Development of a Novel Biosensor with Quantized "Off-On" Reversible Fluorescent Molecular Switch for the Lipid Phosphatidylserine

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Honors Thesis
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PASS WITH DISTINCTION
Phosphatidylserine is a key component of the mammalian cell membrane that is highly regulated with regards to which leaflet (inner or outer) of the membrane it is located upon. Its position on the inner leaflet of the cytoplasmic membrane has been shown to impact protein localization, regulate osmolarity by maintaining the charge state of the membrane, and various other cellular processes. In many diseased cells, specifically cancerous cells, Alzheimer cells, and apoptotic cells as the major examples, phosphatidylserine is translocated to the outer leaflet of the membrane. In normal cells, this would be a signal for self-initiated cell death (apoptosis) and macrophage or phagocyte (white blood cell) engulfment. However, in some of these diseased cells, the mechanisms underlying induced apoptosis are circumvented by a variety of methods. This results in many of these diseased cells continuing to live and divide, while maintaining an improper phosphatidylserine asymmetry between the inner and outer leaflets of the cytoplasmic membrane. The large amount of phosphatidylserine on the outer leaflet can thus be used as a marker of pathology, and a sensor can be generated to detect this marker and thus identify diseased cells.

Currently there are several biosensors for phosphatidylserine (Annexin-FITC, Lactadherin-FITC, and Lact-C2-FITC) which use proteins coupled with the fluorescent dye FITC (fluorescein isothiocyanate) to detect phosphatidylserine in apoptotic assays. Unfortunately, FITC is prone to rapid photobleaching and the inability to determine whether the protein is actually bound to phosphatidylserine prevents a truly quantitative assay for phosphatidylserine in diseased cells. This study is concerned with generating a photoswitchable fluorescent biosensor which would indicate the phosphatidylserine-bound (red) or unbound (green) states...
of the protein. This study hypothesized that such a biosensor could be made by using the change in conformation of the Lact-C2 protein domain induced by binding to phosphatidylserine to couple the π electrons of two fluorescent molecules. The aromatic dye NBD (nitrobenzodioxazole) was bound to distal (far apart) lysine residues within the protein's unbound state that, when bound to phosphatidylserine, are brought proximal (close) to facilitate the electron coupling.

After the development of the probe through amplification, purification, and derivatization, it was characterized through the use of spectrofluorimetry, epifluorescent microscopy, and confocal microscopy with vesicles and mildly-metastatic prostate cancer cells (DU145 cells). This molecular biosensor shows high binding specificity and low threshold content for binding phosphatidylserine, photoaccelerated binding, much greater photostability when compared to FITC-derivatized biosensors, and a distinct shift from unbound 485nm excitation/540nm emission ("off," green) to a phosphatidylserine-bound 570nm excitation/680nm emission ("on," red). In addition to the ability to determine the amount of bound versus unbound probe, Lact-C2-Lysine-NBD is also able to transverse the cytoplasmic membrane, making possible for the first time studies of phosphatidylserine distribution throughout the cell, as opposed to current probes which are only able to target the outer leaflet phosphatidylserine. This probe represents proof of quantized photoswitching and could lead to the pioneering of new diagnostic and therapeutic techniques specifically targeting phosphatidylserine that are currently not possible. This is a fundamentally powerful approach with many possible applications, including the design of a new family of quantized "off-on" molecular switches for measuring dynamic molecular processes in complex systems.
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Introduction:

Phosphatidylserine (PS), along with phosphatidylcholine (PC) and phosphatidylethanolamine (PE), is one of the largest components of mammalian cell membranes, consisting of up to 30% phosphatidylserine. The membrane structure in mammals is highly regulated by a variety of enzymes which have the ability to translocate these phospholipids to either side of the membrane leaflet, depending upon cellular needs based on charge, permeability, and membrane integrity. Any variation in the normal makeup of the cellular membranes is indicative of pathology (Balasubramanian and Schroit).

Phosphatidylserine is predominantly found on the inner leaflet, helping to balance the charge due to its anionic head group which helps prevent osmotic damage and contributing to the formation of lipid rafts and other structures integral to the survival of the cell (Leventis and Grinstein). In many diseases, such as cancer, Alzheimer's disease, and many viral infections, phosphatidylserine is translocated to the outer leaflet of the membrane. Normally, the surface exposure of phosphatidylserine is a signal for phagocytosis and/or apoptosis of the cell, but through a complex series of changes brought on by these diseases, cell death does not occur. This link between the translocation of a large amount of phosphatidylserine and certain pathologies indicates the possible development of a phosphatidylserine-specific diagnostic tool, which is as of yet relatively unexplored (Zwaal, Comfurius and Bevers). The goal of this research is to develop and characterize an effective and efficient fluorescent molecular probe for phosphatidylserine to facilitate studies of this phenomena.

Central Hypothesis
The main hypothesis is that two identical fluorescent molecules placed at optimum distances on the backbone of a macromolecule with high phosphatidylserine binding affinity will, due to a conformational change, exhibit a substantial shift in the excitation and emission spectra when the macromolecule binds phosphatidylserine.

Lactadherin Protein

The C2 domain of the protein lactadherin and the fluorescent molecule NBD (nitrobenzodioxazole) were chosen to substantiate the central hypothesis. Lactadherin is a protein which is normally found in the milk of mammals bound to the phospholipid bilayer surrounding the triglycerides in milk fat globules. It is also secreted by macrophages to help identify apoptotic cells by binding to phosphatidylserine that has been translocated to the outer leaflet of the membrane and binding integrins on the surface of the macrophages, facilitating the engulfment of the apoptotic cell. Of specific interest is the C2 protein domain, which binds to phosphatidylserine. This domain shares homology with blood coagulation factors V and VIII, proteins containing a similar C domain that bind to phospholipids in the blood, but lactadherin-C2 (Lact-C2) outcompetes both blood coagulation factors for binding affinity and has a lower threshold content for binding phosphatidylserine, making it an obvious choice for the development of a biosensor for phosphatidylserine (Shao, Novakovic and Head).

In order to detect the presence of phosphatidylserine, Lact-C2 must be modified for detection. The fluorescent dye nitrobenzodioxazole (NBD) has been chosen as the marker for detection in this research. NBD is a small aromatic molecule with an excitation wavelength of 470nm (blue) and an emission wavelength of 540nm (green) and was chosen for its relatively small size and its ease of attachment to a variety of amino acids through the use of different
derivatives. The derivative NBD-SE (NBD bound to a succinyl ester) will be used, which will specifically attach this dye to free lysine residues in the Lact-C2 domain. The target lysines are located close to the binding site on the Spike 1, Spike 2, and Spike 3 lysines displayed in Figure 1.

![Figure 1. Lact-C2-Lysine-NBD where the NBD molecules are attached to Lysines 15 and 45 in Spike 1 and Sike 3.](image)

The NBD molecules in Spikes 1 and 3 are far apart when the protein is in solution. After binding phosphatidylserine, however, these arms are brought in close proximity and a shift to a red emission (680nm) due to the coupling of the \( \pi \) electrons of the NBD molecules is expected based on studies with Lact-C2-Cysteine-NBD (Figure 1) in our laboratory. The fluorescence of
Lact-C2-Cysteine-NBD molecules shifts from red in the free form to green in the phosphatidylserine bound form. The Lact-C2-Lysine-NBD molecule investigated in this research exhibits the opposite behavior and is an excellent complimentary phosphatidylserine biosensor. Both Lact-C2-Lysine-NBD and Lact-C2-Cysteine-NBD molecules are to our knowledge the first examples of distance sensitive probes where the proximal to distal movement (Lact-C2-Cysteine-NBD; red to green shift) and distal to proximal movement (Lact-C2-Lysine-NBD; green to red shift) of the fluorescent probe molecule signals the binding of the protein to the target molecule. This fluorescence shift is quantized as indicated by the fluorescence maxima of 680 nm and 540 nm for the red and green emissions respectively. This is, as indicated in the following sections, a powerful approach for sensitive detection and quantitation of target molecules.

**Project Objective**

The objective of this study is to generate a quantized fluorescent molecular probe that can be used as a diagnostic tool for the identification of diseased human cells. To accomplish this goal, two stages of development were required, the first being the generation of the probe in a reproducible and verifiable way and the second being its characterization utilizing vesicles and cells employing spectrofluorimeter, epifluorescence, and confocal microscopy techniques.

**Methodology:**

**Expression and Purification of Lactadherin C2 (Lact-C2)**

The Lact-C2 plasmid inserted into the pET28 bacterial expression vector was purchased from Hematologic Technologies and the sequence was confirmed by sequencing. Transformed BL21 (DE3) *Escherichia coli* containing the plasmid with the Lactadherin C2 domain with
histidine tag under Lac operon control and kanamycin resistance were used to isolate purified protein. The transformed cells were grown in LB media with kanamycin (25 μg/mL) overnight at 37°C in a 10 mL culture with shaking. A 2.5 mL volume of the overnight culture was used to inoculate 1L of LB-kan media in a 2.5L flask, which was grown at 37°C with shaking until A_{600} value of 1 was obtained. The cells were then induced with 10 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to ensure robust expression of Lact-C2 for 3 hours at 25°C with shaking at 175 rpm. The cell solution was then centrifuged at 5000 g for 15 minutes at 4°C and the cells were pelleted out of the solution. The supernatant was then removed and the cells were stored overnight at -20°C (-80°C for long term storage). The cells were then resolubilized and lysed in Bacterial Protein Extraction Reagent (B-PER) according to the manufacturer's guidelines. The resulting solution was once again centrifuged to pellet out cellular contaminants, leaving the protein in the supernatant.

This lysate was filtered before being run on a nickel liquid chromatography column (Ni-NTA Superflow Column), which interacts with the histidine tags on the protein and allows its isolation from the bulk of other proteins, and eluted with 400 mM imidazole. The imidazole was removed through dilution with 300 mM NaCl and 20 mM Tris (pH 7.4) buffer and filtration using an Ultrafree-15 Amicon filter centrifuge column with a 10000 kDa filter to ensure the protein remains above the filter, which also serves to concentrate the protein. As shown in the SDS-PAGE separation, Figure 2 the protein obtained in this manner had an impurity associated with the desired Lact-C2 with 20 kDa molecular weight. This protein was further purified by FPLC.
The protein solution obtained after batch Ni-NTA Superflow column separation was run on a nickel affinity Fast Protein Liquid Chromatography (FPLC) column to further purify Lact-C2 from contaminant proteins. The FPLC program utilizes a step-wise gradient of 10 mM, 30 mM, 50 mM, 70 mM, 140 mM, and 400 mM imidazole in 300 mM NaCl/20 mM Tris (pH 7.4) buffer to remove all other contaminant proteins which interacted with the nickel affinity column and were eluted in the initial purification. The desired Lact-C2 protein was obtained with 400 mM imidazole elution and, as indicated by Figure 2, the protein was pure, exhibiting a single 20 kDa band when run on SDS-PAGE. The FPLC purified protein was then concentrated and the imidazole was removed utilizing the same method explained previously. The FPLC purified protein was then dialyzed in bicarbonate buffer (pH 9.4) to further remove imidazole, as well as tris(hydroxymethyl)aminomethane (Tris) which has a primary amine, and may reduce the

\[ \text{Ni-NTA Superflow column} \quad \text{FPLC} \]

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<tr>
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\[ \text{Figure 2. SDS-PAGE of Lact-C2 after purification by Ni-NTA Superflow Column and FPLC employing Ni-NTA column.} \]
labeling efficiency due to the similarity of their structure to lysine (an amino acid containing an amine group, where the dye will be attached). The concentration of the protein was determined by absorbance at 280 nm utilizing the calculated molar extinction coefficient of 44,620 M\(^{-1}\) cm\(^{-1}\).

**NBD Derivatization**

The purified, dialyzed protein was then reacted with the dye derivative NBD-SE, added in 20 fold molar excess of the protein and dissolved in dimethyl sulfoxide (DMSO), and the reaction was conducted for 24 hours at 4°C with shaking before being removed the following day and dialyzed once again in bicarbonate buffer to remove free, unreacted dye molecules. The protein was then subjected to another round of concentration using the centrifuge filter to further remove free dye and concentrate the remaining dye-labeled protein, which was kept in dark Eppendorf tubes to prevent photobleaching.

**Preparation of Vesicles**

The lipids dipalmitoylphosphatidylcholine (DOPC) and dipalmitoylphosphatidylserine (DOPS) were obtained from Avanti Polar Lipids. 50 μL of 10 mg/mL DOPC/DOPS in a 7:3 ratio were dissolved in chloroform and methanol in a 2:1 ratio. 300 mM NaCl/20 mM Tris buffer was added to the mixture such that the final concentration of lipids was 1 mg/mL. Giant unilamellar vesicles were prepared by evaporation of a mixture of the lipids at 65°C for 10 minutes while gently passing nitrogen gas through the solution. The resultant solution was allowed to sit for 30 minutes before use in vesicle experiments at room temperature. A large population of vesicles ranging from 0.5-30 μm were obtained and were freshly prepared before each set of experiments spanning at most 24 hours. Phosphatidylcholine (100% DOPC) vesicles were used
as controls, as the Lact-C2-Lysine-NBD should not bind to PC head group, though any free NBD (small non-polar molecule) would diffuse through the non-polar portion of the lipid bilayer and indicate that further dialysis is needed. 30% Phosphatidylserine, 70% Phosphatidylcholine vesicles were used for the bulk of the vesicle experiments.

**Cell Culture**

All cell cultures used in this study were initiated from cultures purchased from the American Type Culture Collection (ATCC). Culturing procedures were as follows: RWPE1 and RWPE2 cells were cultured in Keratinocyte Serum Free Media (KSFM) supplemented with antibiotics penicillin and streptomycin (100 units/mL of each). The DU145 and PC3 cells were cultured in RPMI media supplemented with penicillin and streptomycin and 10% V/V Fetal Bovine Serum (FBS). Cells were grown to 80% confluence, treated with Lact-C2-Lysine-NBD for 24 hours, and de-adhered from the culture plates with 2mM EDTA before being placed in a well slide. The cells were then imaged by epifluorescence and confocal microscopy techniques.

**Fluorescence Spectroscopy and Microscopy**

Fluorescence microscopy images were recorded using the Zeiss Axiovert Imager D1 upright microscope with filters for green and red emission obtained from the Chroma Corporation. Typical exposure times were less than 1 second and images were obtained using bright field, green filter (green emission), and red filter (red emission) for each set of cells or vesicles.

Confocal microscopy images were recorded using the Zeiss LSM 510 META confocal microscope with laser excitation and appropriate excitation/emission filters for both red and
green emissions. Bright field and merged images were also obtained for each set of cells or vesicles.

Fluorescence spectra were taken using a Shimadzu RF-5301 spectrofluorimeter. The spectra were taken by adding 20 µL of 1 µM Lact-C2-Lysine-NBD to 1 µM vesicles in a 100 µL quartz cuvette. The samples were measured at constant excitation wavelength to obtain emission spectra and constant emission wavelength for excitation spectra, typically at 485 nm excitation/540 nm emission for the green fluorescence or 570 nm excitation/680 nm emission for red fluorescence.

**Results and Discussion**

**Degree of NBD Labeling**

The degree of NBD labeling or the extent of derivatization of Lact-C2 with NBD was determined from the UV-Visible spectrum displayed in Figure 3. The degree of labeling was determined by first calculating the protein concentration by dividing the absorbance at 280nm (0.23193) by the molar absorptivity (44,620 M⁻¹ cm⁻¹) and then by dividing the absorbance at 485nm (0.26648) by the protein concentration and molar absorptivity of NBD (26,000 M⁻¹ cm⁻¹). The degree of labeling was 1.97 ± 0.5, indicating that an average of two lysine residues per Lact-C2 were derivatized with NBD by the procedure employed. The Lact-C2-Lysine-NBD has green
fluorescence (540 nm emission maximum) in the unbound form and red fluorescence (680 nm emission maximum) in the phosphatidylserine bound form. This shift indicates that the NBD molecules are distal in the free form and proximal in the phosphatidylserine bound form. Since Spike 1 and Spike 3 domains are involved in binding phosphatidylserine, the fluorescence emission shift indicates that NBD molecules are predominantly attached to Lysines 15 and 45 on Spike 1 and Spike 3 respectively. The binding of Spike 1 and Spike 3 to PS results in a distal to proximal movement of the two NBD molecules on Lysine 15 and Lysine 45 resulting in a green to red fluorescence shift.

**Fluorescence Spectra**

The fluorescence spectra of Lact-C2-Lysine-NBD in the free form in Tris buffer (pH = 7.4) and bound to PS in DOPC/DOPS vesicles were recorded to determine the spectral shift between the unbound and PS bound forms. Typical green and red fluorescence spectra of unbound Lact-C2-Lysine-NBD are shown in Figure 4 and for bound form with DOPC/DOPS vesicles in Figure 5.

![Fluorescence Spectra](image)

**Figure 4.** (a) Fluorescence spectrum (545nm green emission; "off") of unbound Lact-C2-Lysine-NBD obtained by 485nm excitation. (b) Fluorescence spectrum (680nm red emission; "on") of unbound Lact-C2-Lysine-NBD obtained by 570nm excitation.
Figure 5. (a) Fluorescence spectrum (545nm green emission; "off") of phosphatidylserine-bound Lact-C2-Lysine-NBD obtained by 485nm excitation. (b) Fluorescence spectrum (680nm red emission; "on") of phosphatidylserine bound Lact-C2-Lysine-NBD obtained by 570nm

Through the use of time dependent fluorescence spectral measurements over the course of 16 hours (measurements every 5 minutes) with 70% PC : 30%PS vesicles in 300 mM NaCl/20 mM Tris (pH 7.4), it was found that the majority of binding events occur after approximately 3 hours of incubation with Lact-C2-Lysine-NBD. This is evidenced by the characteristic decrease in green fluorescence (unbound state) and gradual increase of red fluorescence (bound state) shown in Figure 6.

Figure 6. Time-dependent spectrofluorimetry obtained by incubation of Lact-C2-Lysine-NBD with DOPC/DOPS (7:3) vesicles in 300mM NaCl/20mM Tris (pH 7.4).
Fluorescence Microscopy--Vesicle Experiments

The Lact-C2-Lysine-NBD sample was tested for both specificity of binding and the presence of excess unreacted dye through the use of 100% DOPC vesicles as control experiments. The DOPC vesicles, while containing a polar head group, should not result in binding when incubated with Lact-C2-Lysine-NBD due to its specificity for the phosphatidylserine head group and, indeed, this was the case, as the DOPC vesicles did not fluoresce when excited with 485nm or 570nm light with appropriate filters, though clumps of the unbound probe were visible as illustrated in Figure 7 (a-c). When coupled with the fluorescence observed in 7:3 DOPC/DOPS vesicles, this indicates that the specificity was not

Figure 7. (a) A bright field image of 100% DOPC vesicles not treated with Lact-C2-Lysine-NBD. (b) A green epifluorescence image of 100% DOPC vesicles not treated with Lact-C2-Lysine-NBD. (c) A red epifluorescence image of 100% DOPC vesicles not treated with Lact-C2-Lysine-NBD. (d) A bright field image of 100% DOPC vesicles treated with Lact-C2-NBD. (e) A green epifluorescence image of 100% DOPC vesicles treated with Lact-C2-NBD. (f) A red epifluorescence image of 100% DOPC vesicles treated with Lact-C2-NBD.
impeded by the attachment of NBD to the lysine residues as seen in Figure 8 (a-c). Additionally, this control indicates that there is no free, unreacted dye, as NBD is membrane permeable and less water soluble and would have been trapped in the lipid component of the vesicles, giving them a green fluorescence, and no green fluorescence of the vesicles was observed.

**Figure 8.** (a) A bright field image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-NBD after 1 hour. (b) A green epifluorescence image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-Lysine-NBD after 1 hour. (c) A red epifluorescence image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-Lysine-NBD after 1 hour. (d) A bright field image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-Lysine-NBD after 48 hours. (e) A green epifluorescence image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-Lysine-NBD after 48 hours. (f) A red epifluorescence image of DOPC/DOPS (7:3) vesicles treated with Lact-C2-Lysine-NBD after 48 hours.

In DOPC/DOPS (7:3) vesicles, fluorescence in both the green and red regions were observed after only 10 minutes of incubation (Figure 8). The red fluorescence noticeably increases over time, though not as quickly as is observed in the time-dependent spectrofluorimetry experiments. This is most likely due to the observed property of photo-accelerated binding found in earlier studies in our laboratory, wherein it was found that light
stimulates and accelerates the binding of Lact-C2-Cysteine-NBD to phosphatidylserine (Patel). There is an appreciable difference due to the shutter in the spectrofluorimeter always remaining open and illuminating the contents of the cuvette, while the microscope light source is turned off between measurements, limiting the amount of light illuminating and stimulating the binding of Lact-C2-Lysine-NBD to phosphatidylserine. The continued fluorescence in the spectrofluorimetry experiments also indicates a great deal of photostability which many other probes, such as annexin-FITC, lactadherin-FITC, and Lact-C2-FITC, lack as they photobleach rapidly within minutes of illumination.

Fluorescence Microscopy--Prostate Cancer Cell Targeting

Figure 9. (a) A bright field image of DU145 cells not treated with Lact-C2-Lysine-NBD. (b) A green epifluorescence image of DU145 cells not treated with Lact-C2-Lysine-NBD. (c) A red epifluorescence image of DU145 cells not treated with Lact-C2-Lysine-NBD.

The moderately metastatic cancer cell line DU145 was used for the fluorescent microscopy experiments utilizing prostate cancer cells. As shown in Figure 9 (a-c), cells not incubated with the fluorescent probe inherently contain a small amount of fluorescence in both the green and red spectrums due to the autofluorescence of common cell proteins. Figure 10 (a-c) illustrates the increase in fluorescence in both green and red regions due to the incubation of the DU145 cancer cells with Lact-C2-Lysine-NBD for a period of 24 hours.
Figure 10. (a) A bright field image of DU145 cells treated with Lact-C2-Lysine-NBD. (b) A green epifluorescence image of DU145 cells treated with Lact-C2-Lysine-NBD. (c) A red epifluorescence image of DU145 cells treated with Lact-C2-Lysine-NBD.

Figure 11 (a-d) and Figure 12 (a-d) are examples of the cells imaged with confocal microscopy excited at 485nm (green emission) and 570nm (red emission), as well as the bright field image and the merged image. The yellow areas in the merged images indicate areas where both red and green fluorescence are observed. The persistence of green fluorescence is due to the ability of the protein to diffuse through the membrane and represents regions where it is unbound to PS. While within the lipid bilayer, the conformational change which allows the switch from the "off" (green) to "on" (red) states is reversed due to Lact-C2-Lysine-NBD no longer binding phosphatidylserine, so the protein returns to green fluorescence. With time, the green fluorescence vanishes as equilibrium is reached and Lact-C2-Lysine-NBD is only bound to phosphatidylserine on either the inner or outer membrane or specific organelles within the cells. The time scale for this equilibration is several days, providing ample time to follow the dynamics of phosphatidylserine binding and migration. These studies are currently underway and will provide fundamental information regarding the distribution, translocation, and quantity of phosphatidylserine in cancer cells.
Figure 11. DU145 cells were incubated with Lact-C2-Lysine-NBD for 24 hours before being placed on the slide and imaged using confocal microscopy. (a) Upper Left—Green fluorescence due to unbound Lact-C2-NBD in DU145 prostate cancer cells. (b) Upper Right—Bright field image of the same cells. (c) Lower Left—Red fluorescence due to phosphatidylserine-bound Lact-C2-NBD. (d) A merger of all three images. Areas that are red exhibit more red fluorescence, areas that are green exhibit more green fluorescence, and areas that are yellow exhibit approximately equal red and green fluorescence.
Figure 12. This contains an image from another region of the slide. (a) Upper Left—Green fluorescence due to unbound Lact-C2-Lysine-NBD in DU145 prostate cancer cells. (b) Upper Right—Bright field image of the same cells. (c) Lower Left—Red fluorescence due to phosphatidylserine-bound Lact-C2-Lysine-NBD. (d) A merger of all three images. Areas that are red exhibit more red fluorescence, areas that are green exhibit more green fluorescence, and areas that are yellow exhibit approximately equal red and green fluorescence.
Comparison to Current Probes

Current protocols utilizing this targeting method, phosphatidylserine binding proteins coupled with fluorescent derivatives, of diseased or apoptotic cells, such as annexin-FITC (fluorescein isothiocyanate), are prone to rapid photobleaching when exposed to light. Additionally, such protocols are generally only able to bind to phosphatidylserine on the outer leaflet of the membrane and have a lower threshold content for the binding of phosphatidylserine than lactadherin. This presents a problem for disease detection and study in that 1) phosphatidylserine must be present in a relatively high concentration on the outer leaflet which, in many cases, is achieved through application of an apoptotic agent such as camptothecin, reducing the number of typable diseases with this type of probe to those cells which can undergo camptothecin-mediated apoptosis; 2) the rapid photobleaching decreases the time in which measurement can occur; 3) there is no indicator as to which sensors are bound versus unbound, which indicates that only qualitative results are possible with these assays; 4) the inability of these probes to transverse the cellular membrane results in an ability to only study phosphatidylserine content on the outer membrane; and 5) in normal cells, phosphatidylserine is found primarily on the inner membrane, so no studies of normal cell phosphatidylserine translocation, distribution, or quantity can be achieved with current probes.

Lact-C2-Lysine-NBD addresses all of these concerns due to the nature of the Lact-C2 domain, the NBD dye, and the binding residues chosen for labeling within the protein structure. Lact-C2 has a higher binding affinity and a lower threshold content for phosphatidylserine, allowing the detection of certain diseased cells which are out of the range of current probes and reducing the need for and apoptotic agent, unless an apoptotic assay is the goal of the
study. The use of NBD instead of FITC increases the inherent photostability of the probes, which retain their ability to fluoresce after more than 24 hours of exposure to the mercury lamp in the spectrofluorimeter as opposed to the rapid photobleaching of FITC. The coupling of the NBD molecules after binding allows for a distinction to be made between the bound and unbound Lact-C2-Lysine-NBD which makes a quantitative assay a possibility, given further development of such protocols and quantification of fluorescence efficiency of the bound form. Finally, Lact-C2-Lysine-NBD is able to transverse the cellular membrane if phosphatidylserine is present. This makes possible for the first time studies of the differences in phosphatidylserine expression and location throughout the entire cell between diseased cells and normal cells, both in their natural states, through the use of confocal microscopic techniques with fully equilibrated cells. Such experiments are currently underway, in which cells will be incubated and grown with the probe to ensure cell viability and full equilibration of Lact-C2-Lysine-NBD before confocal imaging, to study these differences.

**Conclusions**

The central hypothesis that two identical fluorescent molecules placed at an optimum distance of a macromolecular backbone will undergo fluorescence emission shift due to change in interaction of the \( \pi \) electron energy levels has been demonstrated. The Lact-C2-Lysine-NBD exhibits an emission maximum at 540 nm when not bound to phosphatidylserine and undergoes a large red shift to 680 nm when bound to this phospholipid. This fluorescence shift results from the movement of the NBD molecules attached to lysine 15 on Spike 1 and lysine 45 on Spike 3 on the Lact-C2 macromolecular backbone from distal to proximal positions in the unbound and phosphatidylserine bound forms respectively. This shift of 140 nm is quantized,
as it is a constant and reproducible shift, indicating that it is due to the binding event which brings the NBD molecules closer as Spike 1 and Spike 3 bind to phosphatidylserine in a specific molecular conformational change. The Lact-C2-Lysine-NBD is highly specific for phosphatidylserine, has high photostability, membrane permeability, and its binding to phosphatidylserine is photoaccelerated. It effectively targets phosphatidylserine in synthetic DOPC/DOPS vesicles and moderately metastatic prostate cancer cells from the DU145 cell line. In the case of DU145, regions of the cell where Lact-C2-Lysine-NBD is free and bound to PS could be clearly discerned from the green and red fluorescence with epifluorescence and confocal microscopy techniques.

Future Directions

The Lact-C2-Lysine-NBD is a unique photoswitching biosensor for the lipid phosphatidylserine, which is an important biomarker for several diseases including Alzheimer’s and cancer. The membrane permeability characteristic of this biosensor makes it a powerful probe for monitoring phosphatidylserine translocation events triggered by apoptosis, mechanical stress, and biochemical events resulting in cell phenotype change, such as normal cells becoming cancerous. Many fundamental questions need to be answered to fully understand the mechanism of the binding of this biosensor to phosphatidylserine and the photoswitching and photoaccelerated binding effects observed. This study also provides the impetus to attach other fluorescent molecules to determine if the central hypothesis can be further substantiated and employed to generate a new family of photoswitching fluorescent probes for various biomarkers that are important for understanding biological processes at the molecular level.
References


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Author: Tyson Todd

Title: Development of a Novel Biosensor with Quantized "Off-On" Reversible Fluorescent Molecular Switch for the Lipid Phosphatidylserine

Date: Spring 2011

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