Video Article

Real-time Monitoring of Ligand-receptor Interactions with Fluorescence Resonance Energy Transfer

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Abstract

FRET is a process whereby energy is non-radiatively transferred from an excited donor molecule to a ground-state acceptor molecule through long-range dipole-dipole interactions[1]. In the present sensing assay, we utilize an interesting property of PDA: blue-shift in the UV-Vis electronic absorption spectrum of PDA (**Figure 1**) after an analyte interacts with receptors attached to PDA[2,3,4,7]. This shift in the PDA absorption spectrum provides changes in the spectral overlap (*J*) between PDA (acceptor) and rhodamine (donor) that leads to changes in the FRET efficiency. Thus, the interactions between analyte (ligand) and receptors are detected through FRET between donor fluorophores and PDA. In particular, we show the sensing of a model protein molecule streptavidin. We also demonstrate the covalent-binding of bovine serum albumin (BSA) to the liposome surface with FRET mechanism. These interactions between the bilayer liposomes and protein molecules can be sensed in real-time. The proposed method is a general method for sensing small chemical and large biochemical molecules. Since fluorescence is intrinsically more sensitive than colorimetry, the detection limit of the assay can be in sub-nanomolar range or lower[8]. Further, PDA can act as a universal acceptor in FRET, which means that multiple sensors can be developed with PDA (acceptor) functionalized with donors and different receptors attached on the surface of PDA liposomes.

Video Link

The video component of this article can be found at http://www.jove.com/video/3805/

Protocol

A. Synthesis and Characterization of PDA Liposomes[4,5,6]

Note 1: Protect the PDA solution from light using aluminum foil wrapping on every container throughout all the experimental steps.

Note 2: Two different sets of liposome solution (B and C) were prepared following procedure A (Synthesis and characterization of PDA liposomes).

1. Synthesis of N-hydroxysuccinimide Diacteylene (NHS-PCDA)

1. To prepare liposomes, an essential ingredient PCDA-NHS is required. We have synthesized PCDA-NHS using following procedure:
2. Add 10,12-pentacosadiynoic acid (PCDA) (0.267 g, 0.713 mmol), N-hydroxysuccinimide (0.0914 g, 0.786 mmol) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.144 g, 0.713 mmol) in dry CH_2Cl_2 (20 ml).
3. Stir the solution at room temperature for 2 hr.
4. Carefully remove the solvent by using rotary evaporator to yield a dry thin film.
5. Use separating funnel to extract the residue with diethyl ether (25 ml) and water (25 ml) three times.
6. Dry the organic layer with $MgSO_4$ (1.0 g) for half an hour. Filter and remove the solvent by rotary evaporation to obtain a white solid powder (0.24 g, >90%).
7. Analyze final compound under Nuclear Magnetic Resonance (NMR).
8. ^{1}H NMR (300 MHz, DMSO), δ (ppm): 0.893 (t, 3H), 1.268 (m, 26H), 1.512 (m, 4H), 1.754 (m, 2H), 2.252 (t, 4H), 2.365 (m, 1H), 2.610 (m, 1H), 2.842 (s, 2H).

2. Liposome preparation[5,6,7]

1. Dissolve PCDA: PCDA-NHS: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) :: 8 : 1: 1 in 20 ml of dichloromethane.
2. Filter the solution with a filter paper to remove aggregates.
3. Evaporate the solvent completely to yield a thin film of monomers.
4. Dry the thin film overnight under vacuum.

5. Hydrate the film with deionized water (50 ml) to make a liposome solution of desired concentration (0.65-1 mM).
6. Sonicate the resultant suspension with a probe sonicator at 76 °C for ~18 min.
7. Carefully pass the solution through a paper filter to remove the lipid aggregates
8. Cool the solution at 4 °C overnight to promote self-assembly of the monomers. The final solution should be optically clear.
9. Polymerize the self-assembled diacetylene monomers (liposome) by irradiation with 254 nm of UV radiation for ~2 min using a Pen Ray UV source (4.5 mW/cm^2) in air.
10. The liposome solution was stable at room temperature for at least two weeks. The solution was more stable when refrigerated.

B. Preparation of Rhodamine-tagged Bovine Serum Albumin (BSA-Rh) modified PCDA liposomes

1. Binding of BSA-Rh to the liposome surface

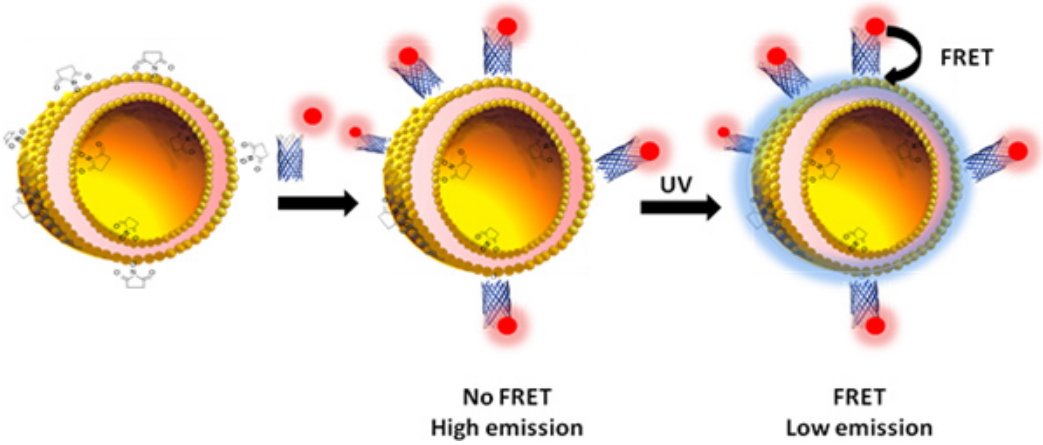
1. Dissolve BSA-Rh in PBS buffer (ionic concentration was 0.01M, pH 7.2) to make the final concentration of 1.2 μM of BSA-Rhodamine solution.
2. Add 2 ml (1.2 μM) of BSA-Rhodamine to 10 ml of liposome solution prepared in step **A.2**. (See above) at room temperature.
3. Classical reaction for the binding of amine groups from the lysine residue of proteins to carboxylic acid activated by NHS group has been followed (Reaction scheme in **Figure 2**). NHS-PCDA was designed (**Figure 2, step 1**) for covalently binding protein molecules with liposomes using NHS-amine reactions (**Figure 2, Step 2**). NHS is an excellent leaving agent that drives the amine-carboxylic acid reaction in the forward direction. The yield of this reaction under appropriate condition should be quantitative.
4. ***Removal of free BSA-Rh*:** Soak a Spectra/Por Biotech Cellulose Ester (CE) membrane (MW*CO*: 100,000) in deionized water for 15 min. This membrane is used for dialysis of unreacted BSA-Rhodamine (Molecular weight ~66000 Da) in deionized water.
5. Carefully transfer the solution in to the dialysis membrane.
6. Change the water at 2 hr, 8 hr, 14 hr, 24 hr, and 36 hr during dialysis.
7. Collect the final solution in a vial covered with aluminum foil.

C. Preparation of SR-diamine and Biotin-tagged Liposomes

1. Instead of using DMPC in step 2.1, we will use biotin-tagged- (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (biotin-DOPE).

1. Follow all the steps through 2.2 to 2.9.
2. Instead of BSA-Rh in step B, use Diamine tagged Sulphorhodamine (SR-diamine).
3. Follow all further steps in B.
4. The liposomes in this preparation contained biotin and SR-diamine on their surfaces. Subsequent steps were similar to A and B (see above) with one exception: streptavidin was added to the solution to investigate biotin-streptavidin interactions through changes in the FRET efficiency.

D. Representative Results



Title Figure. A cartoon explaining the reaction and FRET process occurring at the liposome surface (prepared by following step B).

A. Monitoring of protein attachment to liposomes using FRET[1]

Monitoring of FRET between Rhodamine and PDA liposomes prepared in step B.

Excitation and emission spectra of BSA-Rh and absorption spectrum of PDA are taken (**Figure 3A**). We can clearly see that the emission spectrum of BSA-Rh overlaps with the absorption spectrum of PDA. This satisfies the resonance requirement for the FRET mechanism. BSA-Rhodamine tagged liposomes before and after polymerization were analyzed with UV-Vis and fluorescence spectroscopy. For the isolated

donor and acceptor, the FRET efficiency is highly dependent on donor-acceptor distance (r) and J[1] (j-value). The quenching in the emission is observed (**Figure 3B**) because of FRET between rhodamine and PDA due to the appearance of electronic absorption spectrum of blue PDA after photopolymerization. In our case, FRET efficiency is zero for unpolymerized liposomes and Rhodamine because J=0 for unpolymerized liposomes in the visible region.

We performed similar experiments with only PDA liposomes that did not contain NHS on their surface. In these cases, BSA-Rh was not tagged to the surface of the liposomes. In that case, the average distance between rhodamine and PDA ($r_{average}$) was much larger than Forster radius (R_0 = 2.8 nm). Thus, we didn't observe a large decrease in the fluorescence intensity. This observation also suggests that the fluorescence quenching is dominant when r $\leq$ 2.8 nm.

J and R_0 values were calculated using following formulas:[1]

$$J(\lambda)=\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

$$R_0=0.211[k^2 n^{-4} Q_D J(\lambda)]^{1/6}$$

The extinction coefficient (ε) of blue-PDA is in the range of ~ 2000-10000 M^{-1} cm^{-1} prior to addition of streptavidin. The extinction coefficient of blue-PDA is dependent on the photo-polymerization condition. [2] For example, ε values will be larger for a solution that was polymerized a longer time. Further, the self-assembly of the diacetylene monomers may also affect the ε values. The J calculations take into account of these changes, and they reflect changes in the PDA extinction coefficients due to biotin-streptavidin molecular interactions. J values are calculated using experimental PDA absorption and SR-101 emission data. The changes in the J values are due to shift in the electronic absorption spectrum of PDA following the addition of streptavidin to the solution (**Figure 4C**). The unit of J values is $M^{-1}cm^{-1}nm^4$.

B. Monitoring of FRET with addition of streptavidin to the biotin-tagged liposome solution

Note 1: Monitoring of FRET between Rhodamine and PDA liposomes prepared in step C above.

Excitation and emission spectra of Sulphorhodamine-tagged diamine (SR-diamine) and absorption spectrum of PDA are taken (**Figure 4A**). Unpolymerized and polymerized biotin tagged liposomes were analyzed using UV-Vis and fluorescence spectroscopy. The emission of rhodamine (SR-101) is decreased by about 45% after polymerization (**Figure 4B**) suggesting emission quenching due to FRET. Add 40 µl aliquots (1 µM) of streptavidin solution to 2 ml of liposome solution. With the addition of streptavidin to the solution, changes in J value is observed (**Figure 4C**). As biotin binds to streptavidin, the blue peak intensity of PDA liposome (centered at ~645 nm in **Figure 1**) was decreased whereas an increase in the absorption peak at 540 nm is observed (F**igure 1S**). **Figure 4D** shows changes in the FRET efficiency. The FRET efficiency decreased with the increase in streptavidin concentration is also consistent with our prediction.

Record the SR emission after every 40 µl aliquot addition of streptavidin. We observed a steady increase in the rhodamine emission after the addition of streptavidin (**Figure 5**). This increase in rhodamine emission is due to a decrease in the J value for the sulphorhodamine emission spectrum and PDA absorbance spectrum following biotin-streptavidin interactions. At the molecular level, the biotin-streptavidin interactions lead to subtle changes in the effective conjugation length of the PDA which results in a decrease in the blue-PDA form to a more thermodynamically stable red-PDA form[2]. This is the basis of changes in J values. Interestingly, the subtle differences in the molecular interactions for covalently or non-covalently bonded biotin to PDA liposome can be probed using our sensing assay[4].

We have also performed control experiments and have monitored the emission of the control samples under the same experimental conditions as those for streptavidin-biotin system. The control experiments consist of: (1) Liposome solutions that contained biotin on their surface were added buffer solution of same volume and concentration; and (2) Liposome solution without biotin receptors on their surface were added streptavidin of same volume and concentration. The intensity of the biotin-tagged liposome solution after the addition of streptavidin showed enhanced intensity but the intensity of the liposome solution of the control experiments (for example, please see **Figure 2S**), on the other hand, exhibited a decreased in emission intensity. This is attributed to dilution of the solution. These experiments clearly indicated that the enhanced emission of the solution was due to specific molecular interactions.

Forster radius (R_0) for rhodamine and PDA pair is calculated (eq.2) to be ~2.80 nm. This means that for isolated PDA-rhodamine pairs, 50% of the excited state rhodamine molecules will have their energy transferred to PDA when r is 2.80 nm.

We observed that when biotin is covalently attached to the PDA backbone, the emission increase was 2-3 times larger than that of non-covalently bonded biotin to liposomes.[4] These results strongly suggest that our proposed system is sensitive to distinguish subtle differences in the interactions at the transducer (linker between biotin and liposome bilayer) due to covalently and non-covalently bonded receptors attached to liposomes. Depending upon the scanning and data acquisition capabilities of the spectrophotometer, real-time monitoring (in millisecond to second time scale) of protein interactions (in the UV-Vis spectroscopy) are possible with this sensing system.

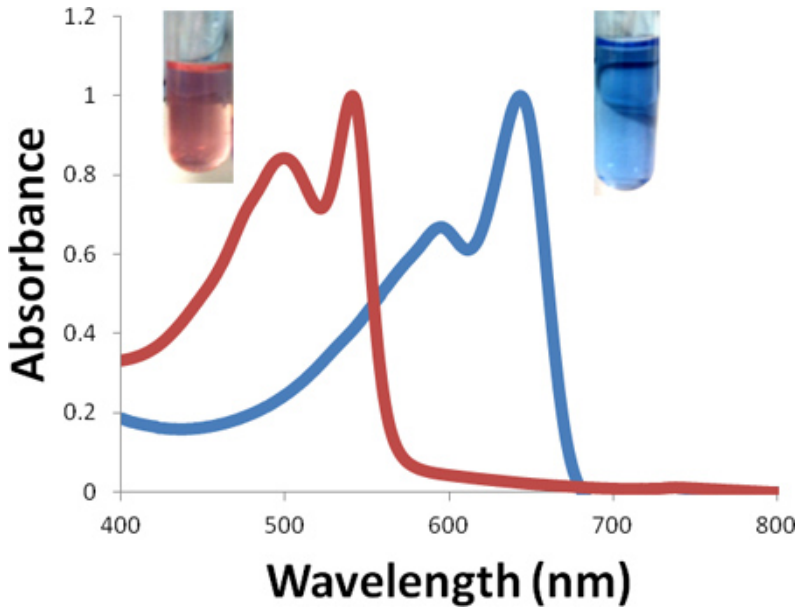


Figure 1. Absorption spectra of blue and red PDA solutions. (Inset) optical micrographs taken with a digital camera.

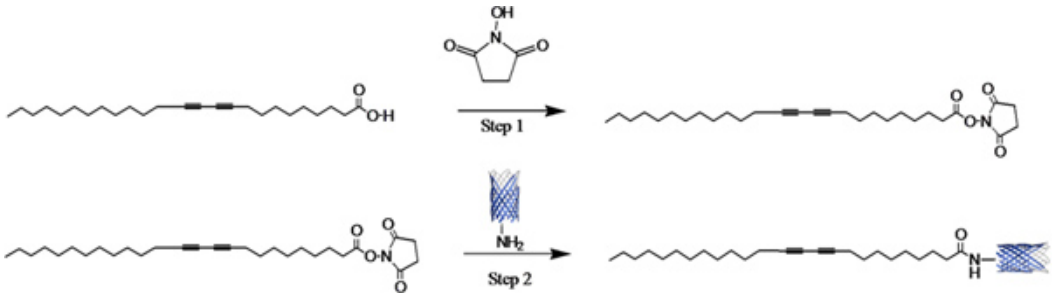


Figure 2. Reaction scheme for the PCDA-NHS synthesis (step 1). Reaction of PCDA-NHS to the amine substituent of proteins (step 2). Step 2 is the basis of binding of lysine residue of proteins to the carboxylic acid of PCDA monomer. Click here to view larger figure.

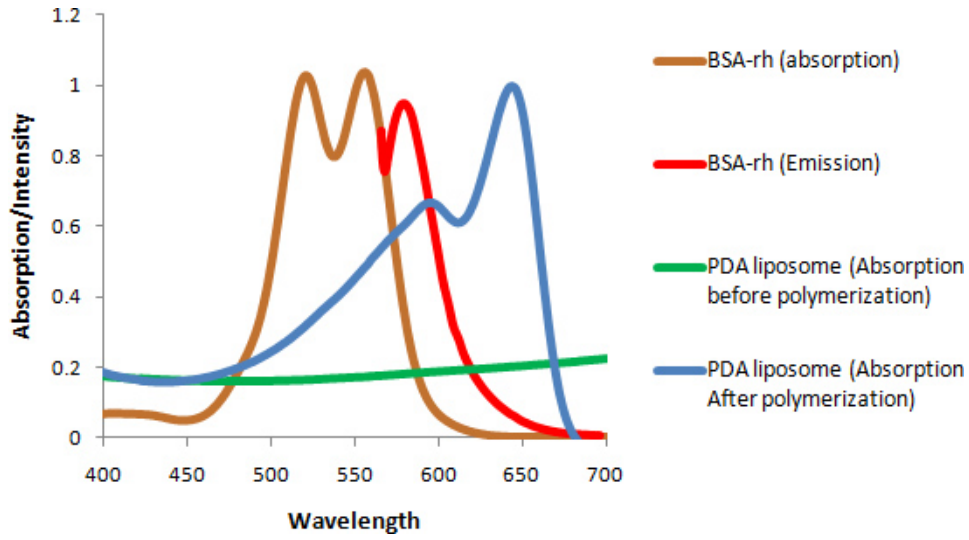


Figure 3A. Change in observed FRET efficiency is due to the changes in the absorption spectrum of the PDA. Before polymerization there is no overlap between BSA-Rh emission and PDA absorption but after polymerization PDA absorption overlaps with BSA-Rh emission which is the requirement for FRET. Rhodamine is a donor (red) and polymerized PDA liposomes act as an acceptor (blue).

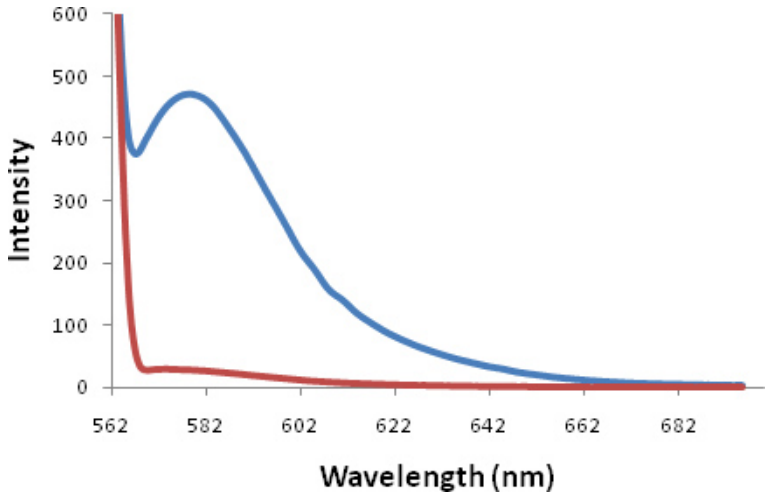


Figure 3B. Fluorescence spectra of BSA-Rh tagged liposomes before (blue) and after PCDA polymerization (red). A large decrease in the rhodamine emission was observed due to FRET between rhodamine and PDA.

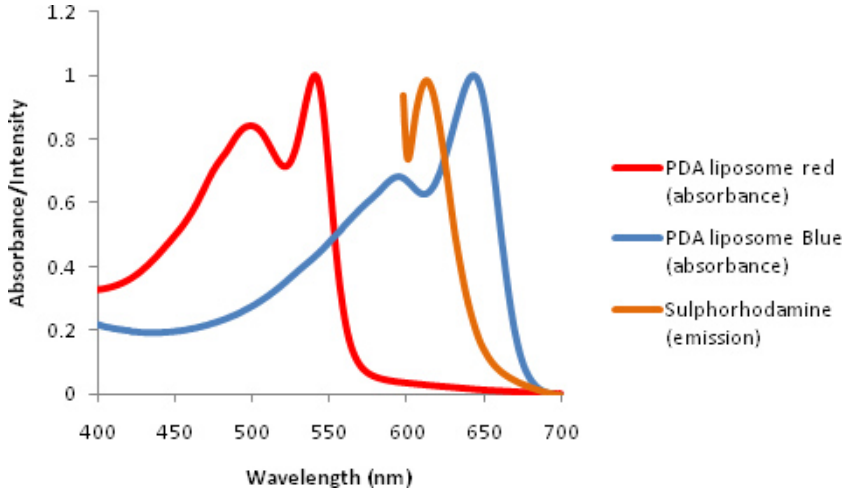


Figure 4A. Spectral overlap (J) change for PDA (blue or red) absorption spectrum and Sulphorhodamine emission spectrum (orange). **Figure 4A** is the representative spectrum for extreme condition; that is, when an excess of streptavidin was added to the solution. Figure 4A shows an almost complete blue-to-red PDA transformation following the addition of an excess of streptavidin. It is clearly seen that J (spectral overlap) increases with blue-shift of the PDA absorption spectrum

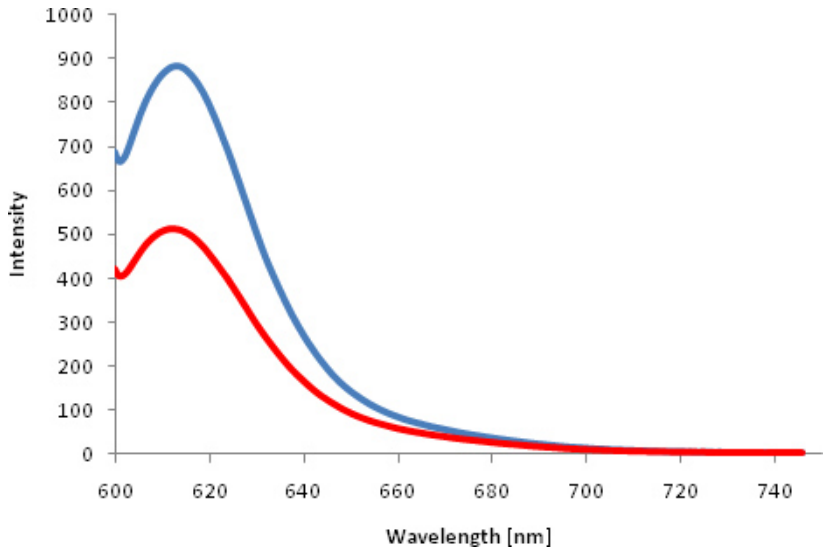


Figure 4B. Fluorescence spectra before (blue) and after (red) liposome polymerization.

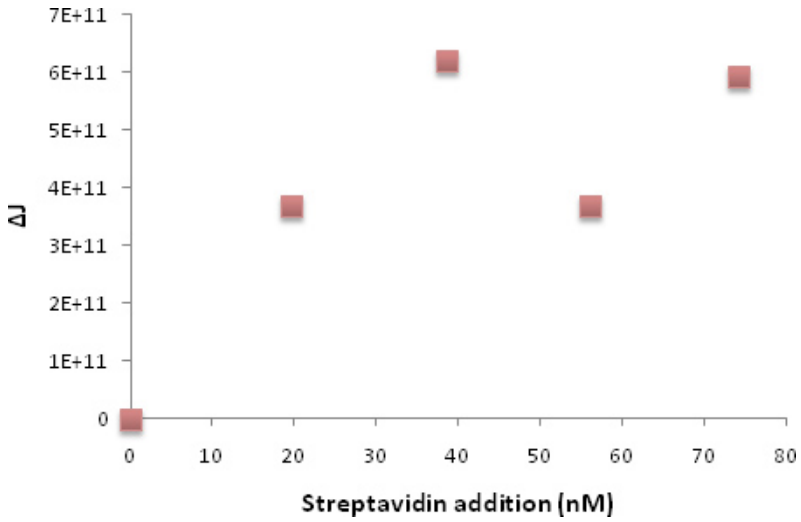


Figure 4C. Condition for FRET: *J* change between donor (sulphorhodamine) and acceptor (PDA) with the streptavidin addition to the liposome solution.

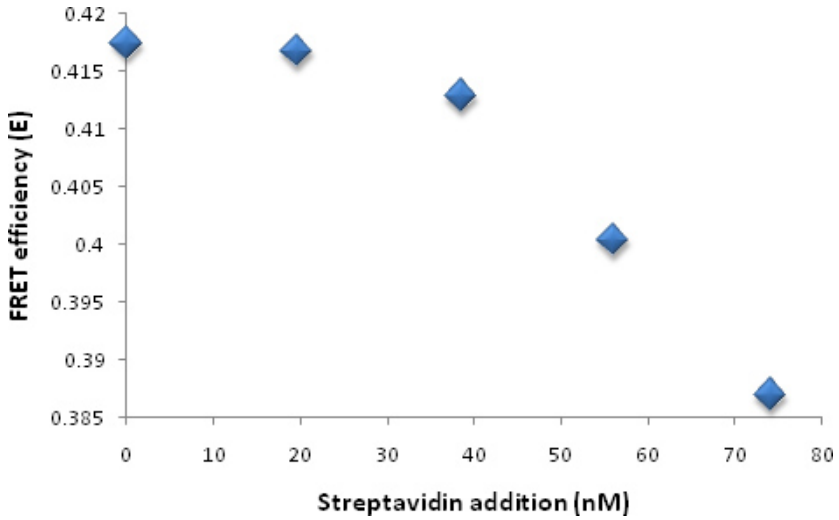


Figure 4D. FRET efficiency change between the donor (sulphorhodamine) and acceptor (PDA) with the streptavidin addition to the liposome solution.

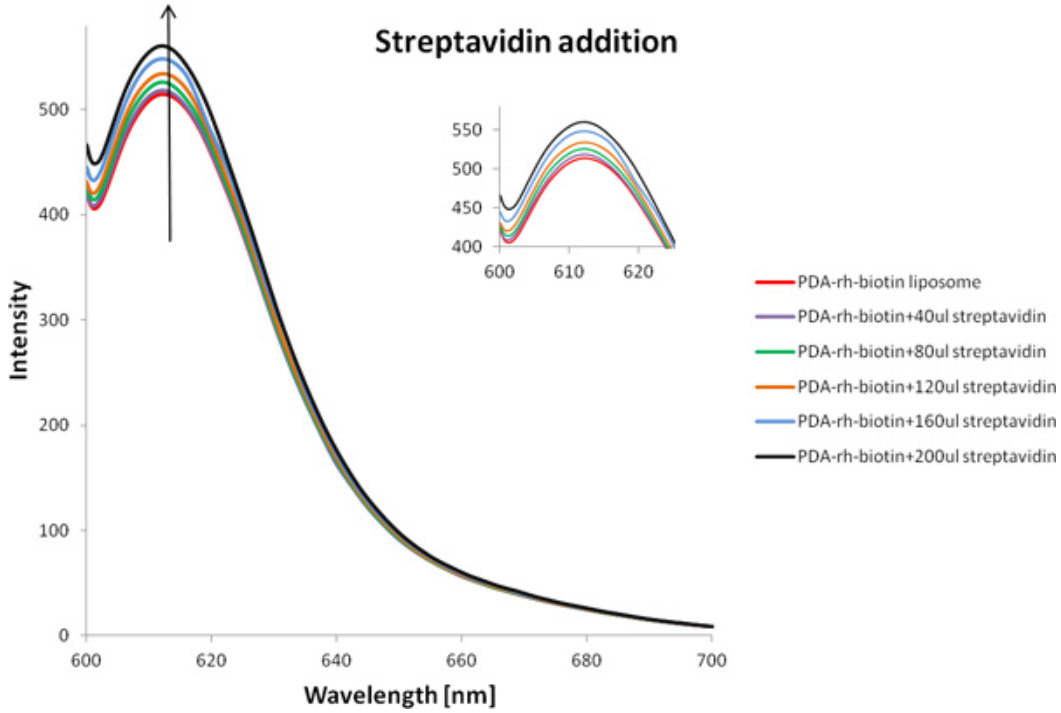


Figure 5. Rhodamine emission spectrum after the addition of streptavidin aliquots to the PDA liposome solution. Inset shows a larger view of emission changes in the SR-101 spectra.

Discussion

We have performed selective binding of lysine residue of protein on liposome surface using NHS-amine reaction. This FRET based method is capable of doing Real-time monitoring of biotin-streptavidin binding and protein (BSA) binding to the liposome surface. Similar procedure can be applied to study the binding dynamics of various protein interactions with their selective receptors. There is flexibility in choosing fluorophores that will provide changes in the *J* values depending upon the spectral characteristics of the fluorophores. PDA is a universal acceptor. Thus, the use of PDA (acceptor) along with multiple fluorophores and receptors raises the possibility of providing us multiple sensors. The sensitivity of our sensors is sub-nanomolar and with optimization, it can be further enhanced. The specificity of the sensors is tuned through the use of molecular interactions between receptors and ligands. These sensors can also be used for larger particles such as viruses and bacteria.

We were also able to gather valuable information like distance between donor-acceptor, FRET efficiency and ***J***-value etc. The distance between the donor and acceptor is calculated to be 2.8 nm. This was consistent with our prediction. As there is a great need of monitoring of insidious viruses, bacteria and other harmful microorganisms, we wish to manufacture a hand held device that can perform real time sensing of hazardous biomolecules.

Disclosures

No conflicts of interest declared.

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