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Fluorescence-Based Zinc Ion Sensor for Zinc Ion Release from Pancreatic Cells

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In this paper, we describe the synthesis and characterization of analytical properties of fluorescence-based zinc ion-sensing glass slides and their application in monitoring zinc ion release from beta pancreatic cells in cell cultures. To fabricate the sensors, the zinc ion indicator ZnAF-2 {6-[*N*-[*N*′,*N*′-bis(2-pyridinylmethyl)-2-aminoethyl]amino-3′,6′-dihydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-3-one} was modified to include a sufficiently long linking aliphatic chain with a terminal carboxyl functional group. The recently synthesized ZnAF-2 zinc ion indicator provided high zinc ion selectivity in physiological solutions containing millimolar levels of calcium and other possible interfering cations. The carboxyl-modified ZnAF-2 was conjugated to the activated surface of glass slides, which then served as zinc ion sensors. It was possible to grow pancreatic cells directly on the zinc-sensing glass slide or on a membrane placed on these glass slides. The sensors were used to monitor zinc ion release events from glucose-stimulated pancreatic cells. The study showed that the zinc ion sensors responded effectively to the release of zinc ions from pancreatic cells at the nanomolar level with high selectivity and rapid subsecond response time.

Zinc is one of the most abundant transition metals in the body. It is an essential element required by all cells, with various functions; for example, the control of gene transcription and metalloenzyme function.[1−7] In pancreatic islets, which contain large amount of zinc compared to other tissues, zinc is involved in insulin synthesis, storage, and secretion.[8] Insulin is costored as a hexamer complexed with two zinc ions. It is found in a crystalline state and stored within vesicles.[9] When pancreatic cells are stimulated by elevated glucose concentration, insulin is coreleased with zinc through exocytosis. The dissociation of the insulin−zinc complex occurs as a result of exposure to the extracellular pH.[8,10] The dissociation results in the formation of insulin monomers, the biologically active form of insulin.[8,11,12] The role of zinc ions in insulin secretion and in the pathology of diabetes is not entirely understood; however, numerous reports have suggested that diabetes affects zinc homeostasis.[13] Abnormally low levels of zinc, which is typically associated with a poor renal zinc ion reuptake, have been found in many diabetes patients.[13] Zinc-sensing glass slides could find application in studies aiming to understand the role of zinc in the pathogenesis and pharmacology of diabetes.

A number of analytical techniques have been used to detect and quantify zinc in biological samples, including inductively coupled plasma atomic emission spectroscopy,[14,15] atomic absorption spectrophotometry,[16,17] X-ray fluorescence[18] and radioisotope detection.[19] However, none of these techniques can be used for real time zinc ion detection in cellular systems. Fluorescence sensors that were developed in the last two decades have provided a new analytical tool for the detection of intracellular ion levels. Most studies have concentrated on the fabrication of sensors for pH and calcium ion measurements in cells.[20,21] This is due to the

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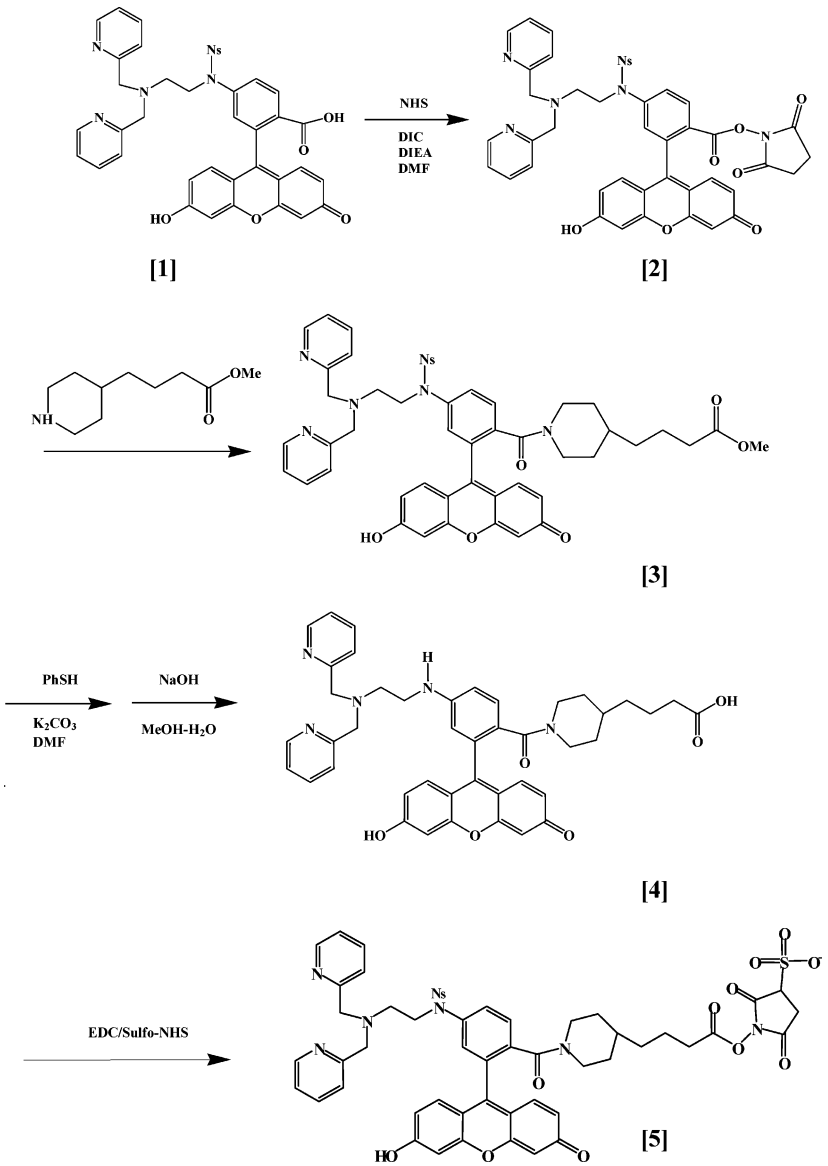

Figure 1. Schematic diagram describing the synthesis of carboxyl-functionalized ZnAF-2 and its activation with EDC/NHS.

availability of fluorescent probes for pH and calcium ion level determination. Zinc ion fluorescent indicators that were used for the analysis of zinc ion in cells include ultraviolet excitable quinoline-based dyes, such as 6-methoxy-8-quinolyl-*para*-toluenesulfonamide (TSQ),[22] Zinquin,[23] and visible light-excitable fluorophores, such as Fluozin-3.[24] A common limitation of these dyes is their limited zinc ion selectivity, particularly in the presence of calcium. In a recent study, Chang et al. developed a new zinc ion-sensitive dye named Zin-naphthopyr 1 (ZNP1).[25] It is based on a hybrid seminaphthofluorescein platform and affords single-excitation, dual-emission ratiometric imaging of intracellular zinc ions through controllable zinc ion-induced switching between a fluorescein and naphthofluorescein tautomeric forms. The tautomeric chemosensor features excitation and emission maxima in the visible range; excellent selectivity for zinc ions over ubiquitous intracellular metal ions such as sodium, potassium, calcium, and magnesium; and a dissociation constant (K_d) for zinc ions of <1 nM. Recently, Kikuchi and co-workers synthesized a new zinc-sensitive fluorophore, {6-[*N*-[*N*′,*N*′-bis(2-pyridinylmethyl)-2-aminoethyl]amino-3′,6′-dihydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthene]-3-one} (ZnAF-2).[1] This fluorophore is structurally and spectroscopically similar to fluorescein. The excitation and emission wavelengths of ZnAF-2 are 492 and 514 nm, respectively. The fluorescence intensity of ZnAF-2 increases with increasing zinc ion concentration. This increase is attributed to photoinduced electron transfer (PET) when ZnAF-2 chelates zinc ions.[1] Whereas similar in zinc-sensing properties to ZNP1, ZnAF-2 exhibits higher long-term stability. The cellular permeability of ZNP1, ZnAF-2, and other zinc ion-sensitive dyes remained a problem, since it is difficult to measure zinc ion release from cells if the zinc ion-sensing dye is cell-permeable. Our current study describes a new

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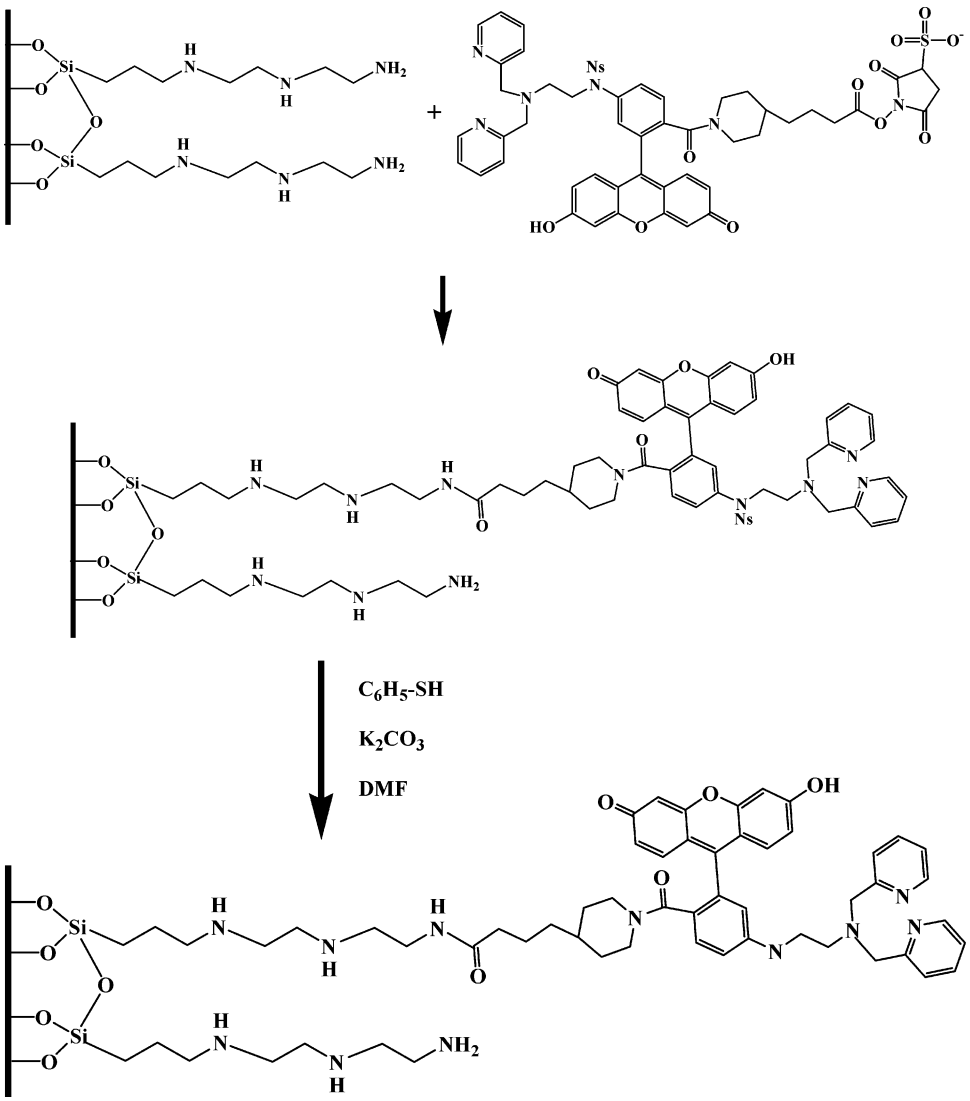

Figure 2. Schematic diagram describing the fabrication of zinc-sensing glass slides, including chemical attachment of carboxyl-functionalized ZnAF-2 to amino-modified silica substrate and the selective deprotection of the nosyl sulfonamide group from the zinc ion binding site.

approach to overcome the cell permeability problem of zinc ion-sensitive dyes in order to facilitate their use in measurement of zinc ion release from cells. It makes use of a modified ZnAF-2 in the fabrication of zinc ion-sensing glass slides. The paper describes the analytical properties of the zinc-sensitive glass slides and their application in the measurement of glucose-stimulated zinc ion release from beta pancreatic cells.

EXPERIMENTAL SECTION

Materials and Reagents. The zinc ion indicator {6-[*N*-[*N*′,-bis-(2-pyridinylmethyl)-2-aminoethyl]amino-3′,6′-dihydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-3-one} (ZnAF-2) was modified to include a carboxyl-terminated aliphatic chain. TPEN (*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine was obtained from Dojindo Laboratories. Trypsin−EDTA was purchased from Gibco. Magnesium chloride, sodium chloride, potassium carbonate, calcium chloride, magnesium sulfate, and potassium phosphate were obtained from EM Industries, Inc. Aqueous solutions were prepared using 18-MΩ deionized water and were conditioned using the water purification system Barnstead Nanopure Diamond. Phosphate buffer (PBS, pH = 7.2) without calcium chloride and magnesium chloride, Dulbecco's modified Eagle's medium, antibiotic−antimycotic and fetal bovine serum qualified for cell cultures were obtained from Invitrogen Corporation. HEPES buffer, *N*-hydroxysulfosuccinimide sodium salt, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, benzenethiol 99%, and zinc sulfate were obtained from Sigma-Aldrich. Trimethoxysilylpropyldiethylenetriamine was purchased from United Chemical Technologies, Inc. Nitrilotriacetic acid was purchased from Spectrum Chemical Mfg. Corp. Cover glasses (22 mm) were purchased from VWR Scientific. All reagents were used as received.

Fluorescence Spectroscopy and Microscopy. Emission spectra were obtained using a PTI international (model QM1) fluorometer equipped with a 75-W continuous Xe arc lamp as a light source. Fluorescence microscopy measurements were carried out using an inverted fluorescence microscope (Olympus IX-70). A 100-W mercury lamp was used as the light source for excitation and a 20× microscope objective was used to collect the fluorescence. The fluorescence microscope filter cube consisted of a 480/30-nm bandpass excitation filter, a 500-nm dichroic mirror, and a 535/40-nm bandpass emission filter. A 250-mm

spectrograph (Roper Scientific, Acton SpectraPro 2300) was attached to the side port of the microscope and used to collect emission spectra of the observed samples. A high-performance charge-coupled device (CCD) camera (Roper Scientific, Photon Max 512B) was attached to the exit port of the 250-mm spectrograph and used for digital fluorescence imaging and spectroscopy of the samples. The Roper Scientific software WinSpec 32 was used for data acquisition and spectral analysis. A typical exposure time was 0.1 s. Each data point was the average of 10 randomly selected fields of view in the obtained images.

Silanization of Glass Slides. Prior to silanization, glass slides underwent acidic treatment to remove particulate matter and organic impurities. The acidic treatment also ensured the release of hydroxyl groups of the silica glass slides.[26,27] Preparation of glass slides for silanization consisted of an immersion in 1:1 concentrated HCl/MeOH for 30 min, rinsing with deionized water, treatment with H_2SO_4 for 30 min, and boiling in deionized water for 5 min. A solution of 1% silane trimethoxysilylpropyl diethylenetriamine (DETA) was hydrolyzed in 1 mM acetic acid for 5 min prior to silanization. The silanization reaction was carried out by immersing the treated glass slides in the DETA solution for 30 min at room temperature. The silanized slides were thoroughly rinsed with deionized water, dried under N_2, and cured at 120 °C for 5 min.

Synthesis of ZnAF-2 Modified with a Carboxyl-Terminated Aliphatic Chain. The synthesis of ZnAF-2 modified with a carboxyl-terminated aliphatic chain is described in Figure 1. Compound 1 was synthesized following Kikuchi's earlier work.[1] Compound 1 (70 mg, 92 μmol) was dissolved in 400 μL of DMF with 50 μL of diisopropylethylamine and 50 μL of diisopropylcarbodiimide. A 50-mg portion of *N*-hydroxysuccinamide was then added to the solution. The reaction mixture was stirred at room temperature for 24 h. It was then evaporated to dryness and resuspended in 0.1% TFA. The product was separated by HPLC with an ODS column. Eluent: initial, 20% acetonitril, 0.1% TFA; final, 80% acetonitril, 0.1% TFA. The collected fractions were lyophilized, and compound 2 was obtained (19 μmol, 21% yield). Compound 2 (16 mg, 19 μmol) was dissolved in 200 μL of DMF with 15 mg of 4-piperidinebutanoic acid. A 8.5-μL portion of triethylamine was then added to the solution. The reaction mixture was stirred at room temperature for 24 h, then the solution was neutralized with 80 μL of 2 N HCl. The solution was separated by HPLC with an ODS column. Eluent: initial, 20% acetonitril, 0.1% TFA; final, 80% acetonitril, 0.1% TFA. The collected fractions were lyophilized, and compound 3 was obtained (13 μmol, 68% yield). Compound 4 was obtained by deprotection of 3, according to the literature.[1]

Binding of ZnAF-2 to Silanized Glass Slides. Figure 2 describes the activation of the carboxyl-modified ZnAF-2 with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The activation resulted in the formation of a highly reactive *O*-acylisourea intermediate that could easily react with the primary amines of the silanized glass slides to form stable amide bonds. The active ester intermediate (*O*-acylisourea) is quite unstable in aqueous solution[28] and could easily hydrolyze to regenerate the carboxyl group. Using sulfo-NHS (*N*-hydroxysulfosuccinimide) increased the stability of the intermediate by forming longer-lived sulfo-NHS ester intermediates. To activate the modified ZnAF-2, we prepared a 500-μL HEPES buffer solution at pH 7.5 that contained 1 μM of the dye, 20 μM EDC, and 50 μM sulfo-NHS. The preparation of ZnAF-2-modified glass slides is shown in Figure 2. The glass slides were incubated in the activated ZnAF-2 solution for 6 h at room temperature. After the coupling reaction, the glass slides were thoroughly rinsed with deionized water and sonicated to remove the adsorbed reagents. It should be noted that the secondary amine of ZnAF-2, which is a part of its zinc binding site, was protected with a nossyl group to preclude the possibility of modifications that would negatively affect the zinc-sensing properties of ZnAF-2 during the fabrication of the zinc-sensing glass slides. The protecting group was removed from the zinc binding site once ZnAF-2 was covalently bound to the glass surface. The selective deprotection of the nosyl sulfonamide group was carried out in aprotic solvent (DMF), with 3 molar equiv of K_2CO_3 salt and 1.5 equiv of thiolate under argon conditions for 6 h at room temperature.

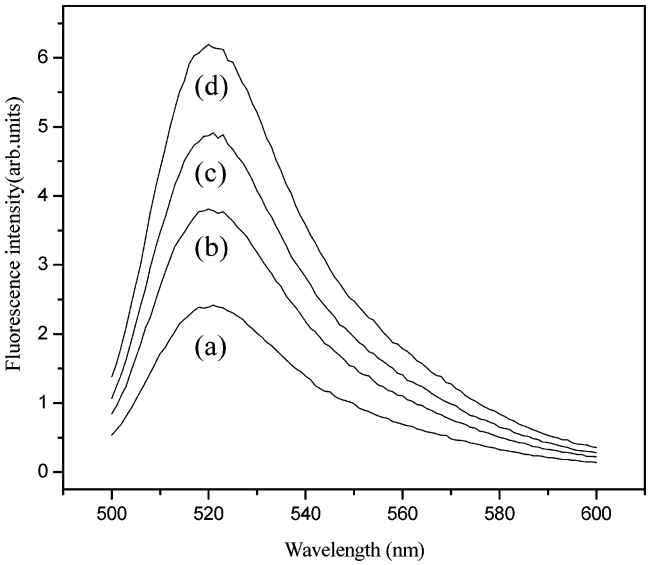

Figure 3. Emission spectra of 5 μM carboxyl-modified ZnAF-2 at increasing zinc ion concentrations: (a) 0, (b) 1, (c) 3, and (d) 5 μM. The excitation wavelength was 498 nm.

Cell Culture and Maintenance. Min-6 cells were kindly provided by Bryan Wolf of Childrens Hospital of Philadelphia. The cells were cultured at 37 °C under 5% CO_2 on polycarbonate membranes of cell culture inserts, which allows media access from both sides of the cell. They were grown in Dullbeco's modified Eagle's medium supplemented with 15% fetal bovine serum, 1% antibiotic−antimycotic, 4% L-glutamine, and 0.06 mM β-mercaptoethanol.

RESULTS AND DISCUSSION

Zinc Ion Fluorescence-Sensing Properties of Modified ZnAF-2. Figure 3 shows the zinc ion concentration dependence of the fluorescence intensity of a 5 μM solution of the modified ZnAF-2 dye. The excitation and emission wavelengths were 498 and 518 nm, respectively. It can be seen that the fluorescence

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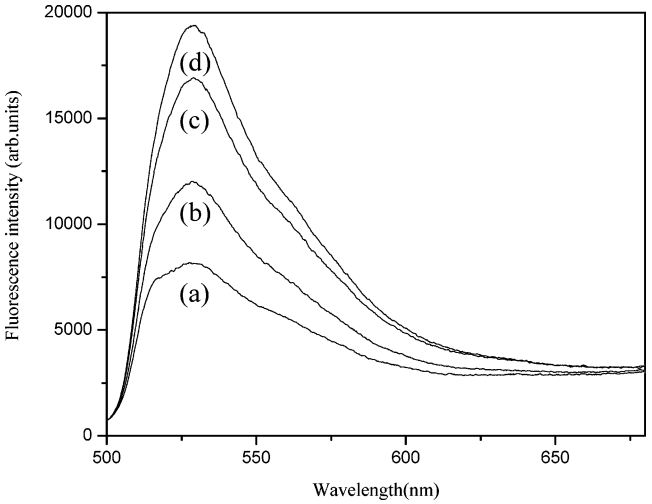

Figure 4. Emission spectra of a zinc ion-sensing glass slide in solutions of increasing zinc ion concentrations: (a) 0, (b) 5, (c) 20, and (d) 80 nM. The excitation wavelength was 498 nm.

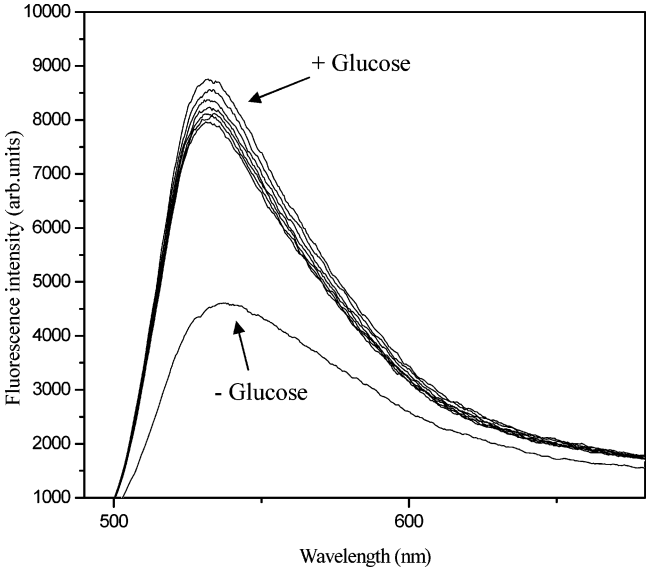

Figure 5. Emission spectra of a zinc ion-sensing glass slide prior to and following glucose-stimulated zinc release from Min-6 cells. The spectra were taken at 1-s time intervals.

intensity increased by ~3-fold when the concentration of zinc in the solution increased from 0 to 5 μM. The excitation and emission wavelengths were very similar to the excitation and emission wavelengths of unmodified ZnAF-2 and other fluorescein derivatives.

Analytical Properties of ZnAF-2-Modified Glass Slides. Zinc ion-sensitive glass slides were fabricated according to the protocol described in the Experimental Section. The concentration of ZnAF-2 in the preparation solution was optimized to realize the maximum zinc ion response. It was found that a solution concentration of 1 μM ZnAF-2 resulted in a maximum response factor of 2.1 ± 0.2. The response factor was defined as F_{max}/F_0 where F_{max} was the maximum fluorescence intensity of ZnAF-2-modified glass slides (obtained at 1 μM zinc ion concentration) and F_0 was the fluorescence intensity in the absence of zinc. Fluorescence intensity measurements of the zinc-sensing glass slides indicated a large variation of up to 25% among different spots on the glass surface. This was attributed to significant variations in the dye distribution on the glass slides, which resulted from the heterogeneous distribution of amine groups on the silanized glass surface. To reduce the variation in the fluorescence intensity measurements, each data point was composed of 10 spectral measurements of randomly selected spots on the glass slide. This decreased the variation in the average reading to ~10%. Photostability measurements were carried out to optimize the experimental conditions in order to avoid photobleaching during fluorescence microscopy measurements. The zinc ion-sensing glass slides exhibited photostability properties similar to glass slides coated with fluorescein. To minimize photobleaching, we employed a 10X microscope objective with a numerical aperture of 0.25, limited the exposure of the glass slides to excitation light by employing a normally closed electronic shutter that opens only during the exposure time of the digital camera, and limited the number of exposures during any given experiment. Under these conditions, we could carry out repeated measurements during 30-min zinc release experiments from cells without significant photodecomposition of the zinc ion-sensing indicator. Selectivity studies were carried out to determine whether the covalent immobilization of ZnAF-2 to the glass slides affected the zinc selectivity of ZnAF-2. The selectivity studies showed that the response factor of the glass slide-based zinc sensors were 1.30 ± 0.21 for calcium, 1.10 ± 0.2 for magnesium, 1.16 ± 0.1 for sodium, and 1.3 ± 0.3 for iron. The affinity of these cations reached saturation at micromolar concentrations. Reversibility measurements were performed to demonstrate the capability of the zinc-sensing glass slides to detect multiple exocytotic events. The glass slide-based sensors were first treated with 1 μM zinc solution, and a 2-fold fluorescence increase was recorded instantly. A fluorescence decrease to the basal level was observed when 1 mM TPEN solution was added to the glass slides. TPEN is an effective zinc ion chelator. At millimolar concentration, TPEN displaced the zinc ions from the glass slides, which resulted in a fluorescence decrease of the sensor. The sample was thoroughly rinsed with a 100 mM HEPES buffer to remove the TPEN and zinc ions from the sensors, then the glass slide-based sensors were exposed again to a zinc-containing solution. As expected, a 2-fold fluorescence increase of the sensor was observed. The response factor decreased gradually with the number of repeated zinc ion exposures, indicating that some of the binding sites were permanently blocked or that the sensing dyes were photobleached. It was possible, however, to run at least five repeated exposures without a significant loss of zinc ion sensitivity. Time-dependent fluorescence measurements of the sensors at 520 nm (λ_{ex} = 498 nm) were carried out to determine the response time of the zinc ion-sensing glass slides. The fluorescence intensity was measured 10 s prior to adding 80 μL of 1 μM zinc ion to the sensor while recording the fluorescence intensity for another 10 s. The fluorescence intensity of the sensor increased due to the addition of zinc in less than 1 s. Figure 4 shows the fluorescence spectra of a glass slide-based zinc sensor at increasing zinc concentration. It can be seen that the fluorescence intensity increased with increasing zinc ion concentration between 0 and 80 nM. The dynamic range of the glass slide-based zinc sensors was between 1.25 and 20 nM. This concentration range is lower than the one observed for free ZnAF-2 solution[1] due to the limited number of ZnAF-2 molecules that are covalently attached to the

glass slide. However, this dynamic range still enables the detection of release events in pancreatic cells.

Application of the Zinc Sensors for the Measurement of Zinc Release from Pancreatic Cells. Min-6 cells were chosen for these experiments since they retain the characteristics of the glucose transport system and glucose metabolism of normal pancreatic beta cells and secrete insulin in response to chemical stimulation by glucose or potassium.[29] We used 500 K cells/mL in our cell experiments. A polycarbonate membrane in which Min-6 cells were grown was placed over the zinc-sensing glass slides. The membrane and glass slides were washed with a solution of Krebs−Ringer buffer at pH 7.4 that contained 500 nM TPEN to complex and remove free zinc ions from the solution. The glass slides and membrane were then washed and placed in a Krebs−Ringer buffer free of TPEN. Fluorescence measurements of the zinc-sensing glass slide under zinc-free conditions were obtained. The cells were then treated with 20 mM glucose to induce zinc release. Figure 5 shows the fluorescence spectra of a zinc-sensing glass slide prior to and following stimulation of the Min-6 cells with 20 mM glucose. An instant increase in the fluorescence of the zinc-sensing glass slide by ~90% is clearly seen. We also employ fluorescence microscopy measurements to monitor the release of zinc ions from Min-6 cells. In these experiments, suspended Min-6 cells were placed directly on a zinc ion-sensing glass slide. Figure 6a shows a transmission image of the cells when placed on the slide. Figure 6b shows the fluorescence image of the cells prior to stimulation with glucose. Only a residual fluorescence is seen. Figure 6c shows the fluorescence image of the glass slide that was taken immediately following stimulation of the cells with 20 mM glucose. A large increase in the fluorescence intensity of the glass slide is observed, which was attributed to zinc release. The fluorescence increase varied largely between cells. Some cells did not register a fluorescence increase when stimulated, whereas others showed a 2-fold fluorescence intensity increase in response to stimulation. This is indicative of a large cell-to-cell variation in the response of pancreatic cells to stimulation.

SUMMARY

Zinc ion sensors were fabricated by the covalent attachment of ZnAF-2, a newly synthesized zinc ion fluorescence indicator, to amino-modified glass slides. Detailed characterization studies indicated that the glass slide-based zinc sensors have the high sensitivity and selectivity and fast response time needed to measure zinc ion release events from pancreatic cells. The sensor was used to measure the release of zinc from Min-6 cells when stimulated by elevated glucose levels. The study revealed large variations in zinc ion release between individual cells. It should be noted that quantitative analysis of released zinc is complicated, since binding of released zinc to free insulin competes to some extent with the binding of released zinc ions to the surface-bound ZnAF-2 molecules. The binding of zinc ions to insulin is pH-dependent. This could result in a pH-dependent negative bias of the measured levels of released zinc ion from the cells. The analytical properties of the newly developed sensors could be further improved by decreasing the heterogeneity of dye distribution on the silanized glass slides. In the future, we will explore alternative approaches to silanization to fabricate more homogeneous sensors.

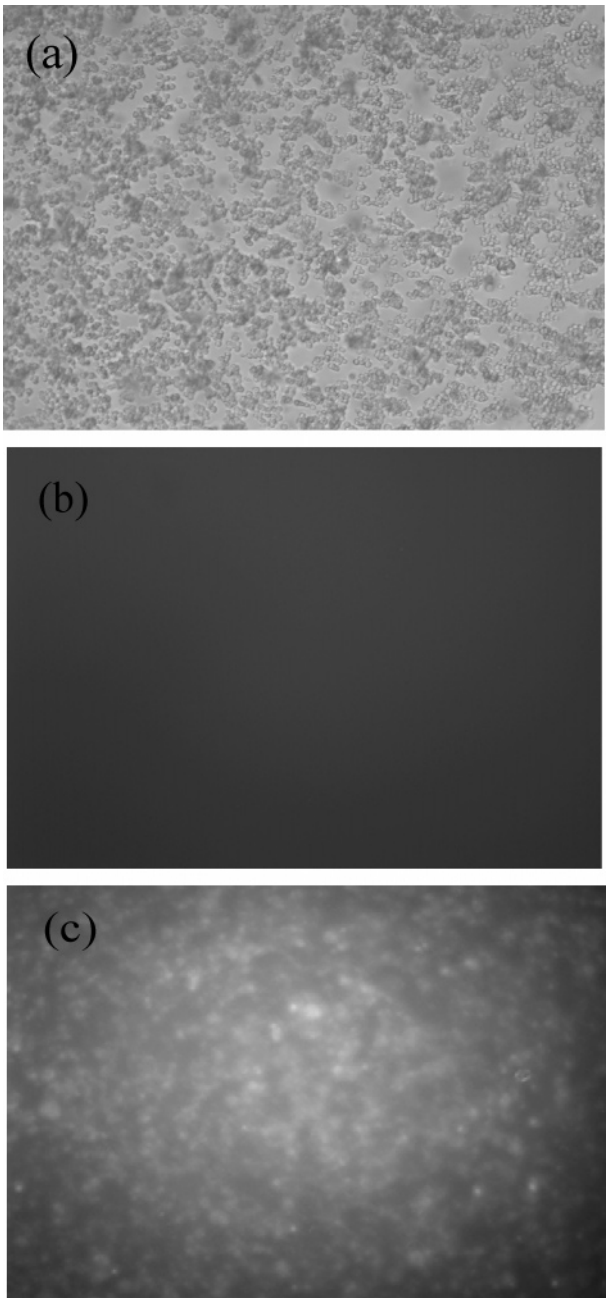

Figure 6. (a) A 10X transmission image of Min-6 cells in suspension, (b) fluorescence image of a zinc ion-sensing glass slide prior to stimulation with glucose, and (c) fluorescence image of the same zinc ion-sensing glass slide following the stimulation and zinc ion release from beta cells deposited on the glass slide.

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