Hindawi Publishing Corporation
Journal of Sensors
Volume 2009, Article ID 235158, 4 pages
doi:10.1155/2009/235158

Research Article

Characterization of PEBBLEs as a Tool for Real-Time Measurement of *Dictyostelium discoideum* Endosomal pH

Everett Moding,[1] Jessica Hellyer,[1] Kevin Rank,[1] Phoebe Lostroh,[2] and Murphy Brasuel[1]

[1] *Department of Chemistry/Biochemistry, Colorado College, 14 East Cache La Poudre Street, Colorado Springs, CO 80903, USA*
[2] *Department of Biology, Colorado College, 14 East Cache La Poudre Street, Colorado Springs, CO 80903, USA*

Correspondence should be addressed to Murphy Brasuel, mbrasuel@coloradocollege.edu

Received 26 May 2009; Revised 21 July 2009; Accepted 4 August 2009

Recommended by Mike McShane

The measurement of intracellular ion concentration change is important for understanding the cellular mechanisms for communication. Recently developed nanosensors, (Photonic Explorers for Biomedical use with Biologically Localized Embedding) PEBBLEs, have a number of advantages for measuring ions in cells over established methods using microelectrodes, unbound fluorescent dyes, or NMR. PEBBLE sensors have been shown to work in principle for measuring dynamic ion changes, but few in vivo applications have been demonstrated. We modified the protocol for the fabrication of pH sensing PEBBLEs and developed a protocol for the utilization of these sensors for the monitoring of dynamic pH changes in the endosomes of slime mold *Dictyostelium discoideum (D. discoideum)*. Oregon Green 514-CdSe Quantum Dot PEBBLEs were used to measure real-time pH inside *D. discoideum* endosomes during cAMP stimulation. Endosomal pH was shown to decrease during cAMP signaling, demonstrating a movement of protons into the endosomes of *D. discoideum* amoebae.

Copyright © 2009 Everett Moding et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Dictyostelium discoideum is a eukaryotic slime mold that undergoes both unicellular and multicellular development with multicellular differentiation occurring after cAMP-dependent aggregation of unicellular amoebae [1]. Many of the mechanisms that the amoeba use during cell-cell signaling, chemotaxis, and cytokinesis are homologous to those in higher, more complex eukaryotic organisms, making *D. discoideum* a well-studied model organism. Additionally, the slime mold completes its life cycle in a matter of days and the development can be stopped and studied at any stage [2, 3]. *D. discoideum* cAMP stimulation has been shown to elicit a proton efflux that generates internal asymmetries in aggregating cells, promoting efficient movement toward chemoattractants [4–6]. While the mechanism by which cytoplasmic alkalinization occurs is not completely understood, H^+ ATPases, Na-H exchangers and acidic vesicles have been implicated in the proton efflux [7, 8], and the purpose of this work was to develop and test novel nanosensors for monitoring this pH change.

Currently, pH-sensitive fluorescent dyes such as fluorescein isothiocyanate (FITC)-dextran are being used to measure endosomal pH in vivo [9–11]. However, photobleaching, free dye to cell interactions, and changes in cell autofluorescence can affect the accuracy of these measurements [12–14]. Furthermore, the nearly neutral pKa of FITC-dextran (5.93) makes the dye poorly suited for measuring pH in acidic endosomes [15]. The drawbacks of FITC-dextran have been circumvented by using internalized aminophosphonates as ^{31}P-NMR pH probes [16] for single measurements of intracellular pH. However, ^{31}P-NMR cannot be utilized for real-time dynamic applications, and it is impractical for most biology laboratories [17, 18].

PEBBLEs and other nanosensors have been established as minimally invasive tools to measure real-time ion concentrations within living cells and have been previously used to make intracellular pH measurements [19–21]. PEBBLEs circumvent the problems with free dyes by encapsulating two or more fluorophores inside a polyacrylamide matrix, permitting ratiometric imaging while preventing differential sequestration by intracellular organelles [22]. The matrix

allows small ions to interact with the dyes while prohibiting macromolecule interference, eliminating fluctuations in fluorescence caused by cell-dye interactions [23]. Additionally, PEBBLEs are slow to photobleach, are cost-effective, and can be easily synthesized by microemulsion polymerization. In this work, we have fabricated probes capable of measuring the pH in acidified endosomes of *D. discoideum*. We have developed protocols for the endocytosis of PEBBLEs into *D. discoideum*. The resultant pH measurements achieved with PEBBLEs are within the 7.3–4.3 published range of endosome pH as measured by ^{31}P-NMR [16]. PEBBLEs have the advantage of following these pHs dynamically over several hours while following the response *D. discoideum* to changes in environmental conditions or during exposure to chemical stimulation.

2. Experimental

2.1. Chemicals. The signal transduction components of the PEBBLE sensors, Q Dot 655 ITK amino (PEG) quantum dots (8 μM solution), Oregon Green 514; 70,000 MW dextran, were purchased from Invitrogen (Carlsbad, Calif, USA). All other chemicals, acrylamide, N, N′-methylenebisacrylamide, (docusate sodium salt) AOT, Brij 30 (polyethylene glycol dodecyl ether), hexanes, sodium metabisulfite, MOPS (3-(N-Morpholino) propanesulfonic acid), Tris (Tris(hydroxymethyl)aminomethane), hydrochloric acid, and cAMP (Adenosine 3′,5′-cyclophosphate) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Contacts for obtaining the AX3 strain of *D. discoideum* were made through dictybase (www.dictybase.org).

2.2. Nanosensor (PEBBLE Construction). Oregon Green was chosen as a pH-sensitive dye with a lower pKa (4.8) than FITC-dextran, allowing more accurate pH measurements in acidic endosomes [24]. Additionally, Oregon Green can be excited with longer wavelength light, reducing the effect of *D. discoideum* autofluorescence on measurements [12]. While Oregon Green is a ratiometric dye, CdSe Quantum Dots were found to increase the accuracy of pH measurements by providing a spectrally resolvable reference peak within the PEBBLEs. CdSe Quantum Dots have high quantum efficiency and emit a narrow fluorescence peak, minimizing overlap of reference and sensing dye signals [25, 26].

Oregon Green 514-CdSe Quantum Dot PEBBLEs were fabricated by microemulsion polymerization. Briefly, 43 mL of hexanes in a 100 ml round bottom flask was positioned in the water bath at 30–35°C. The hexanes were stirred for 20 minutes under nitrogen gas. However, 1.59 g AOT and 3.14 g Brij 30 were added and allowed to dissolve until clear. Also, 1.8 mL of an acrylamide solution (13.5 g acrylamide, 4.0 g bis-acrylamide, 45 ml 10 mM MOPS buffer) was added dropwise and allowed to stir for 5–7 minutes; 140 μL of deionized H_2O was added followed by 30 μL of Oregon Green (1 mg/mL) and 30 μL of Q Dot 655 ITK amino (PEG) quantum dots (8 μM). The reaction was allowed to stir for additional 5–7 minutes. Then 10 μl of freshly made 10% sodium metabisulfite was added and the

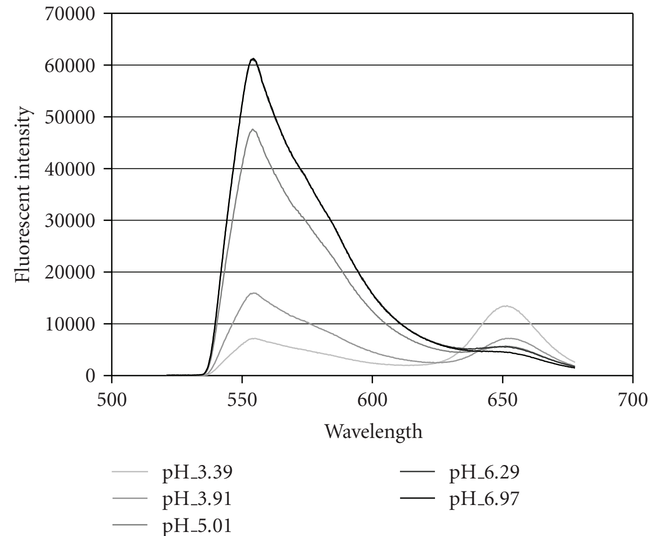

Figure 1: Representative fluorescence emission spectra of Oregon Green 514-CdSe Quantum Dot PEBBLEs, displaying the pH dependence of the Oregon Green peak (554 nm) and the Quantum Dot peak (653 nm) ratio.

round bottom flask was sealed and allowed to continue to stir for 2 hours. The resultant PEBBLE nanosensors were washed by precipitation with 2-propanol, centrifugation, and then repeated (4 times) resuspension in deionized H_2O and re-precipitated with 2-propanol to remove any trace surfactants. After washing the particles are stored dry until used.

2.3. PEBBLE Calibration. The PEBBLEs were calibrated in Tris buffer by adding 0.01 M HCl from pH 7.00 to 2.00 (recorded with a pH meter) while recording the fluorescent spectra. A dynamic range was observed from pH 3.55 to 7.00 with a linear calibration curve of $y = 3.9053x - 13.174$ and an R^2 value of 0.9568 for the ratio Oregon Green 514 emission (554 nm) to the CdSe quantum dot emission (653 nm). Fluorescent spectra (Figure 1) and images were obtained with a Zeiss Axioscope fluorescent microscope, mercury arc lamp excitation source, and a Chroma custom filter cube with a D510/20x excitation filter, a 530 DCLP beam splitter, and an HQ545 LP emission filter. The 510 nm excitation was chosen to minimize cell autofluorescence. Spectra were collected using a PI Acton SpectraPro SP-2156 spectrograph, an Arc-150-030-500 grating, coupled to a PI Acton PhotonMax: 512B EMCCD Camera System.

2.4. Delivery of Sensors to Dictyostelium discoideum. PEBBLEs were introduced into the slime mold via endocytosis. Sussman's medium was inoculated with *Enterobacter aerogenes* and shaken overnight at 37°C, 200 RPM. The bacteria were heat-killed at 80°C for 15 minutes then cooled to room temperature before being inoculated with 5-6 fruiting heads of AX3 axenic strain *D. discoideum* per 5 mL of medium. The medium was shaken at 22°C, 200 RPM for 20 hours. Dried PEBBLEs were resuspended in 1 mL

Sussman's medium at a concentration of 5 mg/mL and allowed to rehydrate overnight. The rehydrated PEBBLEs were added to 5 ml of *D. discoideum* and were shaken at 22°C, 200 RPM for 3–6 hours to allow for endocytotic uptake. The *D. discoideum* were centrifuged at 1900 RPM for 4 minutes to remove unincorporated bacteria and PEBBLEs, and then resuspended in 2.5 ml Bonner's Salt Solution (BSS). The wash was repeated twice and then the amoebae were suspended in 1 mL BSS.

2.5. Monitoring Response to cAMP. For the aggregation experiments, 333 μL of suspended amoebae were placed in a depression slide. Spectra were collected every 10 seconds for a period of 800 seconds; after 200 seconds, a 30 μL aliquot of cAMP was added to the edge of the slide.

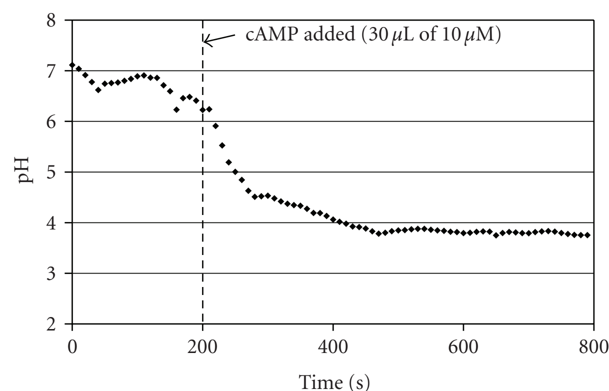

Figure 2: Representative aggregation pH data, multiple experiments give an initial average endosomal pH of 5.9±1 which dropped to an average of 3.9±1 upon addition of cAMP ($n = 4$ experiments). The pH was calculated from the ratio of Oregon Green 514 emission (554 nm) to the Quantum Dot emission (653 nm) based on the calibration of the sensors. The largest source of error is the limits of 95% prediction interval of the linear calibration of the sensors.

3. Results and Discussion

We have used PEBBLEs to measure the change of endosomal pH when *D. discoideum* cells aggregate in response to cAMP. PEBBLEs are particularly suited to this purpose because they avoid problems caused by autofluorescence of the cells.*D. discoideum* autofluorescence overlaps the fluorescence of the deprotonated peak of Oregon Green 514. The error that this would introduce to the pH measured using Oregon Green 514 is minimized by utilizing quantum dots as a reference signal and a long pass filter that selects only the fluorescence of the protonated form of Oregon Green 514. Autofluorescence is a common challenge in biological measurements. The use of quantum dots and indicator dyes colocalized in a PEBBLE nanosensor can be used to achieve the advantages of ratiometric measurement shifted to spectral windows with minimum autofluorescence.

Oregon Green 514-CdSe Quantum Dot PEBBLEs were used to make the first real-time measurements of *D. discoideum* endosomal pH after stimulation of aggregation-competent cells with cAMP. A decrease in endosomal pH during cAMP signaling is observed, suggesting the movement of protons into the endosomes of *D. discoideum* cells. Analysis of the aggregation data shows an average decrease in endosomal pH of approximately three pH units (Figure 2). Control experiments in which BSS was added in lieu of cAMP demonstrated no change in pH, nor was cAMP by itself shown to have an effect on PEBBLE signal outside of cells. This study established PEBBLEs as a novel tool for ion measurement within *D. discoideum*.

4. Conclusions

This experiment demonstrates that the endosomal pH of *D. discoideum* can be successfully quantified in real time using pH-sensitive PEBBLEs. Although previous studies have mapped pH fluctuations during endosomal development, no work has been done to quantify the endosomal pH changes that occur after chemotactic stimulation. The observed decrease in pH is consistent with previous work that identified acidic vesicles as the main sources of protons for the cAMP-induced proton efflux [7]. Future research will seek to track the endosomal development stage where the PEBBLEs are localized in to more fully understand the mechanisms of endosomal acidification and proton efflux during cAMP stimulation. Work will then turn to measuring ion concentrations in organisms where free dyes have proven ineffective.

Acknowledgments

The authors thank the Beland Fund and the Colorado College Venture Grant for research funding, Jakob Franke for the XMC7 mutant *D. discoideum* used in preliminary trials (not reported on here), Rex Chisholm for the AX3 strain, and Steve Burt for constructing the microscope support.

References

[1] P. J. M. Van Haastert, B. Jastorff, J. E. Pinas, and T. M. Konijn, "Analogs of cyclic AMP as chemoattractants and inhibitors of *Dictyostelium chemotaxis*," *Journal of Bacteriology*, vol. 149, no. 1, pp. 99–105, 1982.

[2] D. M. Bozzone, "Tested studies for laboratory teaching," in *Proceedings of the 14th Conference of the Association for Biology Laboratory Education (ABLE '93)*, C. A. Goldman, Ed., 1993.

[3] W. F. Loomis, *The Development of Dictyostelium Discoideum*, Academic Press, New York, NY, USA, 1982.

[4] R. J. Aerts, R. J. W. De Wit, and M. M. Van Lookeren Campagne, "Cyclic AMP induces a transient alkalinization in *Dictyostelium*," *FEBS Letters*, vol. 220, no. 2, pp. 366–370, 1987.

[5] P. Devreotes and C. Janetopoulos, "Eukaryotic chemotaxis: distinctions between directional sensing and polarization," *Journal of Biological Chemistry*, vol. 278, no. 23, pp. 20445–20448, 2003.

[6] B. Van Duijn and K. Inouye, "Regulation of movement speed by intracellular pH during *Dictyostelium discoideum* chemotaxis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 11, pp. 4951–4955, 1991.

[7] H. Flaadt, R. Schaloske, and D. Malchow, “Mechanism of cAMP-induced H^+-efflux of *Dictyostelium* cells: a role for fatty acids,” *Journal of Biosciences*, vol. 25, no. 3, pp. 243–252, 2000.

[8] H. Patel and D. L. Barber, “A developmentally regulated Na-H exchanger in *Dictyostelium discoideum* is necessary for cell polarity during chemotaxis,” *Journal of Cell Biology*, vol. 169, no. 2, pp. 321–329, 2005.

[9] M. Fechheimer, C. Denny, R. F. Murphy, and D. L. Taylor, “Measurement of cytoplasmic pH in *Dictyostelium discoideum* by using a new method for introducing macromolecules into living cells,” *European Journal of Cell Biology*, vol. 40, no. 2, pp. 242–247, 1986.

[10] K. Inouye, “Measurements of intracellular pH and its relevance to cell differentiation in *Dictyostelium discoideum*,” *Journal of Cell Science*, vol. 76, pp. 235–245, 1985.

[11] G. A. Jamieson Jr., W. A. Frazier, and P. H. Schlesinger, “Transient increase in intracellular pH during *Dictyostelium differentiation*,” *Journal of Cell Biology*, vol. 99, no. 5, pp. 1883–1887, 1984.

[12] R. Engel, P. J. M. Van Haastert, and A. J. W. G. Visser, “Spectral characterization of *Dictyostelium* autofluorescence,” *Microscopy Research and Technique*, vol. 69, no. 3, pp. 168–174, 2006.

[13] E. J. Park, M. Brasuel, C. Behrend, M. A. Philbert, and R. Kopelman, “Ratiometric optical PEBBLE nanosensors for real-time magnesium ion concentrations inside viable cells,” *Analytical Chemistry*, vol. 75, no. 15, pp. 3784–3791, 2003.

[14] I. Tatischeff and R. Klein, “Extracellular lumazine from aggregating *Dictyostelium discoideum* cells. Influence of pH on its fluorescence,” *Hoppe-Seyler’s Zeitschrift für Physiologische Chemie*, vol. 365, no. 10, pp. 1255–1262, 1984.

[15] B. Hoffmann and H. Kosegarten, “FITC-dextran for measuring apoplast pH and apoplastic pH gradients between various cell types in sunflower leaves,” *Physiologia Plantarum*, vol. 95, no. 3, pp. 327–335, 1995.

[16] F. Brenot, L. Aubry, J. B. Martin, M. Satre, and G. Klein, “Kinetics of endosomal acidification of *Dictyostelium discoideum* amoebae. 31P-NMR evidence for a very acidic early endosomal compartment,” *Biochimie*, vol. 74, no. 9-10, pp. 883–895, 1992.

[17] J. E. Jentoft and C. D. Town, “Intracellular pH in *Dictyostelium discoideum*: a 31P nuclear magnetic resonance study,” *Journal of Cell Biology*, vol. 101, no. 3, pp. 778–784, 1985.

[18] C. D. Town, J. A. Dominov, B. A. Karpinski, and J. E. Jentoft, “Relationships between extracellular pH, intracellular pH, and gene expression in *Dictyostelium discoideum*,” *Developmental Biology*, vol. 122, no. 2, pp. 354–362, 1987.

[19] H. A. Clark, M. Hoyer, M. A. Philbert, and R. Kopelman, “Optical nanosensors for chemical analysis inside single living cells. 1. Fabrication, characterization, and methods for intracellular delivery of PEBBLE sensors,” *Analytical Chemistry*, vol. 71, no. 21, pp. 4831–4836, 1999.

[20] H. Sun, A. M. Scharff-Poulsen, H. Gu, and K. Almdal, “Synthesis and characterization of ratiometric, pH sensing nanoparticles with covalently attached fluorescent dyes,” *Chemistry of Materials*, vol. 18, no. 15, pp. 3381–3384, 2006.

[21] J. Peng, X. He, K. Wang, W. Tan, Y. Wang, and Y. Liu, “Noninvasive monitoring of intracellular pH change induced by drug stimulation using silica nanoparticle sensors,” *Analytical and Bioanalytical Chemistry*, vol. 388, no. 3, pp. 645–654, 2007.

[22] H. A. Clark, R. Kopelman, R. Tjalkens, and M. A. Philbert, “Optical nanosensors for chemical analysis inside single living cells. 2. Sensors for pH and calcium and the intracellular application of PEBBLE sensors,” *Analytical Chemistry*, vol. 71, no. 21, pp. 4837–4843, 1999.

[23] J. P. Sumner, J. W. Aylott, E. Monson, and R. Kopelman, “A fluorescent PEBBLE nanosensor for intracellular free zinc,” *Analyst*, vol. 127, no. 1, pp. 11–16, 2002.

[24] C. Delmotte and A. Delmas, “Synthesis and fluorescence properties of Oregon Green 514 labeled peptides,” *Bioorganic and Medicinal Chemistry Letters*, vol. 9, no. 20, pp. 2989–2994, 1999.

[25] J. M. Dubach, D. I. Harjes, and H. A. Clark, “Ion-selective nano-optodes incorporating quantum dots,” *Journal of the American Chemical Society*, vol. 129, no. 27, pp. 8418–8419, 2007.

[26] C. Xu and E. Bakker, “Multicolor quantum dot encoding for polymeric particle-based optical ion sensors,” *Analytical Chemistry*, vol. 79, no. 10, pp. 3716–3723, 2007.