Optochemical Nanosensors and Subcellular Applications in Living Cells

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Abstract. What may be the smallest anthropogenic devices to date, spherical sensors (wireless and fiber-less) with radii as small as 10 nm have been produced. This class of optochemical PEBBLE (Probe Encapsulated By Biologically Localized Embedding) sensors covers a wide range of analytes (pH, calcium, oxygen and potassium included here) with excellent spatial, temporal and chemical resolution. Examples of such sensors for the monitoring of intracellular analytes are given. Methods, such as pico-injection, liposomal delivery and gene gun bombardment, are used to inject PEBBLE sensors into single cells. These PEBBLEs have caused minimal perturbation when delivered and operated inside single mammalian cells, such as human neuroblastoma, mouse oocytes or rat alveolar macrophage.

Key words: optodes; nanosensor; cellular probes; sensors.

Optochemical and electrochemical sensors are playing an increasingly greater role in biological and medical science [1–4]. The common denominator of such chemical sensors is the interface that separates sample chemistry from indicator chemistry. This separation is provided by the matrix in which novel combinations of indicators, sensitizers, catalysts and stabilizers can act in concert. For intracellular applications, the challenge has been to reduce the sensor's size, and thus minimize the biological invasiveness, reduce the detection limit and shorten the response time [5, 6]. To date, the smallest sensors have been microelectrodes and submicrometer fiber-based optodes. Owing to the encasing capillary or fiber-

optic tip (which taper in shape) both micropipettes and fibers have penetration volumes of at least many cubic micrometers, and this cone-shaped volume usually grows as the third power of the penetration depth. Thus, to reach into the nucleus of an 80 μm sized mouse oocyte cell, with a penetration depth of about 40 μm, the fiber-tip penetration volume is typically $1000\,\mu m^3$, but the cell volume is about $3\times10^5\,\mu m^3$. Such penetration may induce severe biological perturbation and seriously endanger the viability of the cell. In contrast, the volume of a 0.6 μm diameter PEBBLE is only $0.2\,\mu m^3$, i.e. about a millionth of the cell volume, and for a 60 nm PEBBLE the penetration volume is only about a billionth of the cell volume, a rather negligible perturbation. This point is particularly significant for smaller mammalian cells, such as the neuroblastoma discussed later. Apparently, even common bacterial cells could now be investigated.

Historically, the PEBBLE sensors are a development of fluorescent microspheres, beads and nano-particles [7]. The first use of a *single* nano-particle as a sensor may have been that by Sasaki et al., where a pH sensing nano-particle was manipulated from the bulk of a solution into the interface by a laser tweezer (light trap) [8]. Compared with simple fluorescent beads that contain a single kind of dye indicator, the optochemical PEBBLE sensors discussed here can be more complex in their structure and function (Fig. 1). The PEBBLE composition, including the matrix, the fluorophore and other components are optimized for the task at hand, the same as for fiber-based optodes (e.g., ion correlation optodes [9, 10]). In addition to the usual consideration (detection limit, selectivity, reversibility, reproducibility, stability, etc.), PEBBLE

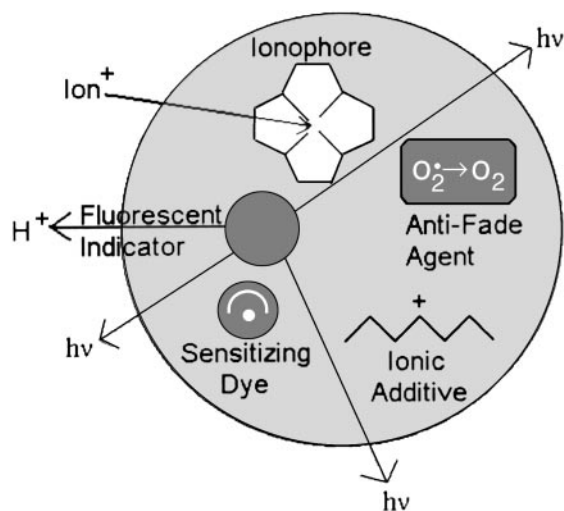* To whom correspondence should be addressed

Fig. 1. Schematic representation of a PEBBLE sensor, containing various active ingredients within the boundaries of a biocompatible polymeric matrix

deliverability into the cell and biocompatibility are of great importance.

PEBBLE sensors consist of mainly two types of devices: a single or a combination of indicator molecules in an acrylamide matrix, or multi-component multifunction devices in a liquid polymer. The chemical and spectroscopic properties of PEBBLE sensors are analogous to those of fiber-optic sensors, based on the same sensor design, i.e., ionic and optically active components within a polymeric matrix. The wide array of fiber-based optodes produced to date have been, or are in the process of being, adapted to PEBBLE sensors. Some consist of an indicator dye or a pair of indicator dyes entrapped in the pores of a hydro-gel. Such sensors use commercially available fluorescent ion indicators for analytes such as pH, calcium and oxygen. For instance, a pH PEBBLE has been produced with linear pH response in the range of 6.8–7.5 (slope $= 0.32$ for fluorescence intensity plotted *vs* pH). This PEBBLE was adapted from a pH optode described previously [11], so the matrix behavior of the indicator could be predicted. The polymer matrix provides advantages in this type of sensor due to its biocompatibility as well as its chemically and physically controlled microenvironment.

As another example of a hydro-gel sensor, a ratiometric calcium sensor has been developed which uses two calcium-selective dyes that are kept in close proximity within the environment of the polymer matrix. Without the matrix, the indicators would move freely throughout the cell and undergo differential sequestration, making ratiometric measurements more difficult. Since the two dyes are used together, the sensor is considerably less prone to measurement inaccuracy arising from photobleaching or position within the cell.

The more complex liquid polymer PEBBLE is minimally composed of an ionophore and a pH indicator that work synergistically (Fig. 1) [10, 12, 13]. For example, if a potassium ion penetrates the polymer and is bound by the ionophore, a positive charge is ejected from the matrix to preserve charge neutrality. This positive charge is in the form of a hydrogen ion, which induces a change in the fluorescence spectrum (intensity) of the pH indicator. In this way a sensor can respond with high selectivity to an ion such as potassium. Thus the sensor utilizes selective ionophores developed over many years for ion-selective electrodes, rather than relying on less selective fluorescent molecular probes. The same principle holds for both fiber-optic and PEBBLE sensors.

Experimental Methods

Preparation of Acrylamide PEBBLEs (pH, Oxygen and Calcium)

The polymerization solution consisted of 0.4 mM fluorescent ionophore (for pH PEBBLEs the ionophore was 5- and 6-carboxynaphthofluorescein, for calcium PEBBLEs the ionophore was Calcium Crimson or a combination of Calcium Orange and Fura Red (Molecular Probes, Eugene, OR) and for oxygen PEBBLEs the indicator was ruthenium tris-(4,7-diphenyl-1,10-phenanthroline), 27% acrylamide and 3% N,N'-methylenebis (acrylamide) in 0.1 M phosphate buffer, pH 6.5. One mL of this solution was then added to a solution containing 20 mL of hexane, 1.8 mmol of sodium dioctyl sulfosuccinate and 4.24 mmol of Brij 30. The solution was stirred under nitrogen for 20 min while cooling in ice bath. The polymerization was initiated with 24 μL of a 10% ammonium persulfate solution and 12 μL of N,N,N',N'-tetramethylethylenediamine (TEMED), then the solution was stirred at room temperature for 2 hours. Hexane was removed by rotary evaporation, then the probes were rinsed free from surfactant with ethanol, to yield a product consisting of 20–200 nm probes.

Preparation of Decyl Methacrylate PEBBLEs (Potassium)

The polymerization solution, which was based on that of a novel macrosensor matrix [14] consisted of 55 mg of decyl methacrylate and 75 mg of hexanediol dimethacrylate combined, then washed three times with 5% sodium hydroxide solution and three times with water. To the washed monomer were added 100 mg of dioctyl sebacate (DOS), 5 mg of benzophenone, 2.5 mg of benzoyl

peroxide, 4 mg of valinomycin, 1.55 mg of sodium tetrakis([3, 5-bis(trifluoromethyl)phenyl]) borate (NaTFPB), and 2 mg of chromoionophre III or 9-dimethylamino-5-[(2-octyldecyl)imino]-benzo[a]phenoxazine (ETH 5350). This solution was added to 1 ml of water and sonicated for 1 hour until uniform. The spheres were then purged with nitrogen for 20 min, and polymerized with a UV lamp for 15 min.

Gene Gun Delivery

A Biolistic PDS-1000/He system (bench-top model) from BioRad (Hercules, CA) with grade 5 helium was used to inject cells with sensor probes. Sample preparation for the particle delivery system required dispersion of the PEBBLEs in water, and the careful application of a thin film of PEBBLEs onto the target membrane. Low firing pressures were used, best results being obtained at 900 psig, with a vacuum of 25 torr on the system. Culture medium (Dulkbecco's Modified Eagle Medium, (DMEM) was removed from the cells by pipet before delivery. Following biolistic delivery of FEBBLEs, neuroblastoma cells were rinsed three times with Dulbecco's phosphate buffered saline (DPBS) and incubated with DPBS during analysis. The cells were analyzed within two or three hours.

Liposomal Delivery

PEBBLEs were incubated with ESCORT™ (Sigma, St. Louis, MO) and DMEM for 15 min, then the mixture was introduced into culture of neuroblastoma cells. The cells were maintained in a 5% CO_2 atmosphere in a 37 °C incubator for 5 hours before removal of the excess PEBBLE/liposome mixture from the cells. Neuroblastoma cells were incubated in fresh medium overnight, then rinsed before analysis.

Transmission Electron Microscopy (TEM)

Cells were fixed immediately after FEBBLE delivery with 2.5% glutaraldehyde in 0.1% sodium cacodylate buffer and postfixed with 1% osmium tetroxide. Cells were embedded in Epon-Araldite, sectioned, stained with lead citrate, counter-stained with uranyl acetate and visualized by use of a JEOL transmission electron microscope.

Mouse Oocytes

These were obtained from superovulated C57BL/6 mice and were maintained in M16 medium in a 5% CO_2 atmosphere in a 37 °C incubator. PEBBLE suspensions (20 mg/ml) were pico-injected into one- and two-celled mouse oocytes, which were allowed to recover for several hours, then analyzed by fluorescence microscopy. Experimental animals were maintained in accordance with University of Michigan Laboratory Animal Medicine and NIH Guidelines.

Alveolar Macrophages

These were recovered from rat lung lavage with Krebs–Henseleit buffer. They were maintained in a 5% CO_2 atmosphere in a 37 °C incubator in DMEM containing 10% fetal bovine serum and 0.3% PSN (penicillin, streptomycin and neomycin). PEBBLE suspensions ranging from 0.3 to 1.0 mg/ml were prepared in DMEM and incubated with alveolar macrophage overnight.

Neuroblastoma Cells (Cell Line SH-SY5Y)

These were maintained in Dulbecco's Modified Eagle medium containing 10% fetal bovine serum and 0.3% PSN (penicillin, streptomycin and neomycin) and subcultured into 60 mm Petri dishes and incubated at 37 °C in a 5% CO_2 environment. The cells were used when they reached a confluency of about 80%.

The Optical Path

This included an Olympus BX50WI fluorescence microscope fitted with Nomarski optics (Lake Success, NY), an Olympus mercury arc lamp, an Acton 150 mm spectrograph (Acton, MA), and a charge coupled device (CCD) optical detector.

Results and Discussion

Hydrogel PEBBLEs were fabricated in a microemulsion process [15] to produce spherical sensors ranging from 20 to 200 nm in diameter, as confirmed by transmission electron microscopy (e.g. Fig. 2). Investigations of PEBBLE suspensions were conducted in order to more carefully characterize PEBBLE number densities, morphology and size distribution. Microscopic examination of the PEBBLEs has been complicated by their gelatinous and water-miscible properties. Transmission electron micrographs of PEBBLE suspensions dried on formvar carbon support grids demonstrate that PEBBLEs produced by the microemulsion process range in size from 20 nm to approximately 250 nm. Initial characterization by this technique indicates that the smaller PEBBLEs (20–50 nm diameter) are present in the highest concentrations, while the larger
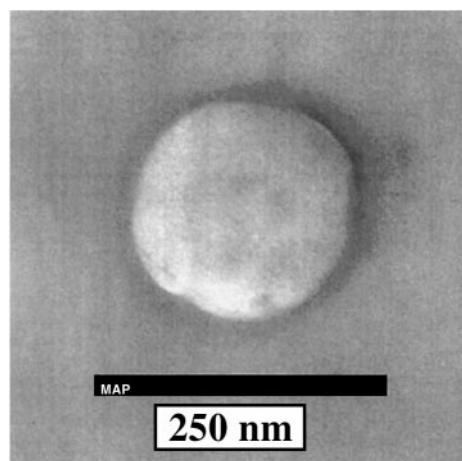
250 nm

Fig. 2. Transmission electron micrograph of a single 200 nm PEBBLE sensor dried on a formvar grid

Table 1. Number densities for PEBBle sensors over the range 20–200 nm (reported as relative numbers of PEBBLEs)

20–50 nm PEBBLEs	50–100 nm PEBBLEs	200–250 nm PEBBLEs
100	50	1

200 nm PEBBLEs are present in lower numbers (Table 1). We note that the smallest PEBBLEs weight only about 4 attograms and contain only about one zeptogram of active material.

PEBBLE sensors can be used as individual probes, or as clusters of single function (single analyte) PEBBLEs, or in sets of many function (multi-analyte) PEBBLEs, or in ensembles including sets of clusters. While PEBBLE clusters are appropriate for imaging single analyte gradients and their dynamics, the sets or ensembles may either give an overall multi-analyte measurement or may provide simultaneous real-time multi-analyte chemical images, describing the spatio-temporal dynamics of complex chemical reactions and events. Thus some of our studies emphasize individual PEBBLE performance while others emphasize PEBBLE cluster or ensemble characteristics. Each of these options for utilizing PEBBLEs requires different delivery methods. One method was developed based on "biolistic" embedding into the cell by a gene gun system (BioRad, Hercules, CA). The gene gun delivers only one or two PEBBLEs to a cell, making single PEBBLE measurements possible. An alternative method is to use liposomes to transport a large number of PEBBLEs into the cytoplasmic space, which allows the user to monitor multiple regions of a cell simultaneously. Two other delivery methods

consist of (1) pico-injection, which allows for controlled PEBBLE delivery and therefore measurements in the cell nucleus, and (2) phagocytosis, which provides for the ability to monitor the chemistry in specialized compartments of immune competent cells, such as macrophages. An example of each of these methods is given below.

A gene gun uses a burst of helium to drive PEBBLEs into adherent cells, much like a shotgun blast. The technique injects many cells at once, and delivers approximately one or two PEBBLEs to each cell that it injects. TEM images of neuroblastoma cells that have been injected with PEBBLEs by the gene gun indicate that a single PEBBLE or a pair of PEBBLEs is shot into the cytosol of the cells (Fig. 3). Both 200 nm and 20 nm PEBBLEs can be injected by this method. To assess the viability of cultured neural cells after pebble delivery with the gene gun, neuroblastoma cells were bombarded with PEBBLEs and immediately tested for their ability to exclude the dye Trypan Blue (as a measure of cell viability). This assay indicated that minimal cell death (1.4% and 2.6% in excess of controls) occurred with biolistic delivery of PEBBLEs (Fig. 4).

Liposomal delivery uses commercially available lipid vesicles to transport PEBBLEs into neuroblastoma cells. The liposomes interact with the PEBBLEs, then fuse to the cell membrane, where they release the PEBBLEs into the cytoplasm. This is a very gentle method, and many PEBBLEs can be introduced into the cytoplasm at once. A distribution of PEBBLE sensors throughout the cytosol provides a method for monitoring ion concentrations at distinctly different locations, thus imaging ion concentrations across the
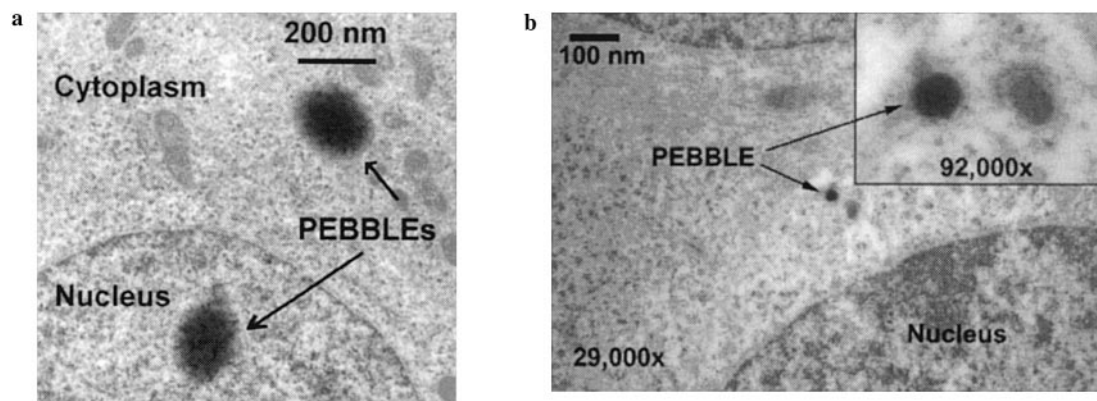

Fig. 3. Transmission electron micrographs of PEBBLE sensors embedded biolistically (via gene gun), at 900 psig, into the cytoplasm of neuroblastoma cells: (a) two 200 nm PEBBLEs, near or inside the cell nucleus, (b) one 20 nm PEBBLE next to a primary lysosome in the cell cytoplasm. Original magnification is indicated on the figure and the inset
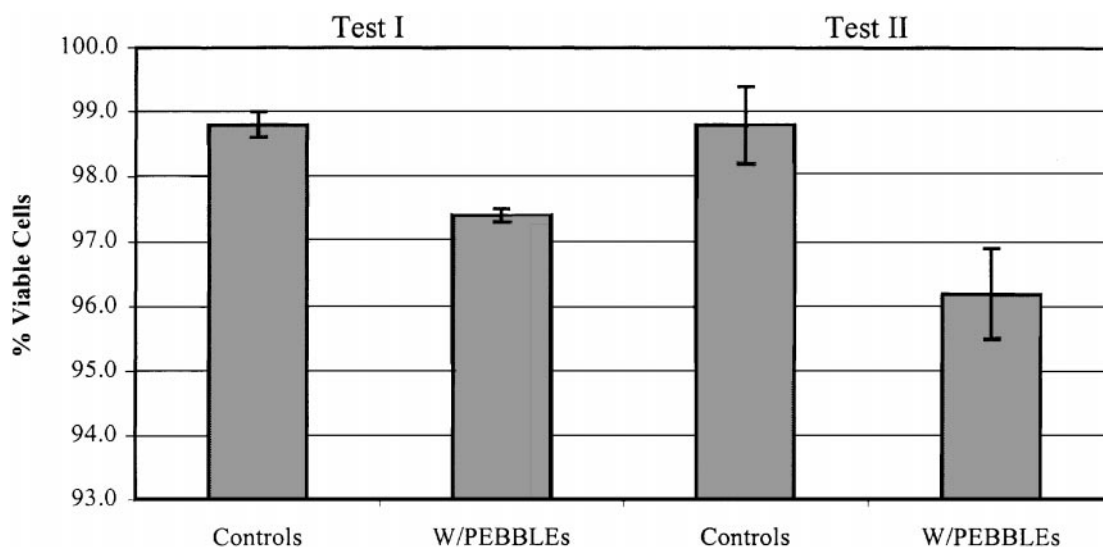
Fig. 4. Trypan Blue viability assay: untreated controls and neuroblastoma cells bombarded with PEBBLEs at 900 psig. Variation of 3 results shown as error bars
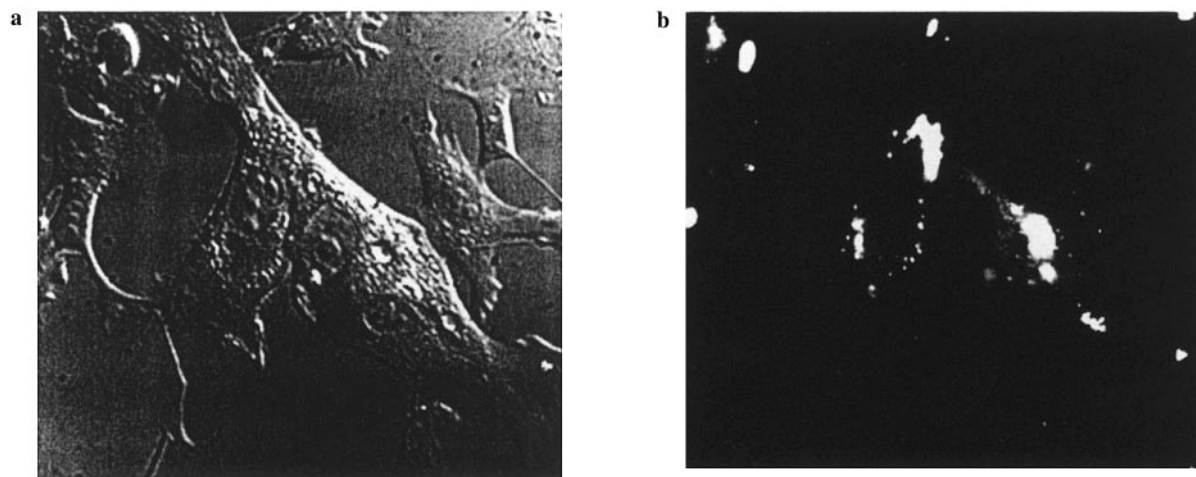

Fig. 5. Calcium PEBBLEs delivered to neuroblastoma cells by liposomal delivery. (a) Nomarski illumination, (b) fluorescence image with excitation at 590 nm. Note that the PEBBLEs remain in the cytoplasm of the cells, and are not contained in the nucleus

cytosol is possible (Fig. 5). Another advantage is that many cells can be injected at once, unlike some methods (such as pico-injection) where delivery takes place on a cell-by-cell basis.

Pico-injection has been shown to be an effective method for controlled delivery of PEBBLEs, or sets of PEBBLEs, to single cells. Fine control of the injection tip allows insertion of PEBBLEs into the nucleus of a cell or the cytoplasm, or both. Single areas of a dividing cells can be selectively injected (Fig. 6). In this example, a single cell of a divided mouse oocyte

was injected with pH-sensitive PEBBLEs. The green fluorescence can be easily observed against the background of the cell.

Pico-injection can also be used for inserting ensembles of PEBBLEs into single cells. Figure 7 (a) shows a mouse oocyte with a two-analyte sensor set, containing a cluster of oxygen-selective PEBBLE sensors as well as a cluster of calcium PEBBLE sensors. Using two distinct excitation wavelengths resulted in distinct fluorescence images [Fig. 7(b) and (c)] as well as fluorescence spectra [Fig. 7(d)]. In this
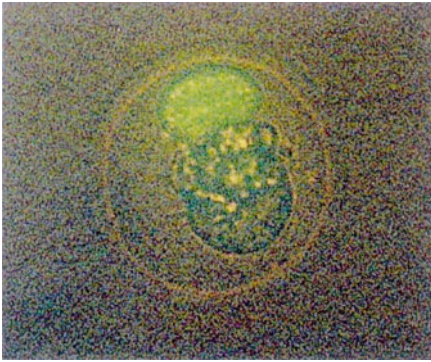
Fig. 6. Mouse oocyte that has divided into two cells, with pH sensitive PEBBLEs injected into the top cell only. The oocyte is being illuminated with 488 nm excitation, and the green PEBBLEs are fluorescing

defined even after some hours, owing to the absence of leaching across the cell membrane.

PEBBLEs were also used to monitor calcium in phagosomes within rat alveolar macrophage. Macrophage that had phagocytosed calcium-selective PEBBLE sensors were challenged with a mitogen, Concanavalin A (Con A), inducing a slow increase in intracellular calcium, which was monitored over a period of 20 min (Fig. 8). PEBBLE clusters confined to the phagosome enabled correlation of ionic fluxes with specific functions of this organelle. In contrast, conventional fluorescent calcium indicators tend to monitor concentrations throughout the cell simultaneously (including the nucleus), making resolution of individual compartments difficult.
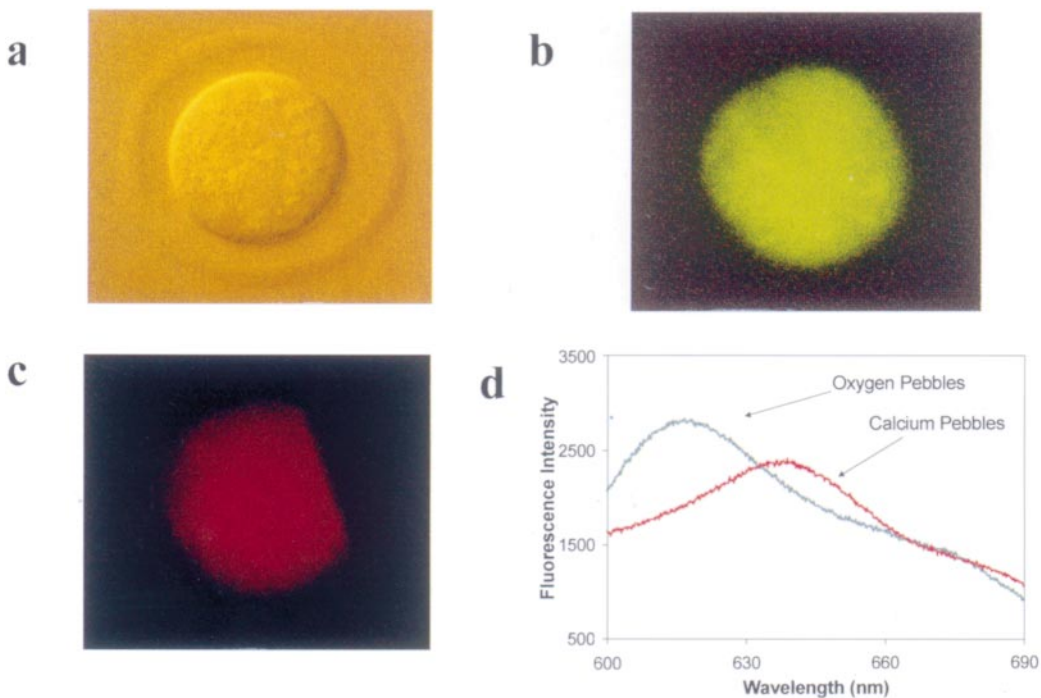

Fig. 7. Mouse oocyte that has been injected with both calcium PEBBLEs and oxygen PEBBLEs. (a) Nomarski illumination, (b) fluorescence image using 488 nm illumination to excite the oxygen PEBBLEs, (c) fluorescence image using 560 nm illumination to excite the calcium PEBBLEs, (d) spectra from oxygen PEBBLEs and calcium PEBBLEs with excitation wavelengths indicated in (b) and (c)

way, different analytes can be measured and imaged without spectral overlap. It is noted that, in contrast to fluorescent indicator dyes, here there is no physical interference between the two fluorescent indicators (no binding, quenching or energy transfer), as each indicator is separately embedded in its own protective PEBBLE matrix. Also, the cell can be highly loaded, thanks to the absence of quenching of the indicator dyes. In addition, the cell boundaries are sharply

Despite their small size, PEBBLE sensors were found to be highly stable, both physically and photophysically. The effects of photobleaching are minimized by using ratiometric measurements and short exposure times (100 ms or less). Leaching of the indicator dyes from the PEBBLE sensor was investigated and did not present a serious problem. Oxygen PEBBLEs localized in the harsh environment of phagosomes of rat alveolar macrophages for five days
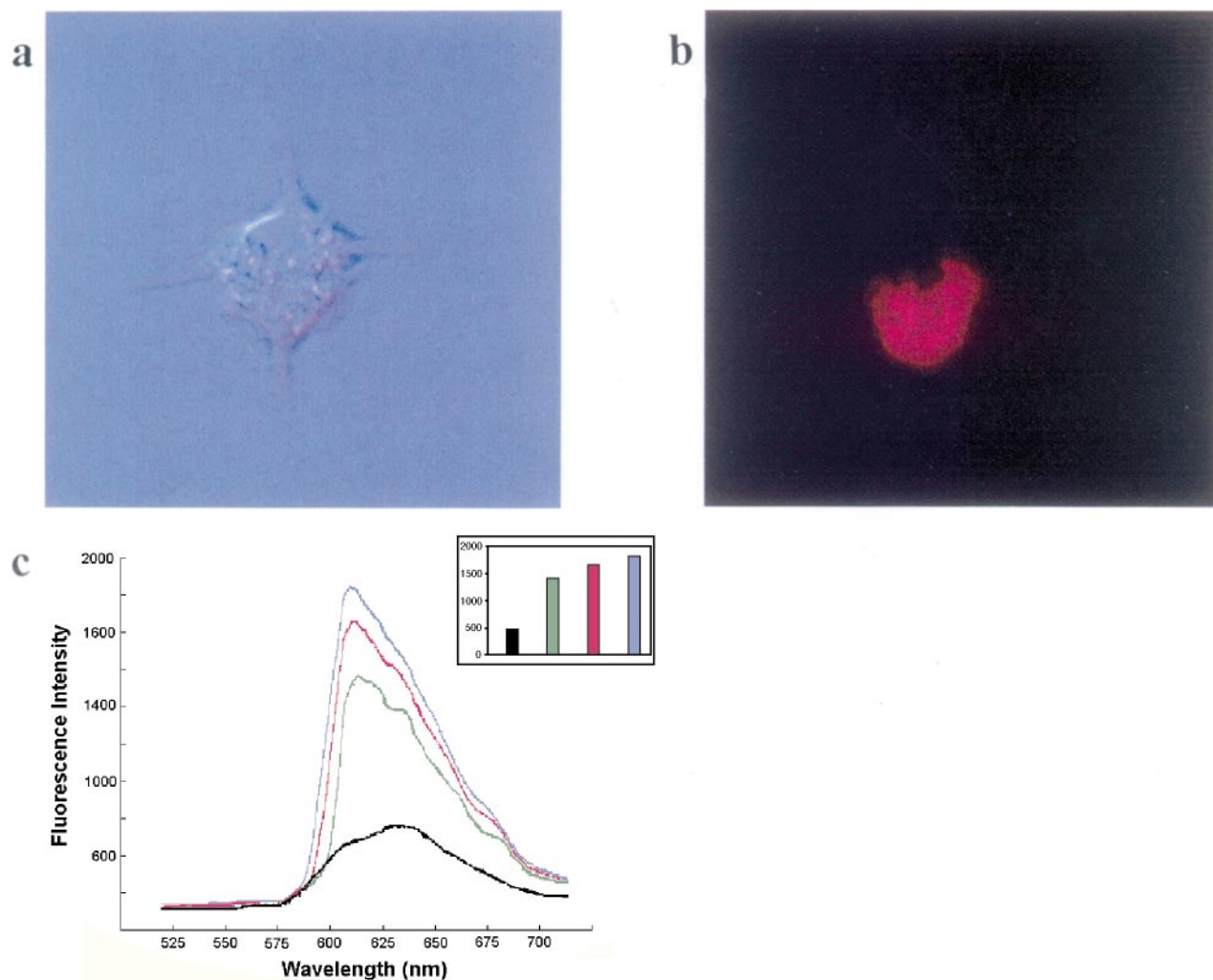
Fig. 8. Rat alveolar macrophage with phagocytosed calcium selective PEBBLE sensors (60 x). (a) Nomarski illumination, (b) fluorescence illumination, (c) the increasing intracellular calcium level, monitored by calcium PEBBLEs in alveolar macrophage following stimulation with 30 μg/ml Concanavalin A (Con A). Black, no PEBBLEs in macrophage; green, PEBBLEs contained in phagosomes of macrophage, no stimulation; red, 10 min after stimulation of PEBBLE containing macrophage with Con A; blue, 20 min after stimulation with Con A

did not display physical degradation or leaching of the fluorophore. This suggests that PEBBLEs can withstand acidic and oxidative biological conditions and remain functional. The small size is a distinct advantage when it comes to response time, since diffusion time through the matrix becomes inconsequential. When compared to the free dye, PEBBLE sensors were found to have comparable response times, suggesting that the diffusion time through the matrix was indeed minimal (less than 1 ms).

The small size and smooth shape of the sensor further enhances biological compatibility. The biocompatibility of PEBBLE sensors was investigated by assessing the viability of cells in a culture, using a variety of histological and biochemical techniques (e.g. Trypan Blue exclusion, lactate dehydrogenase leakage and energy change). The viability of cultured neuronal cells containing PEBBLE sensors was comparable to that of control samples or cells containing other often used submicron particles, such as gold colloids and latex spheres.

Calculations show that fewer than 10^3 analyte ions are measured by a 200 nm PEBBLE (with 1% fluorescent molecules, by weight), putting the absolute detection limit in the zeptomole range. This low detection limit, combined with millisecond acquisi-

tion and response times, as well as submicrometer spatial definition, has provided the ability to detect real-time intracellular ion fluxes. PEBBLE sensors for sodium, chloride, nitrite, magnesium and glucose are currently under development. Other PEBBLE matrices under study include protein and enzyme-tagged gold colloids (similar to a recent nitric oxide fiber-optic sensor [16]), and sol–gel based PEBBLE sensors. Advanced optical imaging methods are expected to transform such PEBBLE-based chemical analysis ensembles into streamlined biomedical tools.

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