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Nanoparticle PEBBLE Sensors for Quantitative Nanomolar Imaging of Intracellular Free Calcium Ions

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Abstract

Ca^{2+} is a universal second messenger and plays a major role in intracellular signaling, metabolism and a wide range of cellular processes. To date, one of the most successful approaches for intracellular Ca^{2+} measurement involves introduction of optically sensitive Ca^{2+} indicators into living cells, combined with digital imaging microscopy. However, the use of free Ca^{2+} indicators for intracellular sensing and imaging has several limitations, such as nonratiometric measurement for the most sensitive indicators, cytotoxicity of the indicators, interference from non-specific binding caused by cellular biomacromolecules, challenging calibration and unwanted sequestration of the indicator molecules. These problems are minimized when the Ca^{2+} indicators are encapsulated inside porous and inert polyacrylamide nanoparticles. We present PEBBLE nanosensors encapsulated with rhodamine based Ca^{2+} fluorescence indicators. The here presented rhod-2 containing PEBBLEs show a stable sensing range at near-neutral pH (pH 6–9). Due to the protection of the PEBBLE matrix, the interference of protein non-specific binding to the indicator is minimal. The rhod-2 PEBBLEs give a nanomolar dynamic sensing range for both in-solution (K_d = 478 nM) and intracellular (K_d = 293 nM) measurements. These nanosensors are a useful quantitative tool for the measurement and imaging of the cytosolic nanomolar free Ca^{2+} levels.

Keywords

Ca^{2+}; nanoparticles; rhod-2; confocal microscopy; PEBBLEs (Photonic Explorers for Bioanalysis with Biologically Localized Embedding)

Ca^{2+} is a universal second messenger that transmits and processes information inside a variety of cells. It plays a major role in intracellular signaling, muscle contraction, metabolism and a wide range of cellular processes.[1–3] The total calcium content in resting cells is typically 1–7 mM,[3] but most of the cellular calcium (>99.9%) is stored in organelles or bound to membrane components, cytosolic metabolites and proteins. Cells strictly regulate the internal free Ca^{2+} at the optimal 100–200 nM level,[2] which is 20,000 times lower than the extracellular Ca^{2+} level. Changes in cytosolic Ca^{2+} concentration are transitory, as increases in cytosolic Ca^{2+} dissipate rapidly because cytosolic ligands quickly bind up the free Ca^{2+}. Monitoring of the intracellular Ca^{2+} signals necessitates direct measurements in intact living cells. To date, the most successful approach for intracellular Ca^{2+} measurement is by the introduction of optically sensitive Ca^{2+} probes into living cells, combined with digital imaging microscopy. In addition, fluorescence lifetime and polarization are also utilized to determine metal ion concentrations.[4,13,14]

The development of BAPTA-based fluorescent probes has been a breakthrough for monitoring Ca^{2+} inside live cells.[5,6] A fluorescence change is induced when Ca^{2+} binds to

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the carboxylic cage of BATPA moiety in these probes, revealing the free Ca^{2+} level. Indo-1 and fura-2 are the most commonly used ratiometric probes. However, the use of these two dyes requires UV excitation which causes much cell or tissue autofluorescence in many situations. It also has been reported that both dyes degenerate into Ca^{2+}-insensitive fluorescent compounds due to the UV induced photobleaching,[7] which can lead to inaccurate measurements. Indicators with visible excitation, such as fluo-3 and rhod-2, have also been widely used for intracellular Ca^{2+} measurements, because their visible excitation causes less autofluorescence and photodamage to the cells or tissues. Rhod-2 is often used for *in situ* measurement of Ca^{2+} level in brain cells, cardiac cells or in organs, such as perfused mouse or rabbit heart.[8–11] Its long excitation wavelength gives better tissue penetration and induces less autofluorescence. Additionally, rhod-2 has been used for measurements of mitochondrial Ca^{2+} levels, because the AM (acetoxymethyl) ester form of rhod-2, which is positively charged, tends to be accumulated in mitochondria.[12]

The major limitation of rhod-2 is the lack of shift of either the absorbance, excitation or emission wavelength upon Ca^{2+} binding;[6] therefore the dye by itself is not ratiometric. Ratiometric measurement cancels out variations in dye concentration, optical path-length and optical instabilities. Therefore, it is probably the best way for fluorescence intensity (rather than lifetime) based methods, for achieving accurate monitoring of calcium levels in live cells. Although the dye is highly sensitive by itself, without retiometric measurements, the fluorescence intensity cannot be accurately converted to Ca^{2+} levels, unless the dye concentration, path-length, quantum efficiency and instrumental sensitivity are precisely known. For solution tests in cuvettes, these parameters can be established and controlled; but it is almost impossible to reproduce them in single cell tests without lysing the cells and titrating the dye in the supernatant. An alternative route,[10–12] is to monitor the fluorescence change over time during cell stimulation or other manipulation. Such a method at least cancels out variations in dye concentration and path-length, and enables accurate measurements of the change in Ca^{2+} concentration. However, the absolute levels of intracellular Ca^{2+} cannot be calculated in this manner, only values relative to the pre-treatment values.

Ratiometric measurements can also be achieved effectively through the use of fluorescence lifetime imaging (FLIM), as reported by Lakowicz and co-workers.[13,14] The local decay times can be resolved into a composition of free and bound forms of the dye molecule and therefore reveal the free Ca^{2+}. The FLIM method allows images to be generated by the local lifetime, which is independent of dye concentration, rather than by the local fluorescence intensity. For this reason, the need for ratiometric probes can be bypassed. Unfortunately, for dyes like rhod-2 or fluo-3, their lifetimes are in the picosecond range for both the free and bound form.[6] Consequently, for this method, an instrument with picosecond resolution is required, which is not easily available with standard confocal microscopes.

Other problems encountered when measuring intracellular Ca^{2+} using fluorescent molecular probes are: 1). Cytotoxicity; some probes may be toxic to some types of cells. For example, it has been reported that sea urchin eggs loaded with fluo-3 do not develop normally.[15] 2). Unwanted compartmentalization due to sequestration; one of the most important issues in the use of chemical fluorescence probes is that the indicators are not homogeneously distributed throughout the whole cell but are trapped or sequestered within some organelles.[6,10] The level of Ca^{2+} in a given compartment is usually not the same as in the cytosol, therefore compartmentalization would result in inaccurate measurements of cytosolic Ca^{2+}. 3). Binding to other ions and proteins; many of the probes bind with intracellular proteins and thus undergo changes in their diffusion constant, emission spectra, reaction kinetics, and their K_d for Ca^{2+}.[9,16] Additionally, all of these indicators are affected by pH, to various degrees,[8,17] or by other divalent cations, such as Mg^{2+}, Mn^{2+}, Co^{2+},

Zn^{2+}.[7,6,9,18] 4). Indicator dye leakage from the cytosol to the extracellular medium; this leakage is regulated by anion transport systems, and the leaking rate is dependent on temperature, the cell type and the dye itself.[7] 5). Standard loading protocols using the AM ester form of the indicators may lead to high concentrations of the intracellular dye. Therefore, the free ions can be substantially depleted and the measurement can be distorted.[19]

The development of PEBBLEs (Photonic Explorers for Bioanalysis with Biologically Localized Embedding) has provided a new type of biological imaging method.[20] By incorporating the fluorescent indicators inside a nano-particle matrix, PEBBLEs have been used for intracellular measurements of pH, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, $Cu^{+/2+}$, OH radicals, oxygen and glucose.[21,22] PEBBLEs have many advantages for intracellular sensing because of their small size, nontoxicity and excellent engineerability: 1) For non-ratiometric probes such as rhod-2, by incorporating both the sensing indicator and a reference dye into the PEBBLEs, ratiometric measurements can be easily achieved. 2) The inert polymer matrix of the PEBBLEs protects the cellular environment from any potential toxicity of the indicators and, vice versa, protects the indicators from cell components. 3) The particle matrix also reduces or eliminates the sequestration of the indicator molecules, as well as any nonspecific binding of the indicator to cell proteins. 4) The PEBBLE matrix is a well defined environment, with a well defined K_d that can be calibrated in a test-tube and still be valid anywhere inside the cell. 5) The high surface-to-volume ratio of the PEBBLE, and its small size, grant the analytes high and fast accessibility to the indicators, thus resulting in a fast response time. 6) The amount of the indicators loaded into cells is limited by the controllable amount of PEBBLEs loaded into cells, thus reducing the depletion of the free ions. 7) The excellent engineer-ability of the matrix enables surface conjugation of biomacromolecules, such as proteins or targeting peptides, so that the PEBBLEs can be delivered to specific cell types or to specific organelles inside the cell.

Previous studies have incorporated calcium sensing probes, e.g. calcium green and fluo-4, inside nanoparticle sensors, and some of the advantages of such calcium sensing PEBBLEs were demonstrated.[23–26] These nanoPEBBLEs have shown a nanomolar linear sensing range. However, these PEBBLEs have only been utilized to monitor cytosolic calcium changes, represented by the changes of fluorescence intensity or intensity ratios, but not the true calcium levels. In order to convert the change of fluorescence intensity or intensity ratios to true calcium levels, the calcium dissociation constant K_d of the sensors must be determined by performing calibrations. The accuracy of the calculated intracellular free calcium levels greatly depends on the accuracy of the K_d for the indicators. It is known that the K_d of calcium for many fluorescent calcium probes is significantly affected by the sensing environment. As for rhod-2, its K_d for calcium in solution and in cellular environment is determined to be 570 nM and 720 nM, respectively.[9] However, when the indicators are encapsulated in a nanoparticle matrix, how much its K_d is affected by the sensing environment is unclear. This may make the calibration of the PEBBLE nanosensors in solution invalid for intracellular measurements. In the present work, Ca^{2+}-sensitive ratiometric PEBBLE nanosensors, loaded with rhodamine based Ca^{2+} indicators, were developed and characterized. The rhod-2 PEBBLEs were introduced into live cells, and intracellular calcium imaging was performed on a Leica laser scanning confocal microscope. High resolution images, with excellent limits of detection, were achieved. Specifically, inside a typical cellular environment (cytosol of 9L gliosarcoma cells), the K_d of rhod-2 encapsulated in PEBBLEs was determined to be 293 nM by the in-cell calibration, which enables conversion of the fluorescence intensity to the absolute calcium levels. In aquous solution, the K_d of rhod-2 encapsulated in the PEBBLEs was determined to be 478 nM.

EXPERIMENTAL SECTION

PEBBLE preparation

The polymerization procedure was as described previously with minor changes.[23] The monomer solution was prepared by first dissolving 711 mg acrylamide in 1.2 ml MOPS buffer (pH=7.2), then dissolving 55 mg N-(3-aminopropyl) methacrylamide hydrochloride (APMA) and adding 480 µl glycerol dimethacrylate (GDMA) at last. Calcium indicators were added into the monomer solution for preparing the calcium sensing PEBBLEs. This monomer solution was added to an argon deoxygenated solution that contained 45 ml of hexanes, 2.6 g Brij 30, 1.6 g Tween 80 and 1.3 g Span 80. The solution was stirred under argon throughout the duration of the preparation. To initiate the polymerization, 100 µl of 20% (w/w) ammonium persulfate solution and 100 µl TEMED were added. The solution was stirred at room temperature for two hours to assure complete polymerization. Hexanes were removed by rotary evaporation. To remove the surfactants, unreacted monomers and dye molecules, PEBBLEs were washed with 500 mL ethanol and 500 ml Milli-Q water in an Amicon ultra-filtration cell (Millipore Corp., Bedford, MA) using a 300 kDa filter under 10 psi of pressure. PEBBLEs were then freeze-dried and collected.

Reference dye was conjugated onto the PEBBLEs. 100 mg PEBBLEs were suspended in 5 ml MOPS buffer yielding a 20 mg/ml particle solution, into which 10 ~ 20 µl of Hylite 647 solution (0.1 mg/ml in DMSO) was added. The mixture was stirred for 2 hours at room temperature, then transferred to an Amicon ultra-filtration cell and washed with 60 ml Milli-Q water.

PEBBLE calibration

A 2 mg/ml PEBBLE solution was prepared by suspending PEBBLEs in 10 mM MOPS buffer pH 7.22 containing 0.1 mM EGTA. The free Ca^{2+} concentrations in the calibration solutions were computed. Spectra were collected on a Horiba FluoroMax-3 fluorometer by exciting the sample at 540 nm and recording the resulting emission from 565 nm to 700 nm in 1 nm increments with an integration time of 0.5 second. An aliquot of $CaCl_2$ was added after each successive spectrum was collected. The samples were prepared and continuously stirred by a magnetic stir bar in a 4.5 ml quartz cuvette which was kept by a temperature controlled cuvette holder. All experiments were performed at 37°C.

PEBBLE calibrations were also performed in the presence of background ions. Samples with 2 mg/ml PEBBLEs in 0.1 mM EGTA 10 mM MOPS buffer pH 7.22 containing (i) 20 mM Mg^{2+}9 ; (ii) 20 mM Mg^{2+}, 20 mM Na^{+} and 120 mM K^{+} were prepared. An aliquot of $CaCl_2$ was added after each successive spectrum was collected.

PEBBLE interference

An assay was conducted on rhod-2, and on rhod-2 PEBBLEs, to monitor the interference due to non-specific binding of proteins to the sensors. Samples containing 0.1 µM rhod-2, or 2 mg/ml rhod-2 PEBBLEs, 1% w/v Bovine Serum Albumin (BSA), 0.1 mM EGTA in 10 mM MOPS buffer, pH 7.22 were prepared. An aliquot of $CaCl_2$ was added after each successive spectrum was collected.

Cell culture

PC-3 human prostate carcinoma cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and penicillin (10 IU/ml). 9L gliosarcoma cells were cultured in RPMI medium containing 10% fetal bovine serum (FBS) and penicillin (10 IU/ml). All cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were plated on 1.0 Borosilicate chambered coverglass one day prior to experiments.

Live cell imaging by confocal microscopy

Cells were incubated with PEBBLEs (0.1~0.5 mg/ml) for 30 minutes to 3 hours in the culture media at 37°C. Rhod-2 was loaded into cells by adding rhod-2 AM, 5 μM from a 1 mM stock in dry DMSO, to the culture media and incubating at 37°C for 30 minutes. After loading, cells were washed 3 times with PEBBLE-free/dye-free HHBSS (20 mM HEPES, 1× Hanks Balanced Salt Solution, 2 g/l D-glucose, pH 7.4) and incubated at 37°C for about 30 minutes prior to mounting on the microscope.

Fluorescence images were obtained on a laser scanning confocal microscope. Scanner and detector were mounted on an inverted microscope (Leica sp5) equipped with oil-immersion, 1.4-numerical aperture, ×40 and ×60 objective (Leica). A white light laser (540 nm) served as a primary light source and fluorescence light (560–620 nm, 660–720 nm) from the calcium sensing dye and the reference dye was detected with a high efficiency photomultiplier tube. Confocal pinhole aperture settings were kept at 2 μm throughout the entire experiments.

RESULTS AND DISCUSSION

PEBBLE Sensitivity and Characterization

The sensitivities of the PEBBLEs were determined as two important parameters were optimized: 1) dye loading, and 2) matrix composition. For intracellular sensing one may encounter various interfering fluorescent signals, thus high dye loading is always preferable since it results in a better signal to noise ratio. However, high dye loading may also result in fluorophore self-quenching and therefore lower the sensitivity. In Table 1, the calcium affinities of rhod-2 encapsulated PEBBLEs with different dye loadings were tested and compared with those of the free dye. Four samples, with various amounts of dye (0.1, 0.2, 0.5 and 1 mg Rhod-2) initially added in the preparation, were calibrated. The dye loading amounts in the rhod-NP-1 and rhod-NP-2 PEBBLEs were high enough, so that these two PEBBLEs exhibited satisfactory fluorescent intensity for intracellular measurements. For all four samples, the K_d values were found to be between 500~600 nM, which is somewhat higher than that of the free rhod-2 dye (317±16 nM in Table 1). This result can be explained by the positive charge of the PEBBLEs matrix, which is considered to be caused by the amino groups of the APMA. Note that both the particle matrix and the analyte, Ca^{2+}, are positively charged; the charge-charge interaction may thus affect the PEBBLE sensitivity. In Table 2, the zeta potentials of the PEBBLEs and the calcium affinities of the rhod-2 encapsulated PEBBLEs with different mol % of APMA were tested. Comparing the PEBBLEs with 0.5% APMA to the PEBBLEs with 2.5% APMA, a 2% increase of APMA is not high enough to dramatically affect the zeta potential of the particle matrix and consequently affect the affinity. The function of APMA is to provide amino groups for conjugating targeting peptide and reference dyes. For that purpose, 2.5% APMA would suffice.

The leaching of rhod-2 from the PEBBLEs was monitored. The leaching behavior of the PEBBLEs could be different in solution and in the cellular environment, since dye molecules can be pulled out from the particle matrix by proteins or enzymes, due to non-specific binding. Two particle samples with 0.5 and 1 mg dye loading were monitored over a 3-day time period. A 1% w/v BSA solution was used to mimic the cellular environment. Leaching mostly occurred during the initial 6 hours, with totally 18% dye having been leached out from the 1 mg dye loaded sample but less than 3% from the 0.5 mg dye loaded sample, over 72 hours (see Figure S-1 in supporting information).

The ratiometric property of the rhod-2/Hilyte PEBBLEs was validated (see Figure S-2 in supporting information). The fluorescence peak ratio of F(rhod-2)/F(Hilyte) does not change

as the PEBBLE concentration increases from 0.1 to 5 mg/ml. Among all the commercially available calcium probes, only a few are ratiometric by themselves. This feature limits the use of the non-ratiometric indicators since ratiometric measurement is the most accurate and reliable method, unless a fluorescence lifetime measurement is used. However, encapsulating the indicator probes inside the matrix of the PEBBLEs can solve this issue, because a reference dye can easily be encapsulated inside or conjugated onto the nanoparticles.

PEBBLE Interference Test

Calibrations of the PEBBLEs without background ions or in the presence of 20 mM Mg^{2+}, 20 mM Na^{+} and 120 mM K^{+} were performed, as shown in Figure 1a. The peak intensities of rhod-2 and Hilyte were ratioed and plotted against the free Ca^{2+} concentration. Figure 1b shows typical spectra of rhod-2/Hilyte PEBBLEs with rhod-2 emission increasing as the free Ca^{2+} concentration increases, while the emission of Hilyte is independent of the free Ca^{2+} concentration. Without background ions, K_d is determined to be 429±38 nM. In the presence of 20 mM Mg^{2+}, the K_d rises to 786±65 nM, indicating a decreasing affinity of rhod-2 for Ca^{2+}. Although the addition of Na^{+} and K^{+} changes the ionic strength of the solution, thereby influencing the equilibrium of EGTA and Ca^{2+}, it does not significantly affect K_d, which remains at 830±87 nM in this case.

As a divalent cation, Mg^{2+} also binds with EGTA and changes the free Ca^{2+} level in solution, although the Mg^{2+} binding with EGTA is much weaker than that of Ca^{2+}. At pH 7.22, ionic strength of 0.15 M, and 37°C, K_d(Ca^{2+}EGTA) is found to be 0.115 μM, while K_d(Mg^{2+} EGTA) is 9.4 mM. As expected, Mg^{2+} also binds with the BAPTA moiety on rhod-2, as it binds with other BAPTA-based calcium indicators, such as fluo-3.[6] Therefore, the binding of Mg^{2+} slightly weakens the affinity of rhod-2 for Ca^{2+}. However, the Mg^{2+} binding has relatively little effect on the rhod-2 fluorescence, compared to the 100-fold enhancement from the Ca^{2+} binding, because Mg^{2+} binds mostly to the half of rhod-2 which is remote from the xanthene chromophore.[6] Besides, although the intracellular free Mg^{2+} concentration fluctuates, intracellular free Mg^{2+} is estimated to be much less than 20 mM and its fluctuation is usually within 1 mM range.[27] As long as the interference caused by Mg^{2+} is taken into consideration in the calibration, while the K_d of the sensor is measured in the presence of Mg^{2+} at cellular level, intracellular free Mg^{2+} should pose no serious problem for intracellular Ca^{2+} measurements.

It has also been found that rhod-2 binds with some transition metal ions and its emission increases, such as by binding with Mn^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}; other transition metal ions, such as Fe^{3+}, Co^{2+}, Ni^{2+} and Pb^{2+} also bind to rhod-2 and quench its fluorescence.[28] Therefore, binding with these transition metal ions poses a potential interference to Ca^{2+} sensing. Fortunately, the typical intracellular concentrations of free transition metal ions are lower than their affinities to BAPTA type indicatiors, resulting in little interference. When interference does occur, it can be identified and controlled by using the selective heavy metal ion chelator N,N,N’,N’-tetrakis(2-pyridylmethyl)-ethylenediamine.[29,30]

Another interference test, with 1% w/v BSA, was conducted on the rhod-2 dye and rhod-2/Hilyte PEBBLEs (see Figure 2). The K_d of the free dye in the presence of BSA is about 200 nM higher than the K_d of the free dye in the absence of BSA (Figure 2b). On the other hand, the PEBBLEs containing rhod-2 are unaffected by the addition of BSA (Figure 2c). This suggests that BSA is unable to enter the PEBBLEs due to its large molecular size and thus unable to come in contact with the dye so as to affect its fluorescence or the Ca^{2+} affinity. Non-specific binding of interferents occurs for many small molecule Ca^{2+} indicators, as reported,[7,9] enhancing or weakening their fluorescence. As shown in Figure 2a, in the presence of 1% w/v BSA, the fluorescence of rhod-2 free dye decreases very significantly.

This trend demonstrates a major drawback for intracellular measurements using free dyes, and thus a key advantage of the PEBBLE nanosensors. In the complex cellular environment, in the presence of numerous proteins and enzymes, free dye molecular probes show at least some change in their binding affinity, if they still remain fluorescent. Evidently, the use of PEBBLEs eliminates this problem, by protecting the sensing elements with a polymer matrix. For that reason, a PEBBLE calibration in solution should remain valid for measurements inside the cellular environment. In addition, the cellular environment is also protected by the PEBBLE matrix from potential interference/toxicity of the dye.

The pH dependence tests (see Figure S-3 in the supporting information) suggest that the PEBBLEs are stable at near-neutral pH (pH 6–9). At low pH, the fluorescence of rhod-2 was quenched due to the protonation of the dye molecule. Previous studies also indicated that the $K_d(Ca^{2+}$ rhod-2) value rises when the pH drops below 6.6.[8] Note that the PEBBLE matrix pores easily allow passage of the small hydroxyl and hydronium ions.[23]

Intracellular Calcium Measurements

A prevalent method used for almost all calcium indicators involves adding a Ca^{2+} ionophore A23187 or Br-A23187 to the cell medium so as to manipulate the intracellular free Ca^{2+} levels.[31] Calcium ionophores A23187 and Br-A23187 are mobile ion carriers that form stable complexes with divalent cations and carry these ions across cell membranes. In this method, fluorescence intensity (or intensity ratio) at the Ca^{2+} depleted state (F_{min}) and the Ca^{2+} saturated state (F_{max}) are determined to calibrate the probes. To reduce cell calcium, cells are first washed with Ca^{2+}-free HHBSS. Then the cells are incubated in Ca^{2+}-free HHBSS containing 1 mM EGTA and 5 μM A23187 for 30 minutes, for Ca^{2+} to be depleted from the cytoplasm. Images are taken at the Ca^{2+} depleted state (F_{min}). Next, the indicators are saturated by adding $CaCl_2$ to a final concentration of 5 mM. Images are taken at the Ca^{2+} saturated state (F_{max}) before cell death occurs, which is indicated by membrane blebbing and change in size. After the probes are calibrated, samples of interest are measured (F), and the free Ca^{2+} level is calculated using the equation:

$$[Ca^{2+}]_{free} = K_d(Ca^{2+}\text{rhod}-2) \times (F - F_{min})/(F_{max} - F).$$

A demonstration of the above method using rhod-2 free dye is shown in Figure 3. The AM (acetoxymethyl) ester form of rhod-2 enters the cells, except for the nucleus, through incubation. A Ca^{2+} depleted state and a saturated state were imaged. Rhod-2 AM has shown signs of compartmentalized loading into mitochondria. The uneven distribution of rhod-2 may lead to over- or under-estimation of the Ca^{2+} level at a certain spot. As discussed previously, rhod-2 is not ratiometric by itself. Therefore, it is difficult to accurately determine the absolute Ca^{2+} levels by using free rhod-2. In many studies, the fluorescence changes during cell stimulations or other manipulations, instead of true Ca^{2+} levels, were monitored.[10–12] Note that, since free dye is used for the measurement, extra efforts have to be made to measure the precise $K_d(Ca^{2+}$rhod-2) in the intracellular environment, due to the weakening of the Ca^{2+} affinity by proteins and enzymes.[9]

However, with ratiometric measurements by PEBBLEs, the above issues are resolved or minimized (see Figure 4a). The rhod-2/Hylite PEBBLEs were delivered into 9L gliosarcoma cells by nonspecific endocytosis. An aliquot of $CaCl_2$ was added into cell media to alter the Ca^{2+} level, and the images were taken at different Ca^{2+} levels. In the presence of A23187, the intracellular free Ca^{2+} level changes with the extracellular free Ca^{2+} level, which was manually manipulated by controlling the concentration of EGTA and total calcium in the cell medium. The emission of rhod-2 (as shown in green) increases with the free Ca^{2+} concentration, while the emission of Hilyte (as shown in red) is independent of the free Ca^{2+}

concentration. Therefore, in the overlaid images of green and red, one would see a color change from red to green as the free Ca^{2+} level changes from low (0.32 nM) to high (5 mM). The fluorescence confocal images were processed by Matlab, and the intensity ratios of rhod-2 (green)/Hilyte (red) at each free Ca^{2+} level were calculated. The intensity ratio was plotted against the extracellular free Ca^{2+} concentration in Figure 4b. We name it an "in-cell calibration". The K_d of the encapsulated rhod-2 binding to Ca^{2+} in the in-cell calibration was determined to be 293±200 nM. An in-solution calibration of PEBBLEs was also performed on the Leica confocal microscope in the same cell media. The K_d was determined to be 478±30 nM. Since the K_d of the encapsulated rhod-2 in the in-cell calibration was calculated from the confocal images, it has a larger standard deviation range. The mean K_d in the in-cell calibration is 185 nM less than the mean K_d in the in-solution calibration, indicating the calcium dissociation constant of the encapsulated rhod-2 is not dramatically affected by the cellular environment due to the protection of the PEBBLE matrix. Moreover, for a non-ratiometric indicator like rhod-2, calculating its K_d in cellular environment is much more complicated.[9] However, by encapsulating the indicator in PEBBLEs which are designed to be ratiometric, the calculation of K_d became simple and straightforward. As the K_d for calcium in cellular environment is determined, it enables an absolute calcium level calibration.

Rhod-dextran and rhod-5N PEBBLEs

The other two rhodamine-based Ca^{2+} indicators, rhod-dextran and rhod-5N, were also encapsulated in PEBBLEs with the reference dye Hilyte Fluor 647. Calibrations of these two PEBBLEs were performed (see Figure S-4 in supporting information). The Ca^{2+} binding affinities of these encapsulated dyes were determined to be at millimolar levels, 698±200 μM for encapsulated rhod-dextran and 1.25±0.06 mM for encapsulated rhod-5N. Because of their lower calcium binding affinity, these PEBBLEs are not sufficiently sensitive for intracellular measurements. Figure S-5 shows the fluorescence confocal images of 9L cells loaded with rhod-dextran and rhod-5N PEBBLEs. As shown in the images, PEBBLEs were delivered into 9L cells by PEBBLE-surface-conjugated HIV-1 TAT peptide.

It has been reported that free rhod-2 tends to be trapped in mitochondria due to its large negative membrane potential,[6,10] Therefore, dye leaching from the PEBBLEs became a concern. However, cell imaging using rhod-dextran/Hilyte PEBBLEs, which contain the much larger molecules of rhod-2 dextran conjugates (10 kDa), also shows a similar distribution pattern, indicating that leaching is not a serious concern (Figure S-5a).

There are several limitations of this work that are important to discuss. Although the particle matrix protects the dye from the cellular protein, small ions such as H^+ and Mg^{2+} can still enter the matrix freely and interact with the dye, which would interfere with the Ca^{2+} measurements, similarly as with the free molecular probes. Fortunately, these interferences do not significantly affect the binding of rhod-2 with Ca^{2+}, and corrections can be made by taking these factors into consideration when calibration is conducted.

Another limitation is that the rhod-2 PEBBLEs were delivered into cells by nonspecific endocytosis. It is argued that after cell entry by endocytosis, particles may stay in endosomes which contain different Ca^{2+} levels and pH than the cytosol, resulting in inaccurate Ca^{2+} measurements.[32] Other delivery methods, such as receptor mediated endocytosis or cell-penetrating TAT peptide, lead to less PEBBLE sequestration and more PEBBLEs can be delivered directly into the cytosol. It has been reported that the translocation of TAT peptide into the cells is unaffected by endocytosis inhibitors, indicating a non-endocytotic pathway.[33] In a matter of fact, targeted delivery is another advantage of PEBBLEs. Targeting peptides or proteins can be surface-conjugated on PEBBLEs, so that

the PEBBLEs can be delivered to specific cell types or to specific organelles inside cells.[21,22] These methods will be introduced in our future work.

The calcium levels in cellular organelles are also of great interest due to the function of calcium as a second messenger. However, as demonstrated in our work, without targeting, nano PEBBLEs cannot be internalized by cellular organelles, such as the nucleus, mitochondria, etc. Although targeted delivery of PEBBLEs into cellular organelles has become part of our ongoing research, currently for the calcium sensing inside organelles, free calcium indicators may be more useful than PEBBLEs. For example, free rhod-2 was utilized to monitor calcium levels in mitochondria,[10–12] while the emergence of mithochondrial calcium into the cytosol was easily observed by PEBBLEs.[23]

CONCLUSIONS

This work details the preparation and characterization of Ca^{2+}-sensitive PEBBLEs, loaded with rhodamine based probes as the Ca^{2+} sensing dye and conjugated with Hilye Fluor 647 as the reference dye. While, as discussed above, PEBBLEs are not a panacea for all the problems associated with intracellular chemical imaging, some of the major advantages due to the use of PEBBLEs are well demonstrated by this work: 1) Ratiometric measurements can be achieved by co-loading a reference dye, allowing the changes in fluorescence ratio to be converted quantitatively to Ca^{2+} levels. 2) The polymer matrix protects the dye from non-specific binding caused by intracellular proteins, because the proteins are simply too big to enter the particle pores and interact with the dye molecules. 3) The dye molecules are trapped inside the nanoparticles, and thus some other issues one may have when using the free dye, such as dye sequestration, leakage out of the cells or over-loading of the AM form of the indicators, are reduced or eliminated. 4) The K_d of the encapsulated dye in PEBBLEs stays well defined inside the cell, so that calibration in a test tube still remains valid for intracellular measurements down to the nanomolar level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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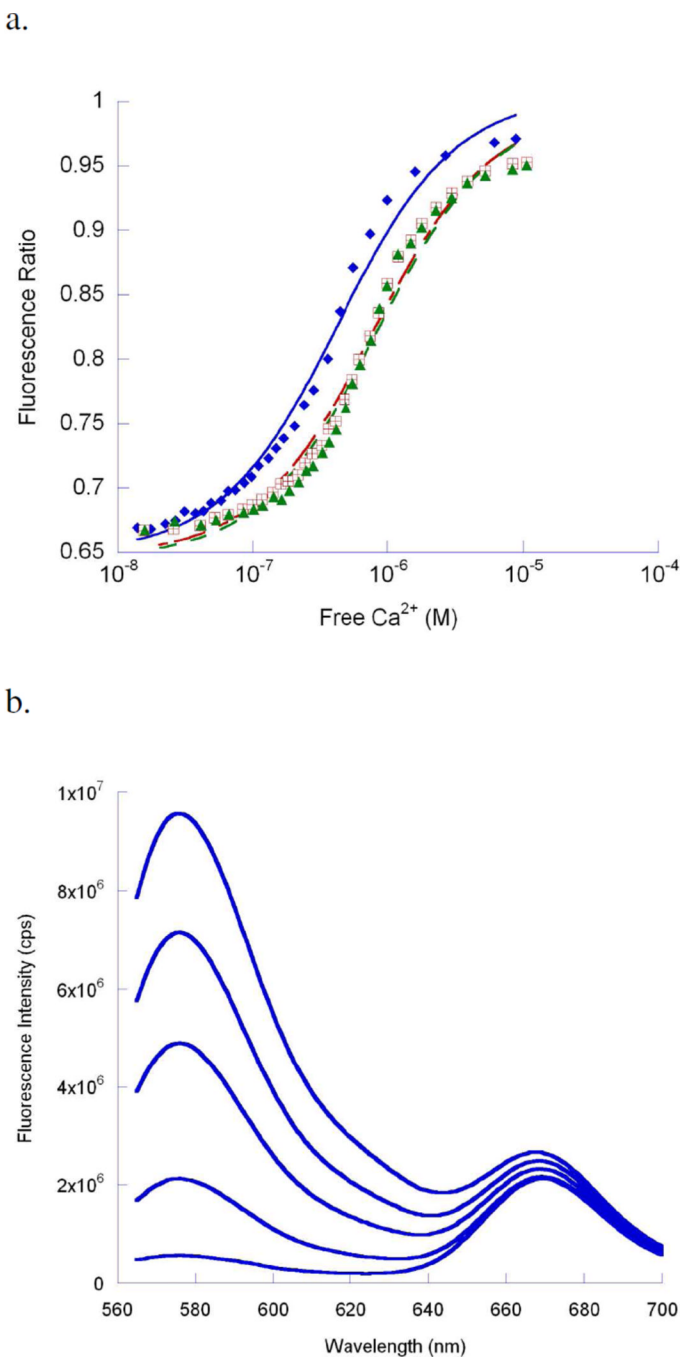


Figure 1.

PEBBLE Interference Test of Metal Ions. (a) Calibration of rhod-2/Hilyte PEBBLEs at 37°C in the presence of different background ions concentrations: zero background ions (blue diamonds), 20 mM Mg^{2+} (red squares), 20 mM Mg^{2+}, 20 mM Na^{+} and 120 mM K^{+} mimicking intracellular environment (green triangles). The peak ratio ($F_{Rhod\text{-}2}/F_{Hilyte}$) is plotted against free Ca^{2+} concentration. (b) Fluorescence emission spectra of rhod-2/Hilyte PEBBLEs without background ions. From bottom to top, free Ca^{2+} was at 10, 130, 350, 775 nM and 3.4 μM.

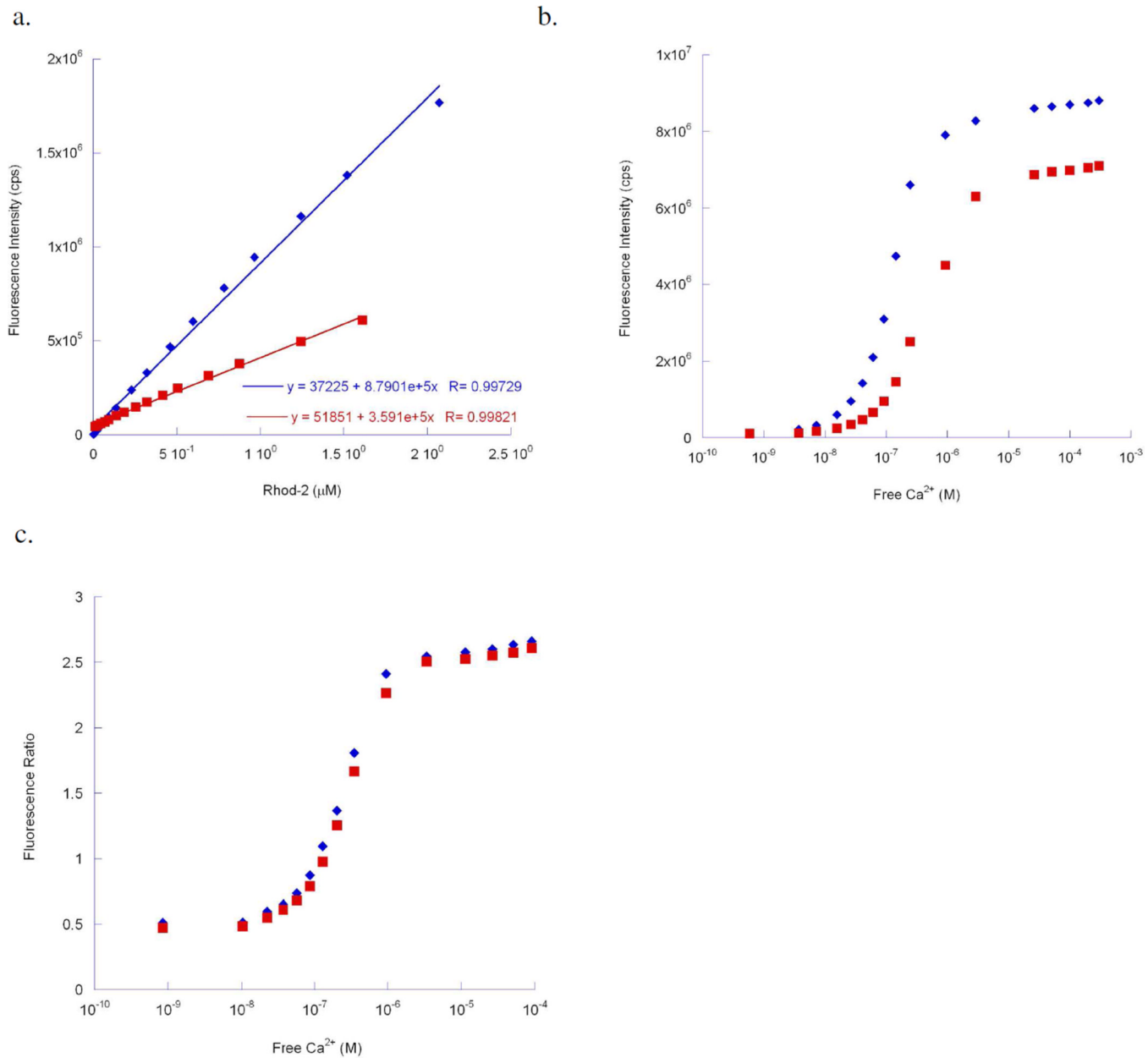


Figure 2.

Interference of 1% w/v BSA to rhod-2 dye and PEBBLEs in solution. Red squares: 1% w/v BSA; blue diamonds: without BSA. (a) Plot of rhod-2 fluorescence versus dye concentration. Based on this graph, the amount of dye loading in PEBBLEs was calculated. In addition, the fluorescence increases observed in the dye leaching test with PEBBLEs in 1% w/v BSA were quantified. Interference of 1% w/v BSA on rhod-2 dye (b) and rhod-2/ Hilyte PEBBLE (c).

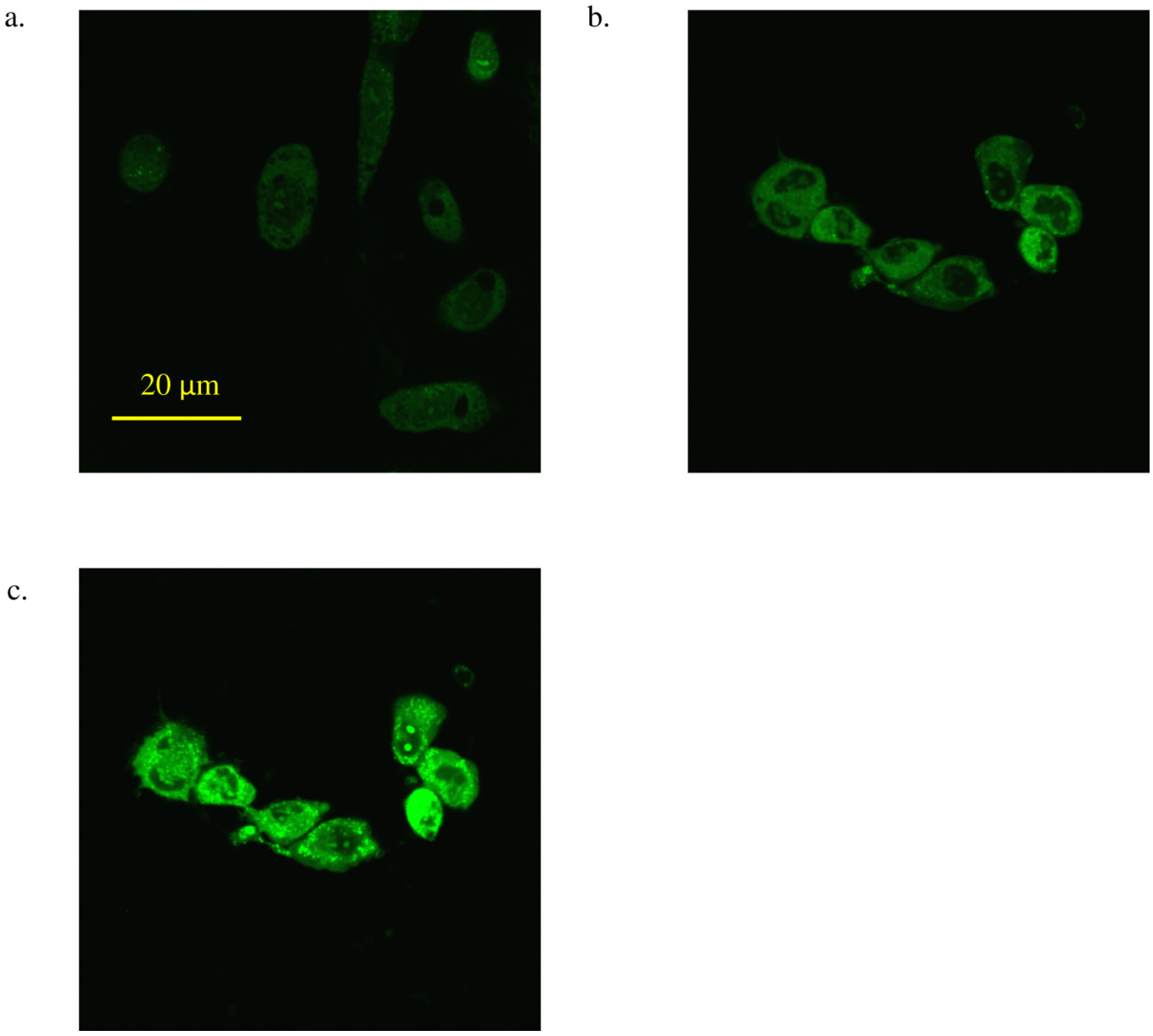
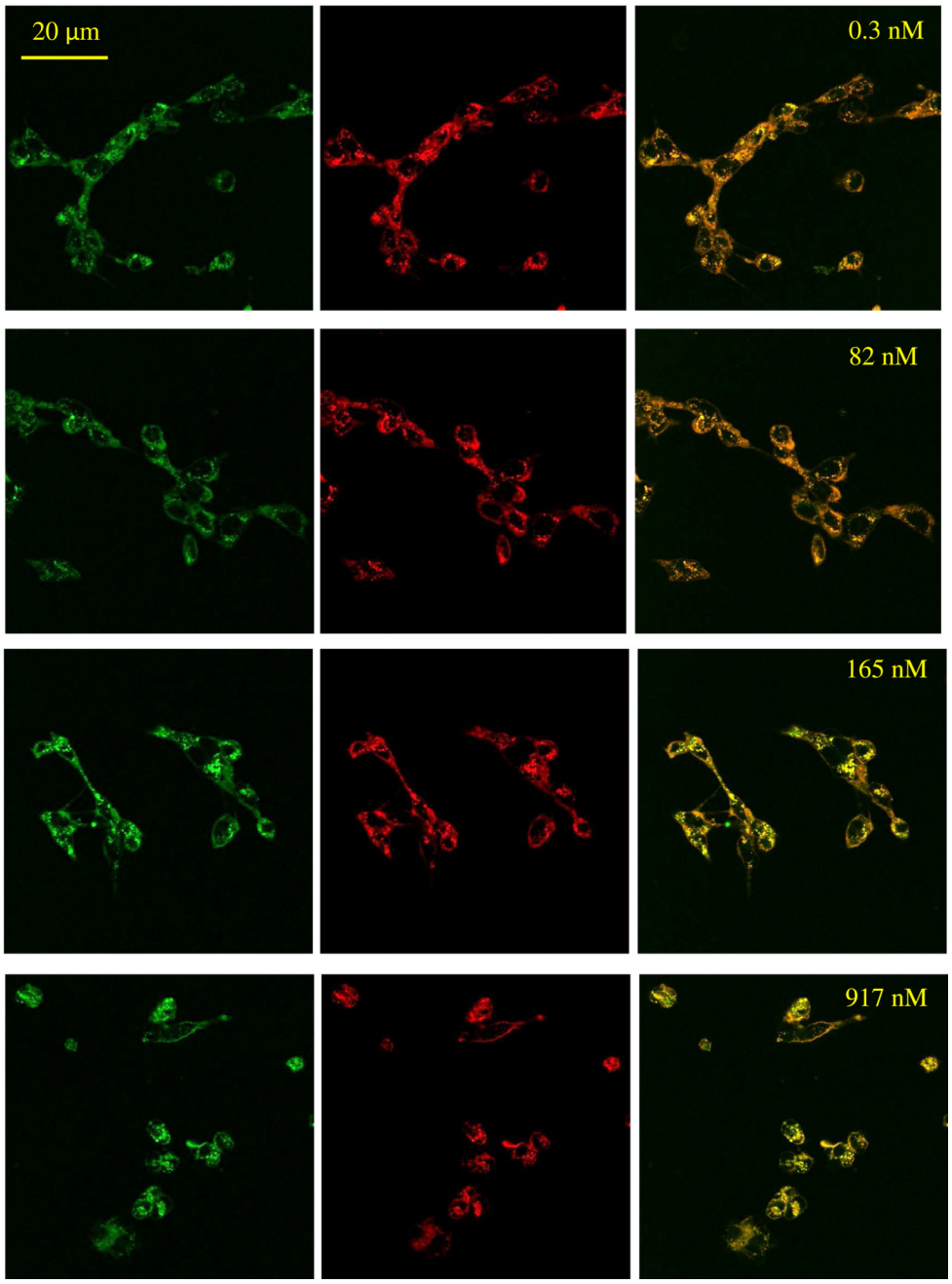
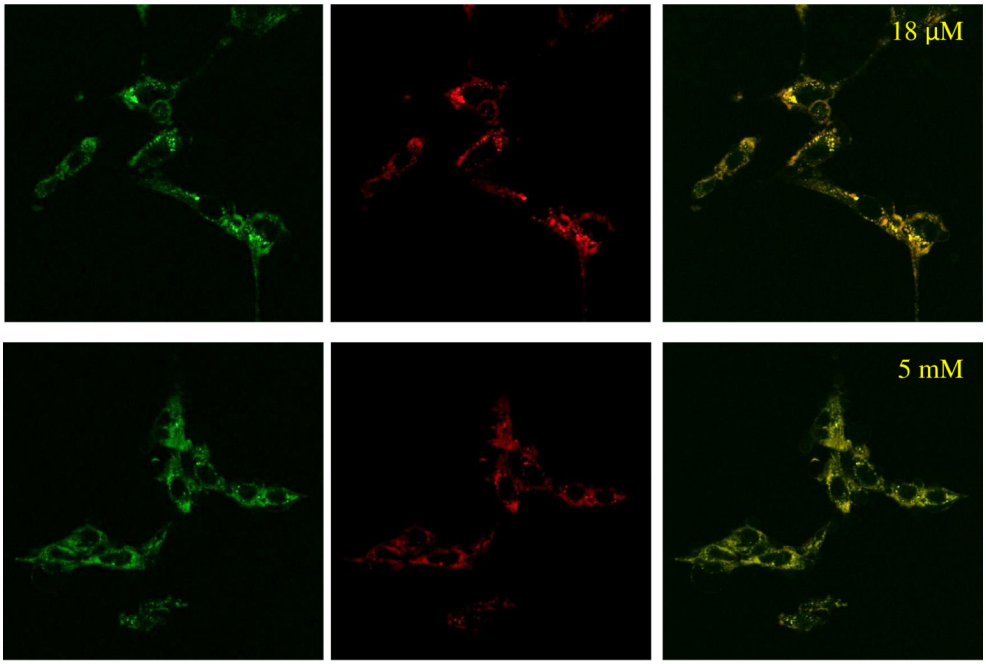


Figure 3.

Fluorescence confocal images of PC-3 cells loaded with rhod-2 AM. Free extracellular Ca^{2+} levels were controlled at (a) < 1 nM, (b) 1 μM, and (c) 5 mM. Here calcium ionophore A23187 (5 μM) was added into the medium transporting the Ca^{2+} through the cell membrane. The sample was excited at 540 nm using a white light laser, and the emission of rhod-2 (560–600 nm) was collected by a PMT and is shown in false color (green). A scale bar is shown in the images.

a.





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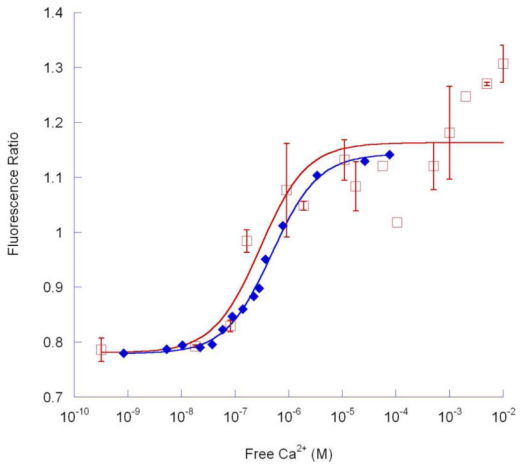

Figure 4.

Intracellular Ca^{2+} Measurements by PEBBLEs. (a) Fluorescence confocal images of 9L cells loaded with rhod-2/Hilyte PEBBLEs. PEBBLEs were delivered by nonspecific endocytosis. Free extracellular Ca^{2+} levels were controlled at 0.3 nM, 82 nM, 165 nM, 917 nM, 18 µM and 5 mM as shown in images. The samples were excited at 540 nm by a white light laser, and the emissions of rhod-2 (560–600 nm) and Hilyte (660–700 nm) were collected by PMTs. Images are shown in false colors as rhod-2 in green, Hilyte in red and overlaid images of rhod-2 and Hilyte. (b) An in-cell calibration (red squares) of rhod-2/Hilyte PEBBLEs conducted on Leica confocal microscope. Intensity ratio of rhod-2/Hilyte was plotted against free extracellular Ca^{2+} concentration. All data were processed by Matlab based on fluorescence confocal images of 9L cells loaded with PEBBLEs. An in-solution calibration of PEBBLEs (blue diamonds) was also performed on Leica confocal microscope

in the same cell media (Ca^{2+}, Mg^{2+}-free HEPES buffered Hanks Balanced Salt Solution containing 10 mM EGTA, pH 7.4) without adding A23187.

Table 1

Dye Loading Efficiency and Ca^{2+} Sensitivity of Various rhod-2 PEBBLEs

PEBBLE ID	dye input (mg)	dye input/total monomer (mg/g)	Dye loading per PEBBLE (wt %)	Dye loading efficiency (%)	K_d (nM)
Free Rhod-2					317±16
Rhod-NP-1	1	0.95	0.025	24	554±44
Rhod-NP-2	0.5	0.48	0.012	23	587±64
Rhod-NP-3	0.2	0.19	0.0065	32	523±22
Rhod-NP-4	0.1	0.10	0.0045	45	526±33

The dye loading efficiency is defined as the percentage of the loaded dye amount with respect to the input amount. Dissociation constant K_d of free rhod-2 or encapsulated rhod-2 in PEBBLEs with different dye loading were computed from experiments performed in 10 mM MOPS buffer pH 7.22 containing 0.1 mM EGTA at 37°C. The mol % of APMA/total monomer of all PEBBLEs is 2.5 %.

Table 2

Zeta Potential and Ca^{2+} Sensitivity of Various rhod-2 PEBBLEs

PEBBLE ID	APMA/total monomer (mol %)	Zeta potential (mV)	K_d (nM)
Rhod-AFPAA-1	0.5	15.2±0.2	593±31
Rhod-AFPAA-2	1	17.0±1.6	588±21
Rhod-AFPAA-3	2.5	18.2±2.3	564±40

PEBBLEs were suspended in Milli-Q water for zeta potential measurements. The K_d values were computed from experiments performed in 10 mM MOPS buffer, pH 7.22, containing 0.1 mM EGTA at 37°C. The total dye input of all PEBBLEs is 0.5 mg.