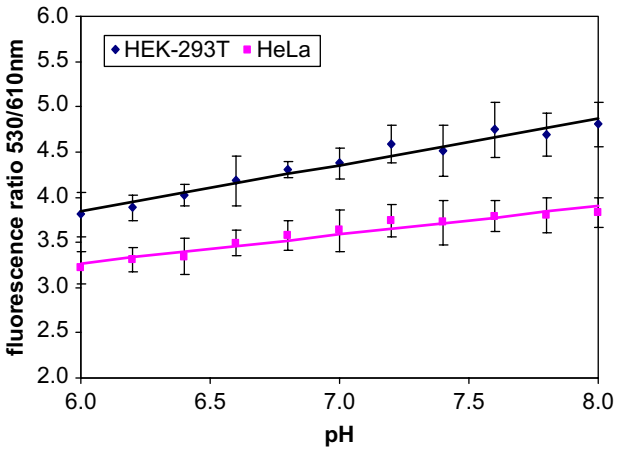
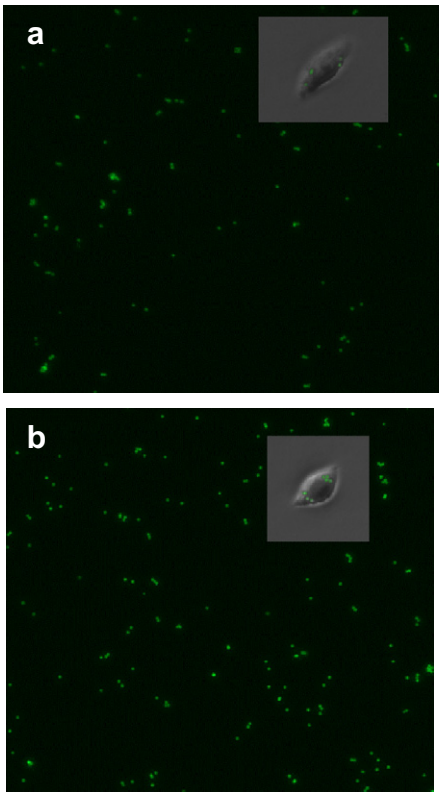


Figure 3. Fluorescence ratio of fluorescein-loaded microspheres **1** in two different living cell lines as a function of pH (obtained by flow cytometry analysis, performed in triplicate).[22]



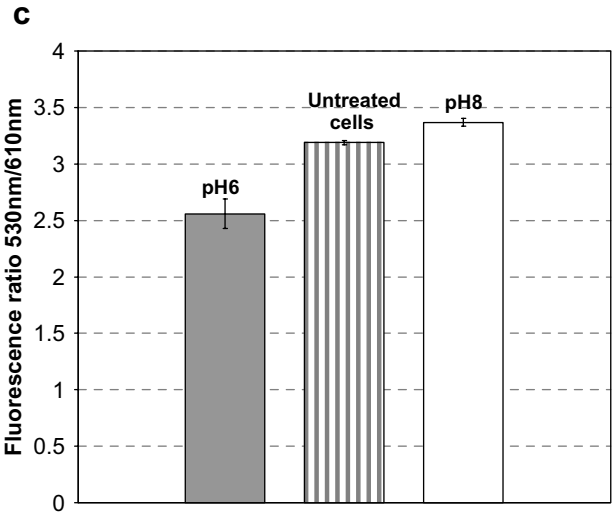


Figure 4. Fluorescence microscopy image of L929 living cells loaded with fluorescein microspheres **1** (green circles) after 12 h of incubation at 37 °C. (a and b) The green fluorescence images obtained for pH 6 and 8, respectively. (c) Quantitative analysis of fluorescence intensity ratio versus pH by microscopy.[22,23]

how the fluorescence ratio increases as a function of intracellular pH (in equilibrium with extracellular pH) from 6.0 to 8.0. The differences in the fluorescent ratio between these two cell lines can be explained by the different capabilities of each cell line to take up microspheres.

Intracellular changes in the pH were also detected globally by microscopic examination of the fluorescein-labelled microspheres in cells. Fluorescence images of cells loaded with fluorescein microspheres were taken at different pH values after treatment with nigericin. The images in Figure 4a and b, which are representative of all the cells that we observed, demonstrate qualitatively the pH sensitivity of these microspheres in cells. The fluorescence intensity increases when the pH increases from 6 to 8. The quantitative results in Figure 4c show that the fluorescence intensity increases by approximately 25% when pH is changed from 6 to 8. As expected, the value of the untreated cells was between pH 6 and 8.

Finally the fluorescein-loaded microspheres were compared with the traditional sensor fluorescein diacetate (FDA) by microscopy and flow cytometry. After 2 h of incubation, the fluorescence intensity for FDA decreased for the same pH value while there was no loss of fluorescence for the microspheres. These results show that the leakage problem was completely solved using the bead approach (see details in supporting information).

The use of fluorescein-loaded microspheres as intracellular pH sensors in living cells has been successfully proved using a number of different techniques such as spectrofluorometry, fluorescence microscopy and flow cytometry. Covalent binding of fluorescein to the microspheres dramatically improves the stability of the indicator overtime and eliminates leakage. At the same time it retains the properties of fluorescein for pH sensing. The fact that these polymeric particles are not toxic, in addition to their cytosolic localisation, makes them perfect candidates for intracellular pH sensing. These fluorescein-loaded microspheres provide a reliable way to perform long-term cell monitoring. These results encourage the possibility of use of this approach for other ions such as K^+ or Na^+ and for other applications such as monitoring of enzymatic activity or drug delivery.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.075.

References and notes

1. Roos, A.; Boron, W. F. *Physiol. Rev.* **1981**, *61*, 296.
2. Frelin, C.; Vigne, P.; Ladoux, A.; Lazdunski, M. *Eur. J. Biochem.* **1988**, *174*, 3.
3. Kulichikhin, K. Y.; Aitio, O.; Chirkova, T. V.; Fagerstedt, K. V. *Physiol. Plant.* **2007**, *129*, 507.
4. Sommer, S.; Jahn, A.; Funke, F.; Brenke, N. *Naturwissenschaften* **2000**, *87*, 283.

5. Koo, M. K.; Oh, C. H.; Holme, A. L.; Pervaiz, S. *Cytometry A* **2007**, *71*, 87.
6. Briggs, M. S.; Burns, D. D.; Cooper, M. E.; Gregory, S. J. *Chem. Commun.* **2000**, 2323.
7. Liu, Y. S.; Sun, Y. P.; Vernier, P. T.; Liang, C. H.; Chong, S. Y. C.; Martin, A.; Gundersen, M. A. *J. Phys. Chem. C* **2007**, *111*, 2872.
8. Bizzarri, R.; Arcangeli, C.; Arosio, D.; Ricci, F.; Faraci, P.; Cardarelli, F.; Beltram, F. *Biophys. J.* **2006**, *90*, 3300.
9. Diehl, H.; Horchak-Morris, N. *Talanta* **1987**, *34*, 739.
10. Klonis, N.; Sawyer, W. H. *J. Fluoresc.* **1996**, *6*, 147.
11. Khodorov, B.; Valkina, O.; Turovetsky, V. *FEBS Lett.* **1994**, *341*, 125.
12. Haworth, R. S.; Fliegel, L. *Mol. Cell. Biochem.* **1993**, *124*, 131.
13. Shimoda, L. A.; Luke, T.; Sylvester, J. T.; Shih, H. W.; Jain, A.; Swenson, E. R. *Am. J. Physiol.* **2007**, *292*, L1002.
14. Kang, M.; Walker, J. W. *Exp. Biol. Med.* **2006**, *231*, 865.
15. Tsien, R. Y. *Nature* **1981**, *290*, 527.
16. Tsien, R. Y. *Annu. Rev. Neurosci.* **1989**, *12*, 227.
17. Sanchez-Martin, R. M.; Cuttle, M.; Mittoo, S.; Bradley, M. *Angew. Chem. Int. Ed.* **2006**, *45*, 5472.
18. Sanchez-Martin, R. M.; Muzerelle, M.; Chitkul, N.; How, S. E.; Mittoo, S.; Bradley, M. *ChemBioChem* **2005**, *6*, 1341.
19. Franck, P.; Petitipain, N.; Cherlet, M.; Dardennes, M.; Maachi, F.; Schutz, B.; Poisson, L.; Nabet, P. *J. Biotechnol.* **1996**, *46*, 187.
20. A set of high [K^+] buffers of varying [H^+] were prepared by mixing appropriate proportions of KH_2PO_4 (135 mM) and K_2HPO_4 (110 mM) in 20 mM NaCl to give pH values in the range of 6.0–8.0.
21. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
22. A set of high [K^+] buffers of varying [H^+] were prepared by mixing appropriate proportions of KH_2PO_4 (135 mM) and K_2HPO_4 (110 mM) in 20 mM NaCl to give pH values in the range of 6.0–8.0. Each cell sample was treated with nigericin (10 μM) for 5 min before flow cytometry analysis. The samples were analyzed in a random order and each experiment was done in triplicate.
23. To perform quantitative analysis images of cells loaded with fluorescein microspheres were taken at two different positions in each well and the fluorescence intensity of every bead in the defined region measured (all experiments were done in duplicate with approximately 500 beads measured per image, giving a total of approximately 1000 fluorescent intensities per well and therefore, 2000 fluorescent intensities per pH value). These intensities were summed and averaged to give the values in Figure 4c.