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Advances in zinc sensors for studying zinc release events from pancreatic cells

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ABSTRACT

This paper describes the fabrication and characterization of the analytical properties of fluorescence–based zinc ion sensing glass slides and antibody based zinc sensors and their application in monitoring zinc release from beta pancreatic cells. The zinc ion indicator ZnAF-2 {6-[N- [N', N'-bis (2-pyridinylmethyl)-2-aminoethyl] amino-3',6'-dihydroxy-spiro[isobenzofuran-1(3H),9'-[9H] xanthene]-3-one} was modified to include a sufficiently long linking aliphatic chain, with a terminal carboxyl functional group. The activated carboxyl-modified ZnAF-2 was conjugated to the amino silanized surface of glass slides and to free amino groups of the A2B5 antibody molecules. The sensors were used to monitor zinc ion release events from glucose-stimulated pancreatic cells.

1. INTRODUCTION

There is a growing interest in exploring the biology of β pancreatic cells (1-5). Zinc is involved in insulin synthesis, storage and secretion. Insulin is co-stored as a hexamer complex with two zinc ions in secretor vesicles located preferentially near the plasma membranes. Exposure to insulin secretagogues induces fusion of vesicles with the plasma membrane and release of zinc-insulin hexamers into the extra-cellular space, where the complex dissociates into zinc ions and insulin monomers. The function of zinc ions following insulin secretion is not well understood. Zinc deficiency may be associated with low efficiency of insulin production and secretion observed in diabetes I and with complications in diabetes II. (4) Given the importance of zinc ions in this cellular system it is not surprising that many research groups have focused their effort on developing new and improved zinc ion indicators. Zinc ion fluorescent indicators were previously used for the analysis of zinc ions release from β-cells. These include ultraviolet excitable quinoline-based dyes like Zinquin (6) and visible light excitable fluorophores like FluoZin-3 (1,7). One of the problems in detecting and monitoring zinc release from β-cells using fluorescence detection is the permeation of free fluorescent dyes into cells. Additionally, zinc ion sensitive fluorophores suffer from interferences, particularly from calcium ions. Recently, Kikuchi and coworkers synthesized a new zinc ion sensitive fluorophore {6-[N- [N', N'-bis (2-pyridinylmethyl)-2-aminoethyl] amino-3',6'-dihydroxy-spiro[isobenzofuran-1(3H),9'-[9H]xanthene]-3-one} or ZnAF-2 (8). The fluorescence intensity of ZnAF-2 increases with increasing zinc ion concentrations. In previous studies Kikuchi and coworkers showed that ZnAF-2 had a limit of detection for zinc ions of sub-nM with a dynamic range between 0 and 5 μM. The selectivity of ZnAF-2 towards zinc was significantly improved compared to previously used zinc sensing fluorophores. Permeability of ZnAF-2 into β-cells still remained a problem. In this paper we present a new approach for monitoring zinc ion release from β-cells. To enable these measurements we immobilized the newly modified ZnAF2 (9) to the amino modified silica substrates or to A2B5 monoclonal antibodies. The new bioconjugate sensor has the capability to specifically recognize the biological system subject to stimulation with insulin secretagogues and detect zinc release following stimulation.

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2. ZINC ION SENSING GLASS SLIDES

2.1 Spectral properties of carboxyl-modified ZnAF-2

The synthesis of carboxyl-modified ZnAF-2 was described in an earlier paper (9). Excitation and emission spectra of 5 μM carboxyl-modified ZnAF-2 (shown below) dissolved in 100 mM HEPES buffer solutions at pH 7.4 are shown in figure 1. The spectra of the modified ZnAF-2 show a slight shift in the excitation and emission peak maxima at 499 nm and 520 nm respectively when compared to ZnAF-2.

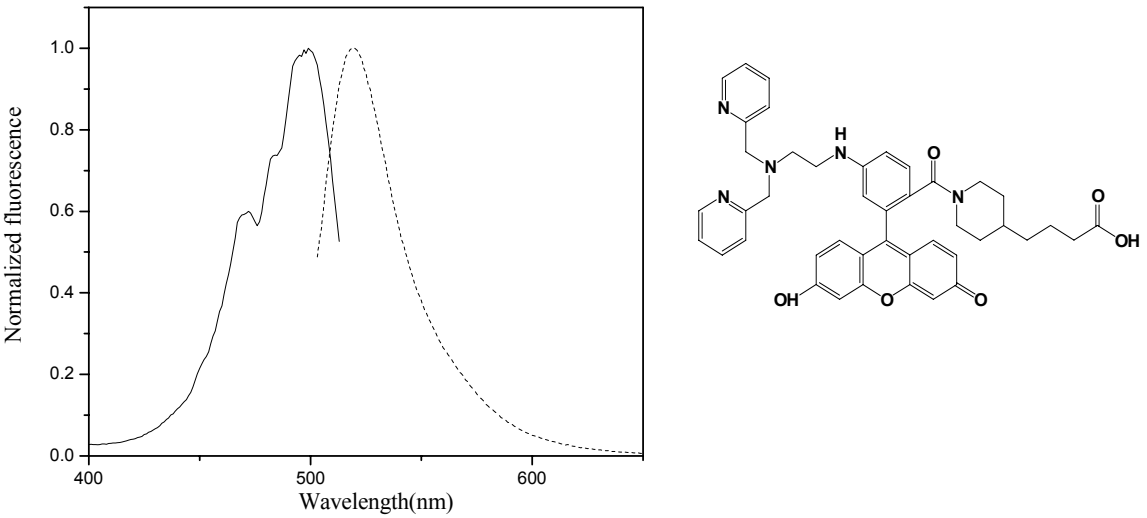

Figure 1 – Normalized excitation and emission spectra of carboxyl-modified modified ZnAF-2

2.2 Fabrication of the zinc ion sensing glass slides

The fabrication of glass slides with zinc ion sensing capabilities involved the silanization of glass slides and chemical attachment of the modified ZnAF-2 to the amino-silanized glass slides. The glass slide silanization was preceded by immersing the glass slides in 1:1 concentrated HCl:MeOH for 30 min, rinsing the slides with deionized H_2O, then immersing them in H_2SO_4 for 30 minutes, and in boiling deionized water for 5 minutes (10). A solution of 1% DETA (silane trimethoxysilyl-propyl-diethylenetriamine) in 1mM acetic acid was hydrolyzed for 5 minutes prior to silanization. The silanization of the rinsed glass slides was performed by treating glass slides with DETA solution for 30 minutes at room temperature. DETA-treated slides were thoroughly rinsed with deionized water, dried under nitrogen and cured at 120°C for 3-4 min (10). The carboxyl moiety of the modified ZnAF-2 was activated with EDC to form a highly reactive O-acylisourea intermediate which reacted with the primary amines of the amino-silanized glass slides. The intermediate was stabilized with sulfo-NHS (N-Hydroxysulfosuccinimide) by forming a sulfo-NHS ester intermediate which hydrolyzes slower in aqueous solution. 1μm of modified ZnAF-2 was activated with 20 μM EDC and 50 μM sulfo-NHS in PBS buffer at pH 7.2. 500 μl of the activated dye was deposited on the surface of glass slides and allowed to react for 6 hours at room temperature. The secondary amine of ZnAf-2 which belongs to the zinc binding site was protected with nosyl groups to prevent the chemical attachment of highly reactive NHS ester intermediate to the zinc binding site which would diminish the zinc ion sensitivity of the slide. After the coupling reaction the glass slides were thoroughly rinsed with deionized water and sonicated to remove loosely adsorbed reagents. The protecting group was removed from the binding site in aprotic solvent (DMF), with a 3 molar equivalent of K_2CO_3 salt and 1.5 equivalent of thiolate under argon for 6 hours.

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2.3 Zinc ion response of ZnAF-2 coated glass slides

The concentration of ZnAF-2 in the solution used to fabricate the zinc ion sensing glass slides 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 (the response factor was defined as F_{max}/F_0 where F_{max} was the maximum fluorescence intensity of ZnAF-2-glass slides (obtained at 1 μm zinc ion concentration) and F_0 was the fluorescence intensity in the absence of zinc (figure 2). The dynamic range was between 1nM and 20nM. This range is sufficient to measure zinc release events in pancreatic cells.

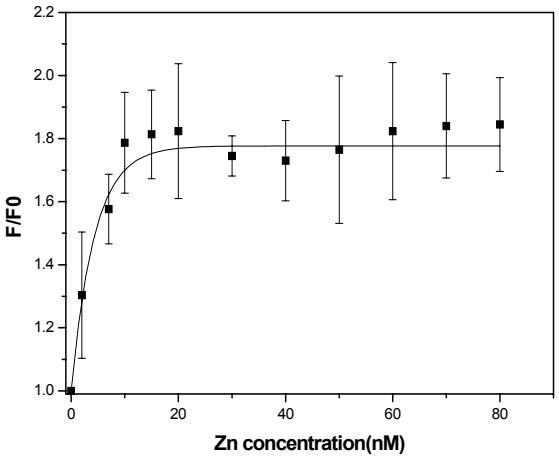

Figure 2 - A calibration curve describing the response of zinc ion sensing glass slides prepared using a solution of 1 μM carboxyl-modified ZnAF-2 to increasing concentrations of zinc ranging from 1to 80nM.

2.4 Zinc release from pancreatic cells

Min-6 cells were kindly provided by Bryan Wolf of Childrens Hospital of Philadelphia. The cells were cultured at 37ºC under 5% CO_2 on polycarbonate membranes of cell culture inserts to allow media access from both sides of the cell. They were grown in Dullbeco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% Antibiotic-antimycotic, 4% L-glutamine and 0.06 mM β-mercaptoethanol. For zinc release measurements, the cell medium was replaced with a Krebs-Ringer buffer at pH 7.4. The basal fluorescence spectrum was recorded. The cells were treated with insulin secretagogues (20mM glucose) and fluorescence spectra of the cells (λex = 450 nm) were recorded in 1 second intervals (see figure 3). The zinc ion sensing glass slides responded to zinc ion release events, which were completed in few seconds.

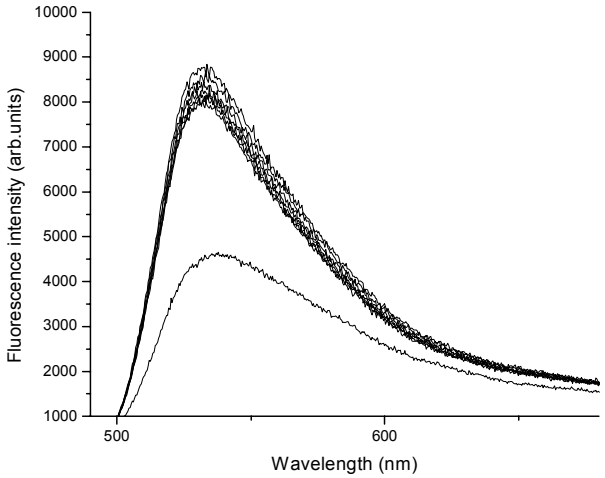

Figure 3 - Emission spectra of zinc release from Min-6 cells. (lex = 450 nm)

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3. FABRICATION AND CHARACTERIZATION OF A2B5–ZnAF-2 BIOCONJUGATE

This part we report the preparation for the first time of antibody A2B5–ZnAF-2 bioconjugate for the targeted measurements of zinc ion release from pancreatic β cells. The sensor was prepared by conjugating mouse A2B5 monoclonal antibody to the carboxyl-modified ZnAF-2 shown in figure 1. The carboxyl modified ZnAF-2 was conjugated to free amino groups of the A2B5 antibody molecules. The fluorescence properties, zinc ion sensitivity, and the binding activity of the newly prepared conjugate were not significantly altered compared to the zinc ion sensing properties of free ZnAF-2 and the binding affinity of free A2B5 antibody molecules. The A2B5-ZnAF-2 bioconjugate attached readily to β-cells in culture and was used effectively for targeted measurements of zinc ion release from β cells in culture. Labeling antibody molecules with fluorophores like fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), Alexa dyes is very common. It enables tracking of antibody molecules when applied as biological probes in cells, tissues or organs or in bioassay development (11-13). However, the conjugation of ion sensing dyes to antibody molecules for the purpose of targeted ion sensing has not been reported. A2B5 antibody molecules recognize specific sialogangliosides and sulfatides on the surface of neurons, neuroendocrine and glial cells. Antibody A2B5 also recognizes sialogangliosides on pancreatic islet cells (14). Expression of antigens to the A2B5 antibody was shown to be directly related to the physiological state of β-cells (14). The binding affinity of A2B5 antibody to β-cells, specifically to the ganglioside GT3 is higher in type 1 diabetes which suggests that gangliosides could be involved in the immunopathology of type I diabetes (15). In our experiments, we used cells derived from a Min-6 mouse insulinoma cell line. Min-6 cells retain β-cell surface antigens and are readily labeled by monoclonal antibody A2B5. Similarly to normal pancreatic cells Min-6 cells secret insulin in response to stimulation by elevated glucose levels (16).

3.1 Synthesis of A2B5-ZnAF-2 Bioconjugate

The conjugation chemistry used to bind fluorophores to antibodies should minimize physical blocking of the binding site of the antibody to its corresponding antigen (11). Previous studies showed that the activity of antibodies is retained when fluorophores are attached to sulfhydrils groups following the reduction of disulfides in the hinge region of antibody molecules. The labeling efficiency of this technique is limited due to the low abundance of sulfhydrils groups in antibody molecules. Another common strategy is to bind the labeling flurophores to glycosylic groups of polysacharrides, which are attached to the Fc region of antibody molecules. This method is limited mostly to polyclonal antibodies that contain polysaccharides in their Fc region (11). Another common technique is to couple the labeling fluorophores to primary amines or carboxylate groups, which are uniformly distributed and easily accessible on the surface of the antibody molecules. Since it is possible that the dye molecules could bind to amino groups in the antibody-binding site, which would result in decreased antibody activity, the resulting bioconjugate must be tested to ensure retention of antibody activity. In our experiments we conjugated the modified ZnAF-2 to A2B5 monoclonal antibody molecules by forming amide bonds between the carboxyl group of the modified ZnAF-2 and the ϵ-amino or N-terminal α–amino groups of lysine residues of the A2B5 monoclonal antibody molecules. Figure 4 describes the preparation scheme of the A2B5–ZnAF-2 bioconjugate. To prevent binding of ZnAF-2 to the antibody molecules through the secondary amines of ZnAF-2, which are a part of its zinc ion-binding site, we saturated the binding sites with 1 mM zinc sulphate prior to the conjugation reaction. A 500 µl solution containing 5 µM modified ZnAF-2 and 1 mM zinc sulfate was used in the conjugation reaction. The modified ZnAF2 was then activated at the free carboxyl group with 3µl 10 mM EDC solution. This step in the EDC coupling reaction leads to the formation of a highly reactive O-acylisourea intermediate. To stabilize the intermediate the reaction took place in the presence of 8µl 10 mM sulfo-NHS. The activated dye was then reacted with 23 µl of 1mg/ml A2B5 antibody molecules under continuous stirring at room temperature for 3 hours. The bioconjugate was separated from excess dye and reaction byproducts using spin dialysis at 14,000g for 10 minutes. The cutoff of the spin dialysis membrane was 100,000Da. The zinc ion-binding site of ZnAF-2 was freed by adding 20µL 50 mM TPEN,

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a strong zinc ion chelator, to remove the zinc ions bound to the ZnAF-2 zinc ion binding site. The bioconjugate was then separated from the TPEN-zinc complex by spin dialysis at 14,000g for 10 minutes and kept in the dark at room temperature until used.

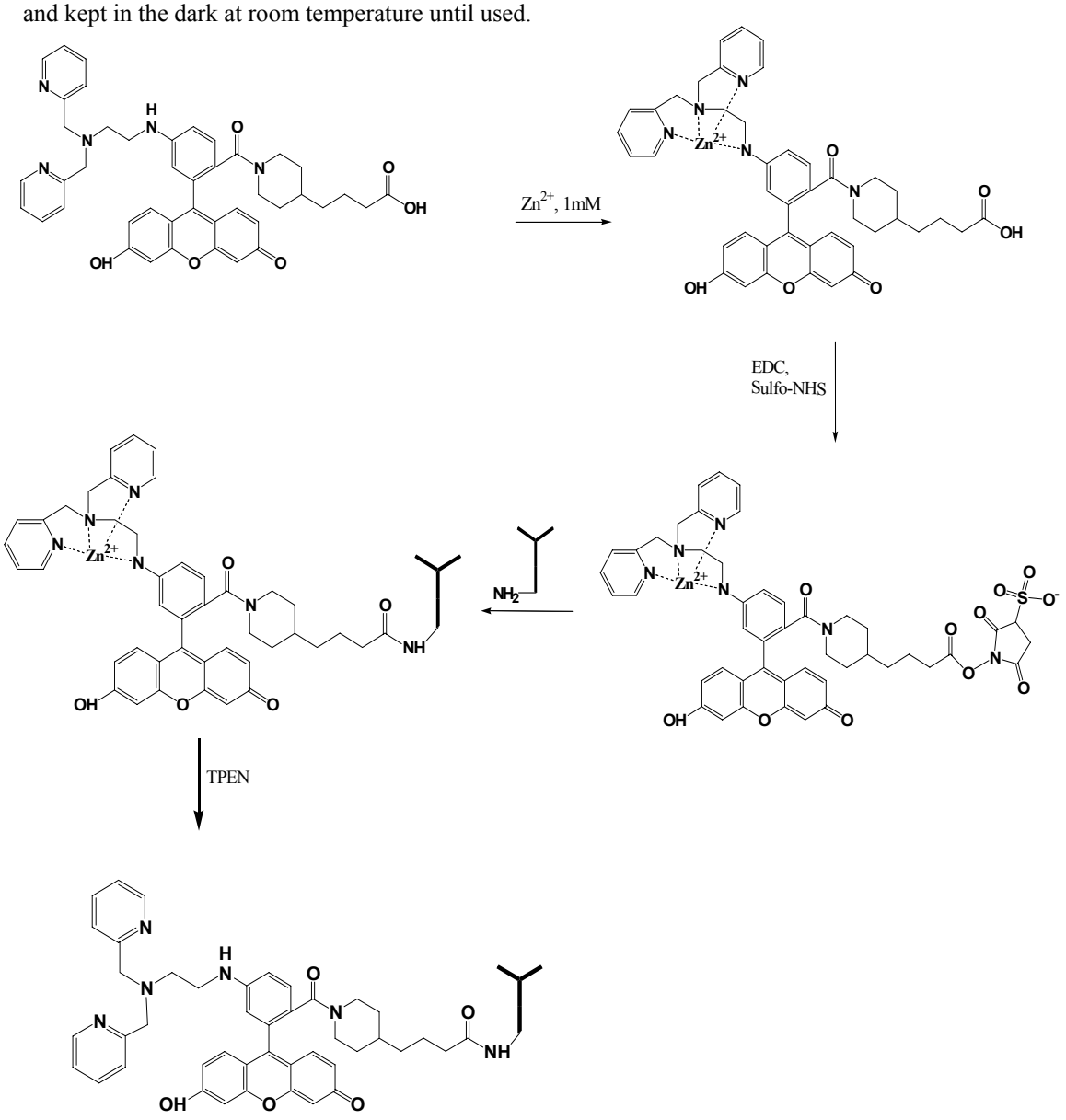

Figure 4 – A schematic diagram describing the synthesis of the A2B5- ZnAF-2 bioconjugate

3.2 Spectral properties of A2B5-ZnAF-2 Bioconjuagte

The spectral properties of the A2B5-ZnAF-2 bioconjugate were measured to study the effect of the conjugation on the spectral properties of ZnAF-2. Excitation and emission spectra of the A2B5-ZnAF-2 bioconjugate show a slight shift in the excitation peak with a maximum at 504 nm which could be attributed to slight changes in the chemical environment of the dye due to conjugation to the antibody molecules. No apparent shift in the emission peak maximum of the bioconjugate compared to free modified ZnAF-2 (excitation at 499 nm and emission peak at 520 nm) was observed. The minimal spectral changes were in agreement with previous studies that mentioned the effect of conjugating fluorophores to

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biomolecules on their fluorescence properties. For example, red shifts in excitation spectra were observed when fluorophores were conjugated to proteins. The extent of these red shifts is often a function of the rate of spectral relaxation, which depends on the specific biomolecule of the bioconjugate (17).

3.3 Targeted attachment of A2B5-ZnAF-2 to MIN-6 Cells and its use for cellular zinc ion release measurements

Min-6 β-cells were grown in 96 well plates to confluence. The cells were then washed with a solution of 500 nM TPEN to complex and remove free zinc ions and were incubated with 50 μL A2B5–ZnAF-2 bioconjugate for 4 hours at 37°C under 5% CO_2 atmosphere. Then, the cells were washed with a PBS buffer solution at pH 7.4 to remove unbound bioconjugate and resuspended in a Krebs-Ringer buffer. The Min-6 cells are at 90% confluence attached to the surface of well plates when the bioconjugates labeled the cells. The signal to background ratio in images of confluent cells was reduced due to the background fluorescence of the plastic surface of the well plate and non specific absorption of the bioconjugate to the extracellular matrix. Min-6 cells were stimulated by exposing to an elevated 20 mM glucose level. This chemical stimulation was previously shown to induce rapid release of insulin and zinc ions from β-cells. The fluorescence spectra of Min-6 cells labeled with the A2B5-ZnAF-2 bioconjugate prior to and following stimulation with glucose is represented in figure 5. A clear and rapid increase in the fluorescence intensity by about 70% is observed.

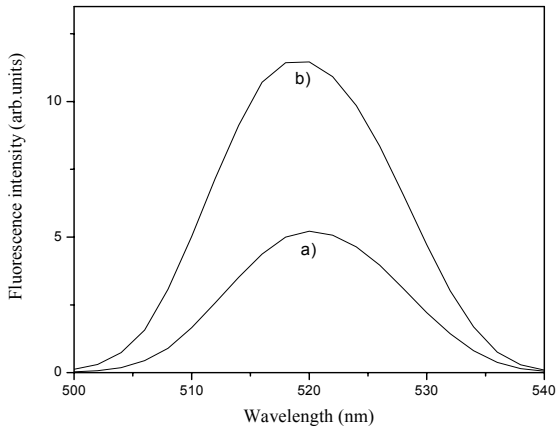

Figure 5 - Fluorescence spectra of the cells prior to a) and following b) stimulation with elevated glucose level

Control experiments in which the cells were treated with glucose free Krebs–Ringer buffer solutions were negative. Only a small fluorescence increase, on an average intensity of 1.09 ± 0.13 was seen, which was attributed to adding other divalent ions like calcium and magnesium to the solution. The binding affinity of the bioconjugate towards Min-6 cells was tested using fluorescence imaging microscopy. Digital fluorescence images of Min-6 cells bound to the bioconjugate are shown in figure 7. Control experiments were carried out using FITC-A2B5 and FITC-AntiGFAP bioconjugates and other non-pancreatic cell types in our laboratory like H9C2. They showed only negligible non-specific binding of the bioconjugates to these cells. The level of non-specific binding was reduced by adding a 1 hour incubation step of the cells with 1% bovine serum albumin (BSA) prior to incubation of the cells with the bioconjugate.

a)

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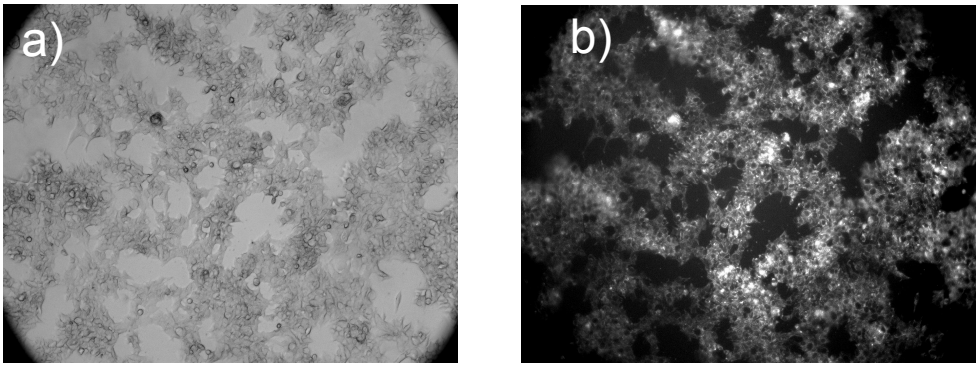

Figure 6 - A phase contrast transmission image (a) and fluorescence image (b) of Min-6 cells labeled with A2B5-ZnAF-2 bioconjugate

SUMMARY AND CONCLUSIONS

The new zinc ion sensors were fabricated by the covalent attachment of ZnAF-2, a newly synthesized zinc ion fluorescence indicator, to amino modified glass slides and amino groups of A2B5 antibody molecules. The zinc ion sensing glass slides monitored effectively zinc release events from immobilized pancreatic cells when stimulated by glucose. It was also possible to conjugate the carboxyl-modified ZnAF-2 to A2B% antibody molecules using the same chemistry. The ZnAF-2-A2B5 bioconjugates effectively target Min-6 pancreatic cells and bind them selectively. The bioconjugate retained its high binding affinity to pancreatic cells and its ability to respond to zinc release events from pancreatic cells. Future studies will focus on the application of the newly prepared zinc ion probes in studies aiming to understand the role of zinc ions levels in the cellular physiology of pancreatic cells and its relation to Diabetes.

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