Biosensors

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Microsphere-Based Real-Time Calcium Sensing**

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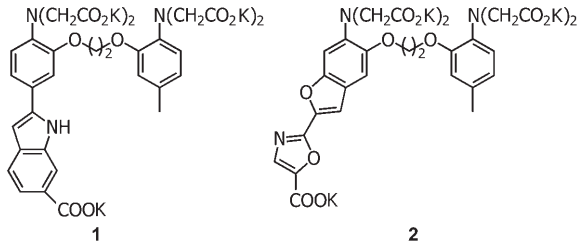
Fluorescent sensors for a variety of ions, such as Ca^{2+} and Na^{+}, are among the most useful tools available for studying ion fluxes within the intracellular environment following the modulation or stimulation of a range of complex biological processes. Two widely used fluorescent indicators for Ca^{2+} ions are Indo-1 (**1**) and Fura-2 (**2**), which complex calcium with high affinity ($K_d = 230$ and 145 nM, respectively), and these reagents have allowed numerous investigations into intracellular free Ca^{2+} concentrations following a range of cellular insults.[1–6] However, the pentacarboxylic acid and salts of Indo-1 and Fura-2 (**1** and **2**, respectively; Scheme 1)



Scheme 1. Structures of the commonly used Ca^{2+} sensors salts of Indo-1 and Fura-2 (**1** and **2**, respectively).

are unable to cross cellular membranes, an essential requirement in allowing such molecules to be used within the cellular environment. Thus, several invasive techniques, such as microinjection[7] or electroporation,[8] are routinely used to allow the introduction of the sensor into the cells, which although highly efficient are time-consuming and laborious and can damage the cells. Another established method for the delivery of these probes into cells is the use of cell-permeable acetoxymethyl (AM) ester derivatives, which can freely enter the cell and are subsequently hydrolyzed.[9] However, this method has disadvantages, such as compartmentalization, incomplete AM ester hydrolysis (five ester groups need to be fully cleaved to give the intact sensor), and cellular leakage over time.[10,11] Additionally, these ester derivatives often need to be solubilized in dimethyl sulfoxide (DMSO), which even at low concentrations can be cytotoxic to cells. 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. New approaches to this problem include the use of polymer-particle-based encapsulation[12] and the use of dextran conjugates of ion indicators that give decreased compartmentalization and leakage of sensor but often need to be loaded into cells using membrane-permeabilization reagents, such as Triton X-100.[13]

We recently demonstrated the efficient cellular delivery of amino-functionalized cross-linked polystyrene microspheres (2 μm) into a broad range of cells, including primary cells, and described the efficient fluorescence-activated sorting of cells that contain either single or multiple beads with one or more colors, thereby allowing the "encoding" of multiple cell types and subsequent cellular multiplexing.[14] The delivery of beads into cells, however, has a number of additional potential applications, and herein we show how the beads can be used not only to deliver "cell-impermeable" compounds, such as the acid salt of Indo-1, but how they can be used as a focal point for cellular examination (without cellular dilution taking place), thus allowing calcium fluxes within single live cells to be followed and determined in real time.

To covalently attach the Indo-1 sensor to the microspheres selectively, the original synthesis of this sensor was modified to allow incorporation of a benzyl ester at the indole 6-position while maintaining four ethyl ester functionalities on the coordinating carboxy groups,[15] thereby generating orthogonality between the acid groups that chelate the Ca^{2+} ions and the additional carboxylic acid group required for resin attachment. The synthesis of the novel derivative **7** was carried out in nine steps (see Scheme 2 and the Supporting Information for further details), with hydrogenation of the benzyl ester **7** to give the tetraethylester indolecarboxylic acid ready for coupling onto the aminomethylated microspheres. Subsequent hydrolysis of the ethyl ester gave the microsphere-immobilized sensor **9** (see Scheme 2 and the Supporting Information for further details).

Fluorescence studies were carried out to evaluate how modification and immobilization of the sensor had affected its sensitivity to Ca^{2+} ions by examining the microspheres under a range of concentrations. The emission maximum shifted from 475 nm in Ca^{2+}-free medium to 400 nm when the sensor molecules were saturated with Ca^{2+} ions, as seen for the original Indo-1 sensor. Figure 1 shows the fluorescence emission spectra of **7**. The value of the dissociation constant for this derivative was calculated ($k_d = 226$ nM; details of the determination of the k_d values are given in the Supporting information).[15] Importantly, the sensor had the same fluo-

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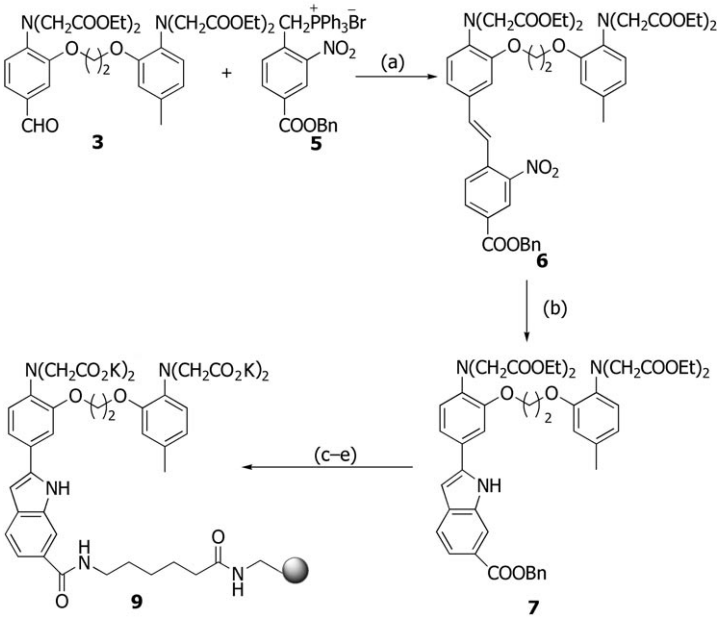

Scheme 2. Synthesis and covalent attachment of modified Indo-1 **7** to the microspheres to give the Indo-1-microsphere sensor **9**. Reagents and conditions: a) K_2CO_3, DMF, 100 °C, 3 h (46%); b) $(EtO)_3P$, 160 °C, N_2, overnight (60%); c) H_2, 10% Pd/C, ethanol, 4 h (91%); d) microspheres derivatized with an aminohexanoic acid spacer,[14] HOBt, PyBOP, 4-ethylmorpholine, DMF, room temperature, 16 h (quant.); e) 1 M KOH, 6 h (quant.). DMF = dimethylformamide, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate

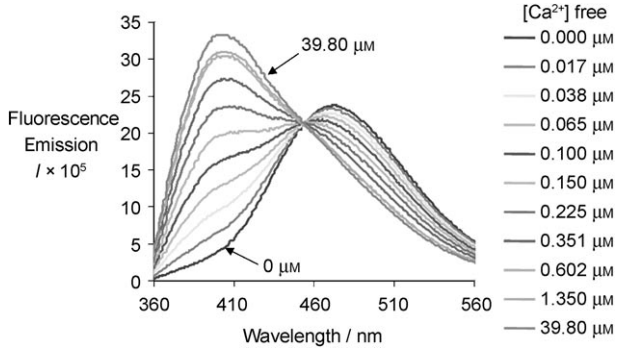

Figure 1. Fluorescence emission spectra (λ_{ex} = 350 nm) for Indo-1 derivative **7** following ester hydrolysis recorded at 22 °C and pH 7.2 as a function of $[Ca^{2+}]$. Solutions of varying $[Ca^{2+}]$ were prepared by mixing together EGTA (10 mM), KCl (100 mM), and MOPS (30 mM) with CaEGTA (10 mM), KCl (100 mM), and MOPS (30 mM) to give $[Ca^{2+}]$ values in the range of 0–39.8 μM. EGTA = ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, MOPS = 4-morpholinepropanesulfonic acid.

rescence profile when bound to the microspheres as in solution.

A population of neuronal cells (ND7) were incubated with 2-μm Indo-1-loaded microspheres (0.1 mg mL^{-1}) for 6 hours. Cells containing the beads (ca. 30%) were isolated by fluorescence-activated cell sorting (FACS; see the Supporting Information for details) and were shown by confocal microscopy after 24–72 h of incubation at 37 °C to be healthy and growing with the sensor microspheres inside the cells (Figure 2). The Indo-1-loaded microspheres were found to be nontoxic at all of the concentrations tested, as verified by methylthiazolyldiphenyltetrazolium bromide (MTT)[16] and trypan-blue assays.[17]

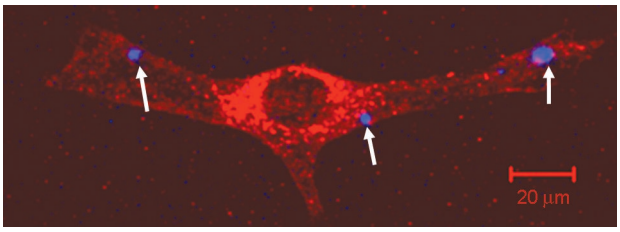

Figure 2. Confocal microscopy image of a single neuronal cell (ND7-immortalized cell line derived from sensory neurons) loaded with Indo-1-microspheres **9** (blue circles) after 24 h of incubation at 37 °C under 5% CO_2 (white arrows indicate the microspheres intracellular location in the cytosol). The cell membrane was stained with a red fluorescent dye (PKH26, Sigma-Aldrich) which allows long term in vitro analysis of live cells.

Intracellular changes in the concentration of free Ca^{2+} ions were detected by microscopy-mediated examination of the Indo-1-labelled microspheres in ND7 cells after stimulation. The measurements were carried out with excitation at 355 nm and with fluorescence emission from the beads monitored at 400 and 470 nm with the levels of free Ca^{2+} ions determined by using the standard ratio method established by Valet and co-workers.[18] Figure 3 shows the results of analysis of cells loaded with microspheres after stimulation of ND7 cells with *N*-methyl-D-aspartate (NMDA) and glutamate (this combination of amino acids is known to active glutamate receptors that gate the flow of Ca^{2+} ions across the membrane of a nerve cell).[19] After approximately six minutes, the average response showed an increase

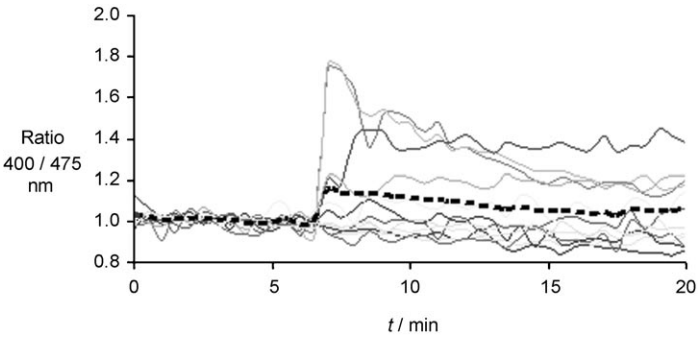

Figure 3. Real-time ratiometric fluorescent analysis (400/475 nm ratio) of the release of Ca^{2+} ions in ND7 cells loaded with Indo-1-microspheres. The cells were incubated with the microspheres for 6 h at 37 °C and under 5% CO_2, sorted (FACS), and regrown for 24 h. Time t = 0 corresponds to the addition of NMDA (10 μM) and glutamate (100 μM) to the microsphere-loaded cells. Each line corresponds to an individual cellular response, while the discontinuous bold line shows the average.

in free intracellular Ca^{2+} ions, followed by a decrease as a result of receptor desensitization, thus clearly proving that the calcium-sensor-loaded microspheres responded rapidly and with good sensitivity to increases in levels of intracellular Ca^{2+} ions.

A technique has thus been developed in which not only is the acid salt of Indo-1 delivered into cells but it is done so in a manner in which dilution and leakage do not occur. An experiment to demonstrate these advantages was performed in which ND7 cells were incubated with either the microspheres **9** and/or the commercially available Indo-1 AM ester. Both sensors were clearly visible within the cell at $t=0$, although the conventional dye was diffuse. The conventional sensor was lost gradually with time, and only the sensors bound to the microspheres were visible after 18 h of analysis (see the Supporting Information). These results indicate that the sensor-based microspheres avoid the problems conventionally associated with cellular leakage and allow intracellular changes to be followed for long-term studies without having to repeatedly load the cells.

Importantly, these beads are nontoxic to cells (see the Supporting Information), do not disrupt cell physiology, and can be introduced with high efficiency with the "capture" of the sensor beads by the cells that allow cell sorting based on bead content and fluorescence. There is of course a number of questions that relate to the uptake of the beads and their location within the cell. Recent reports in the area of derivatized-nanotube uptake[20,21] and bead uptake[22] suggest that the process is both size and material dependant, whereas cellular location and compartmentalization also depend on the amount of time the particles have been within the cell. However, since spectroscopic changes on the beads are observed following cellular stimulation, the sensors are clearly chemically/biologically accessible and respond to changes in the concentration of intracellular Ca^{2+} ions, thus showing that they are located in the cytosol and not trapped and isolated by a vesicular membrane.

By using these beads, we have shown our ability to measure changes in the concentration of intracellular Ca^{2+} ions in single living cells in real time. The fact that the microspheres are stably retained for several days also allows changes in the concentration of intracellular Ca^{2+} ions to be followed in a single cell for much longer periods of time than using traditional sensors. These results open the door to a range of possible applications, with the microspheres acting as carriers of sensors for other metal ions or pH values and for a range of different biological probes.

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