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# Differential Interaction of β-Cyclodextrin with Lipids of Varying Surface Charges: A Spectral Deciphering Using a Cationic Phenazinium Dye

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Interaction of phenosafranin (PSF), a biologically potent cationic dye molecule, has been studied with zwitterionic and anionic lipid membranes of dimyristoyl-L-α-phosphatidylcholine (DMPC) and dimyristoyl-L-α-phosphatidylglycerol (DMPG), respectively. The effect of cyclic oligosaccharide, β-cyclodextrin (β-CD), on the stability of these probe-bound lipid bilayers has also been investigated exploiting steady state and time-resolved fluorescence, steady state fluorescence anisotropy, and dynamic light scattering techniques. An interpretation of membrane destabilization upon interaction of cyclodextrin with the lipids was drawn exploiting PSF as an extrinsic fluorescent probe. The fluorophore showed discernible interactions with DMPC and DMPG vesicles. Experimental results reveal that the extent of interaction of PSF with DMPG is greater compared to that with DMPC. Addition of β-CD into the PSF-bound lipids showed a differential effect for the two lipids of varying surface charge characteristics. In the case of DMPC, addition of β-CD resulted in a preferential interaction of the probe with CD. However, addition of β-CD to PSF-bound DMPG resulted in the selective interaction of DMPG with the added CD leading to the release of the probe into the bulk aqueous medium.

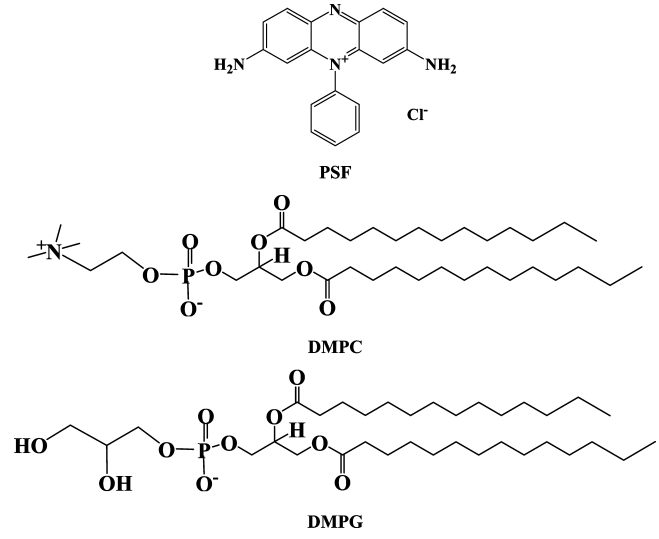
## 1. Introduction

Organized molecular assemblies such as vesicles or lipids can be considered as large cooperative units which are different from the individual structural units which constitute them.[1] Phospholipids are the fundamental matrix of natural membranes and represent the environment in which many proteins and enzymes can display their activity.[2] The problems associated with the wide diversity in composition and structures of biological membranes are avoided if we use synthetic lipids or vesicles which mimic the geometry, topology, and skeletal structure of cell membranes.[3−6] The ability of lipids to mimic the behavior of natural membranes makes them a safe and efficacious vehicle for medicinal applications.[7,8] Lipids have also been effectively utilized in diverse fields like drug loading, immunology, diagnostics, and food industry.[9] Lipid vesicles are also used as selective barriers and nanocapsules to protect the entrapped molecules.[10,11]

Because of their amphiphilic structure, phospholipids organize in the form of bilayers in water. This assembly is stabilized by a lattice of various dipolar and hydrogen bonding forces of interaction between the polar head groups including the solvation, and the hydrophobic effects that enable the fatty acyl chains to aggregate.[12,13] The acyl chains form a hydrophobic core which is expected to have a lower polarity. The polar headgroup region, containing the ester groups of the glycerol backbone, represents an interface between the hydrophobic region and the bulk water phase.[13−15]

Use of molecular probes is certainly one of the best approaches for scrutinizing the membranes in terms of environmental micropolarity. Steady state and time-resolved fluorescence spectroscopic techniques can also provide interesting and often complementary information on the microscopic environment and dynamics of the system, provided that well-suited and solvatochromic probes are used. The minute concentration of the fluorescent probe required for the study does not noticeably perturb the properties of the membranes. Penetration of the probe into the bilayer membrane from the bulk water modifies the photoprocesses, since the polarity and viscosity in the immediate environment around the probe differs significantly from those of the bulk water.[16,17] In the present study, we have used a cationic phenazinium dye, namely, phenosafranin (PSF, 3,7-diamino-5-phenyl phenazinium chloride) as the fluorescent probe (Scheme 1). The dye has been used extensively for various photophysical and photobiological applications as described elsewhere.[18−23] It is red in color with a planar tricyclic phenazinium moiety bearing a positive charge. PSF is a water-

**SCHEME 1: Structures of PSF, DMPG, and DMPC**

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soluble dye with a broad absorption peak in the visible region.[18] Trapping of such biologically potent molecules within different biomimetic environments attracts interest of the researchers because of their ability to achieve specific chemical efficiency as a result of organization in the constrained media. Here, photophysics of PSF has been studied in zwitterionic dimyristoyl-L-α-phosphatidylcholine (DMPC) and anionic dimyristoyl-L-α-phosphatidylglycerol (DMPG) environments (Scheme 1) intending to assess the location of the probe and micropolarity around the probe. The experiments demonstrate the effect of lipid environments on the fluorescence, fluorescence lifetime, and steady state fluorescence anisotropy of the entrapped fluorophore. The interesting effect of addition of $\beta$-cyclodextrin ($\beta$-CD) on these dye-bound lipid bilayers was further explored.

Cyclodextrins are cyclic oligosaccharides formed by bacterial degradation of starch and contain six (α-CD), seven ($\beta$-CD), or eight ($\gamma$-CD) glucose residues linked by glycosidic bonds.[24−26] The conformation and orientation of hydroxyl groups make the external surface of CD hydrophilic, whereas the internal cavity is rather hydrophobic. These molecules are able to form inclusion complexes with a wide range of organic molecules by sequestering them into the cavity.[27] As a result of inclusion, various features including the solubility of the included substance might change. It is also used to remove detergents from ternary detergent/lipid/protein mixtures or desorb sterols from lipid mono- or bilayers.[28−30] Recently, the interaction between CD and biomembranes has been studied in connection with the hemolysis of human erythrocytes. For example, a high concentration of CD has been reported to hemolyze human erythrocytes, the activity being in the order $\beta$-CD > α-CD > $\gamma$-CD.[31,32] Apart from these studies, only a few deal with phospholipid−$\beta$-CD interaction. Moreover, systematic photophysical studies related to the probe−lipid−CD interaction are rather rare. The purpose of the present work is therefore to focus on the interactions between the most common natural CD, namely, $\beta$-CD, and two lipids differing in surface charge characteristics and thereby reveal the differential interaction of $\beta$-CD with the two lipids. This is likely to have relevance in the vesicle stability on one hand and to control the kinetic stability and aggregation behavior of these lipid membranes in aqueous dispersions by uncharged macromolecular oligosaccharides on the other hand.

## 2. Experimental Section

Phenosafranin (PSF), $\beta$-CD, DMPG, and DMPC were purchased from Sigma-Aldrich (USA), and they were used as received. Triply distilled water was used for making the experimental solutions. All of the solvents used were of UV spectroscopic grade (Spectrochem, India). AR grade potassium bromide and Tris−HCl buffer were purchased from SRL, India. The concentration of PSF was kept constant at ca. $7.0 \times 10^{-6}$ mol $L^{-1}$. All of the experiments were performed with Tris buffer solution at pH 7.4. The samples of PSF with lipids and $\beta$-CD were incubated overnight prior to performing the experiments.

For the dynamic light scattering (DLS) measurements, we used a Malvern Nano-ZS instrument employing a 4 mW He−Ne laser ($\lambda = 632.8$ nm) and equipped with a thermostatted sample chamber. The sample was poured into a DTS0112 low volume disposal sizing cuvette of 1.5 mL (path length 1 cm). The operating procedure was programmed by the DTS software in such a way that there were averages of 25 runs, each run being averaged for 15 s, and then a particular hydrodynamic diameter and size distribution was evaluated.

Absorption and steady state fluorescence measurements were performed using a Shimadzu UV-2450 spectrophotometer and a Horiba Jobin Yvon Fluoromax-4P spectrofluorimeter, respectively. The steady state fluorescence anisotropy was also measured using the same spectrofluorimeter. Steady state anisotropy ($r$) is defined by

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where $I_{VV}$ and $I_{VH}$ are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented vertically and horizontally, respectively. The $G$ factor is defined as

$$G = I_{HV}/I_{HH} \quad (2)$$

$I$ terms refer to parameters similar to those mentioned above for the horizontal position of the excitation polarizer. Fluorescence lifetimes were determined from time-resolved intensity decays by the method of time-correlated single-photon counting (TCSPC) using a picosecond diode laser at 403 nm (IBH, UK, nanoLED-07) as the light source and TBX-04 as the detector. The decays were analyzed using IBH DAS-6 decay analysis software. Goodness of fits were evaluated by $\chi^2$ criterion and visual inspection of the residuals of the fitted function to the data. All of the experiments were performed at 25 °C temperature with air-equilibrated solutions.

The DLS spectra were resolved into overlapping Gaussian curves using the MS Origin 7.0 fitting algorithm to obtain the minimum number of reproducible components using the adjustable parameters of the center and width for the resolved bands. Multiple efforts to fit the data with different initial parameters provided a survey of the goodness of the individual attempts. Comparing several such resolutions of an experimental spectrum, a "good fit" was judged from several criteria including a minimum in the goodness of fit parameter $\chi^2$ and the superposition of the convoluted curves on the experimental spectra.[23,33,34] From the statistically acceptable fits, a good fit was further judged by the reproducibility in the values of the centers of the Gaussian curves. A final, *albeit subjective*, criterion was to examine the fits for physically plausible results.

**Preparation of the Lipids.** A defined amount of lipid in 2:1 (v/v) chloroform:methanol solution was dried under a stream of nitrogen and, subsequently, kept overnight under high vacuum. The dry film was hydrated and swelled in Tris−HCl buffer at pH 7.4 containing 20 mM NaCl and vortexed rigorously. The dispersion was then sonicated in ice water using a Vibronics Ultrasonic P1 sonicator until the solution became transparent. Foreign particles (titanium debris, etc.), if any, were removed by centrifugation (Spinwin, MC-02) at 6000 rpm for 10 min.[17,25,35] The size and the size distribution of the lipids were extracted from the dynamic light scattering (DLS) measurements. DLS measurement provides an effective way to investigate the dimension of macromolecular and supramolecular assemblies. For the present lipid systems, namely, DMPC and DMPG, the distribution was found to be fairly monomodal with an average diameter of $30 \pm 3$ nm for DMPC and $13 \pm 2$ nm for DMPG (Figure 1), indicating the formation of small unilamellar vesicles (SUVs) in both cases. The diameters obtained in the present case are consistent with the literature reports.[25,36,37] All fluorescence experiments were performed with freshly prepared vesicles.[25]

## 3. Results and Discussion

There have been only a few studies dealing with cyclodextrin−lipid interaction.[25,38−41] Anderson et al. have shown that

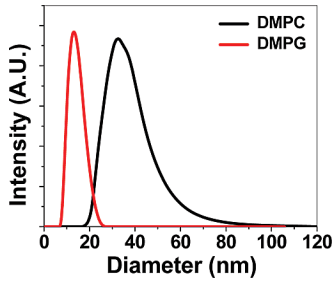



**Figure 1.** Size distribution graph for 1 mM DMPC and 0.6 mM DMPG in aqueous buffer solution at pH 7.4. Concentrations of the lipids were chosen from the saturation of the PSF−lipid interactions as reflected in the fluorometric observations to be discussed in the forthcoming sections. Results of the DLS measurements using a wide range of concentrations for both of the lipids were consistent within experimental limits.

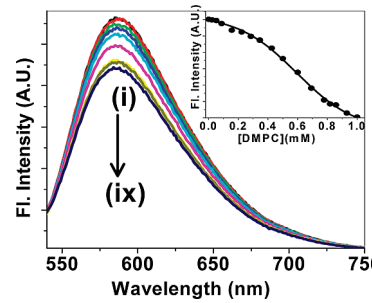



**Figure 2.** Emission spectra of PSF solution with added DMPC ($\lambda_{ex}$ = 520 nm). Curves i−ix correspond to 0, 0.02, 0.09, 0.22, 0.34, 0.50, 0.70, 0.82, and 0.93 mM DMPC. The inset shows the variation of the fluorescence intensity against DMPC concentration at $\lambda_{em}$ = 584 nm. Concentration of PSF = $7.0 \times 10^{-6}$ mol $L^{-1}$.

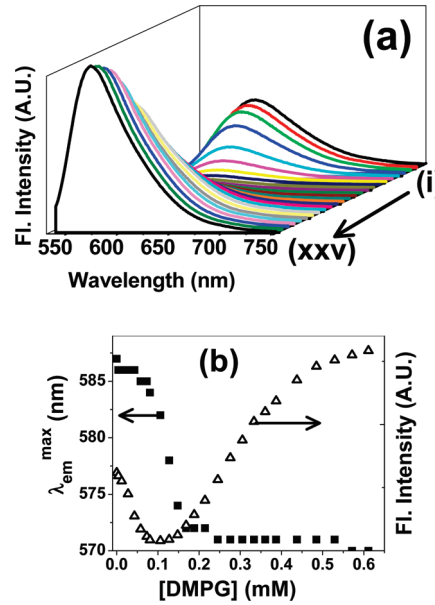



**Figure 3.** (a) Emission spectra of PSF solution with added DMPG ($\lambda_{ex}$ = 520 nm). Curves i−xxv correspond to 0, 0.004, 0.01, 0.03, 0.04, 0.06, 0.07, 0.08, 0.11, 0.13, 0.15, 0.17, 0.19, 0.21, 0.25, 0.28, 0.31, 0.33, 0.36, 0.39, 0.44, 0.48, 0.53, 0.57, and 0.61 mM DMPG. (b) Variation of the fluorescence intensity and wavelength with lipid concentration. Concentration of PSF = $7.0 \times 10^{-6}$ mol $L^{-1}$.

above a certain CD concentration destabilization and destruction of bilayers takes place.[25] Other works revealed that the incorporation of CD into lipid bilayers leads to their solubilization[39] or their protection from UV light.[41] Keeping these reports in mind, we were fascinated to unveil the picture in the present case for the two lipid systems with varying surface charge characteristics.

Although fluorescent probes help in understanding and unveiling the interaction between various components, investigation of the lipid−CD interaction exploiting an extrinsic fluorescent probe is fraught with difficulties, as there are three possible equilibria with respect to the mutual interactions which might operate simultaneously: lipid−cyclodextrin, lipid−fluorophore, and cyclodextrin−fluorophore. The fluorometric behavior might be affected by all of these equilibria, and thus, it is not trivial to correlate the observed changes in the fluorescence parameters directly with the interaction between the CDs and the lipids. The results of such an experiment would depend strongly on the fluorophore used as well as the nature and surface charges of the lipid. This aspect highlights the importance of the suitability of the fluorescent probe to be used to study such systems and their interactions. With this perspective, it will be simpler if we study the individual equilibria and the extent of interaction of the probe with each of the lipids and the cyclodextrin separately. We can then proceed to delve into the interaction between the lipids and $\beta$-CD in the presence of external fluorophore PSF.

**3.1. Interaction of PSF with DMPC and DMPG Lipids.** PSF shows a broad unstructured absorption band at around 520 nm in aqueous buffer solution. On addition of both the lipid solutions to aqueous buffered solution of PSF, the absorbance of PSF decreases slightly at higher lipid concentrations, with no appreciable shift in the case of DMPC and ∼10 nm bathochromic shift in DMPG, indicating that the environment around the probe gets modified in the lipid solutions from that in the pure aqueous phase. The problem arising out of differential salt dependency in the behavior of the two lipids[42−44] was avoided by keeping the ionic strength fixed for both of the lipids by making all of the experimental solutions with Tris−HCl buffer at pH 7.4 containing 20 mM NaCl (*vide* "Preparation of the Lipids" in the Experimental Section). Discussion in the forthcoming sections reveals that a lowering in the polarity in the immediate environment around the probe is responsible for the effect.

The room temperature emission spectrum of the aqueous buffered solution of PSF shows a single broad and unstructured charge transfer (CT) emission band with a maximum at around 587 nm.[18,19] In the presence of DMPC, the fluorescence spectrum of PSF shows a decrease in the intensity associated with a slight blue shift from 587 nm in buffer medium to 584 nm (Figure 2). Gradual addition of DMPG to the aqueous buffered solution of PSF, however, leads to a dramatic modification in the emission spectrum of PSF. The fluorescence spectrum of PSF initially shows a decrease in intensity with an appreciable blue shift to 572 nm followed by a remarkable increase in the fluorescence intensity without any appreciable shift in the emission maximum (Figure 3).

Blue shift in the emission maximum of PSF in the lipid vesicles from that in the buffer solution indicates that the polarity of the microenvironment around the probe is less than the aqueous medium. This is consistent with the previous studies of PSF in biological and biomimicking environments.[15−22] It is well-known that lipids are closed vesicles containing both hydrophobic and hydrophilic regions. Both regions serve important roles so far as the dynamics and functioning of the membrane is concerned. Depending on the nature of the probe, the fluorophore may penetrate in a less polar region of the bilayer (i.e., toward the tail of the lipid) or may stay in the polar part of the bilayer (i.e., toward the aqueous phase).[12] The

fluorometric behavior of the probe here suggests that PSF penetrates slightly in the DMPC membrane. Since the fluorophore contains a cationic charge, it is plausible to argue that the fluorophore fails to achieve a favorable electrostatic attraction with the zwitterionic bilayer as revealed by the negligible blue shift in the emission spectrum of PSF. However, the remarkable hypsochromic shift in the emission maximum observed with an initial decrease in fluorescence intensity with added DMPG indicates a substantial lowering in polarity and a stronger binding interaction of the cationic probe with the anionic lipid resulting from the mutual electrostatic attraction. This is consistent with our studies with PSF in the ctDNA environment, where a significant decrease in the fluorescence intensity was observed with an appreciable blue shift reflecting a lowering in polarity inside the DNA environment together with strong intercalative binding interaction.[22] DMPG vesicles are characterized by unusually low turbidity and high viscosity.[45] The differential scanning calorimetry technique as exploited by Riske et al. showed the presence of several calorimetric peaks, correlated with a decrease in DMPG turbidity and increase in viscosity.[45] From this point of view, we infer that at higher DMPG concentration the large increase in the emission intensity without an appreciable shift indicates an increase in the compactness of the lipid around the PSF, resulting in an increase in the local viscosity of the environment around the fluorophore. It is known that the fluorescence of PSF is very little sensitive to the proticity of the solvent, although it is quite sensitive to the polarity factor.[18] It is pertinent to mention here that the structures of the lipids do not change with increasing concentration.[45] Our DLS study has also revealed the same. That an increase in the viscosity of the medium leads to an enhancement in the fluorescence of PSF has been established independently in a series of water−glycerol mixtures, where the two solvents have comparable polarities (water, $E_T(30) = 63.1$, and glycerol, $E_T(30) = 57.0$) but differ widely in terms of viscosity. It is seen that, with an increase in the viscosity with increasing percentage of glycerol in the solution mixture, the fluorescence intensity shows a remarkable enhancement. This indicates that the increase in the fluorescence intensity of PSF is profoundly sensitive to the viscosity of the medium. Thus, the associated increase in fluorescence intensity at higher DMPG concentration is attributed to an increase in the viscosity. The same does not happen for DMPC because, even at a similar concentration, DMPC is found to be remarkably less viscous than DMPG at a temperature $\leq$35 °C. Above this temperature, however, the microviscosity of the fluid phases of DMPC and DMPG is found to be quite similar.[45]

In order to realize the mechanism of access of a probe on membrane and the role of the lipid environment in the transport phenomenon, knowledge of the partitioning of the probe between the membrane environment and the aqueous phase is important. The partition coefficient of the probe is defined as[46]

$$K_P = \frac{(C_L/C_T)/[L]}{(C_W/C_T)/[W]} \quad (3)$$

in which $C_T$ is the total molar concentration of the drug, $C_L$ and $C_W$ stand for the probe concentration in the lipid and in water media, and [L] and [W] represent molar concentrations of lipid and water, respectively. The partition coefficient of PSF is determined by analyzing the fluorescence data according to the following equation[46,47]

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \frac{[W]}{\Delta I_{max} \times K_p} \times [L]^{-1} \quad (4)$$

where $\Delta I = (I - I_0)$ and $\Delta I_{max} = (I_\infty - I_0)$. $I_0$, $I$, and $I_\infty$ stand for the fluorescence intensities of the fluorophore in the absence, at an intermediate lipid concentration, and at the saturation level of probe−lipid interaction.

The partition coefficients ($K_p$) for the fluorophore in the two lipid environments are obtained as $3.3 \times 10^3$ and $1.03 \times 10^5$ in DMPC and DMPG environments, respectively, from the slopes and the intercepts of the corresponding plots (figure not shown). Since the concentrations of lipids are in the millimolar range whereas that of PSF is in the micromolar range, the possibility of having more than one PSF per vesicle is safely ruled out. A pertinent point to mention here is that, in the case of DMPG, the partition coefficient has been calculated from the first part of the emission spectra at lower DMPG concentrations where there is a decrease in the fluorescence intensity with an associated blue shift in the emission maximum. These observations signify a lowering in the polarity inside the lipid environment together with a binding interaction. The increase in the emission intensity at higher DMPG concentrations is ascribed to an increase in the viscosity of the medium as discussed above. The higher $K_p$ value of PSF in DMPG indicates the remarkably greater partitioning of the fluorophore in the anionic DMPG membrane than in the zwitterionic DMPC membrane. This is rationalized from the stronger binding interaction of the cationic PSF with the headgroup of anionic DMPG compared to that with the zwitterionic DMPC.

At the saturation level of PSF−lipid interaction, we have determined the percentage of probe bound to the bilayers from the following relation using the determined values of partition coefficients:[17]

$$C_0 = C_T/(1 + K_p[L]/[W]) \quad (5)$$

where $C_0$ and $C_T$ are the concentrations of free and total probe, respectively, and $K_p$ is the partition coefficient. Calculation suggests that 17% of the probe molecules are bound to the lipid bilayer of 0.11 mM DMPG (minimum of Figure 3a), whereas in 1 mM DMPC the percentage of binding is only 6%. A higher value of percentage of binding of PSF with DMPG compared to DMPC suggests a stronger binding interaction of the dye with the former lipid. This is interpreted in the light of the surface charge characteristic of the membrane. DMPG bearing an anionic surface charge is able to exert an electrostatic interaction with the cationic dye (PSF) resulting in a stronger binding between the two. DMPC being zwitterionic in nature fails to bind so strongly. The experiments in both the lipids thus reveal that the amount of free probe remains at a much higher level compared to its bound counterpart. The same is also corroborated from the fluorescence lifetime measurements to be discussed later.

**3.2. Interaction of PSF with β-CD.** An aqueous solution of PSF does not show an appreciable change in the absorption spectrum upon addition of β-CD. The emission spectrum of PSF however shows an increase in the fluorescence intensity upon addition of β-CD with a slight hypsochromic shift of ~4 nm (Figure 4). Blue shift in the emission maximum of PSF in the CD environments from that in the aqueous solution indicates that the polarity of the microenvironment around the probe is slightly less in β-CD than that in the bulk water. The small change in the fluorometric parameters of PSF upon addition of

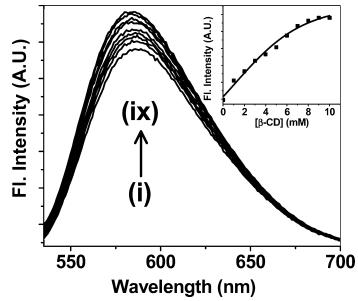



**Figure 4.** Emission spectra of PSF solution with added $\beta$-CD ($\lambda_{ex}$ = 520 nm). Curves i−ix correspond to 0, 1, 2, 3, 4, 6, 7, 9, and 10 mM $\beta$-CD. The inset shows the variation of the fluorescence intensities against $\beta$-CD concentration at $\lambda_{em}$ = 583 nm.

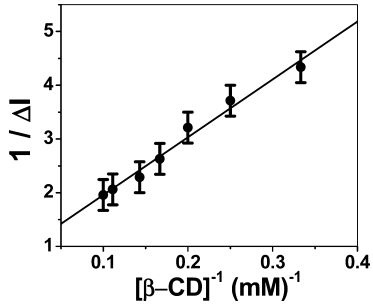



**Figure 5.** Double reciprocal plot for the complexation between PSF and $\beta$-CD.

$\beta$-CD implies only a partial encapsulation of the probe into the $\beta$-CD cavity. Since the overall dimension of PSF is bigger than the opening of the cavity (~6.0−7.0 Å), only a part of the PSF can be accommodated into the CD cavity instead of the entire probe molecule. This proposition is corroborated from the observation of only a slight increase in the fluorescence intensity and a small blue shift of the emission maximum.

In order to see the mode of binding and to establish the stoichiometric compositions of the inclusion complexes, the dependence of the PSF fluorescence on $\beta$-CD concentration was analyzed using the Benesi−Hildebrand equation.[48]

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \frac{1}{\Delta I_{max} \times K_b} \times [\beta\text{-CD}]^{-1} \qquad (6)$$

where $\Delta I = (I - I_0)$ and $\Delta I_{max} = (I_\infty - I_0)$. $I_0$, $I$, and $I_\infty$ stand for the integrated fluorescence intensities of PSF in the absence of $\beta$-CD, at an intermediate $\beta$-CD concentration, and at the saturation level of PSF−$\beta$-CD interaction, with $K_b$ being the binding constant.

A linear plot was obtained using the above equation which substantiates the 1:1 complexation between PSF and $\beta$-CD (Figure 5). The value of the binding constant came out to be 82.0 $M^{-1}$. The low value of $K_b$ indicates only partial encapsulation of the probe inside the $\beta$-CD nanocavity as discussed above.

**3.3. Interaction of PSF with DMPC and DMPG Lipid in the Presence of $\beta$-CD.** After having an understanding relating to the interaction of PSF with the two lipids and $\beta$-CD individually, we endeavored to explore the lipid−$\beta$-CD interaction through the fluorometric behavior of the same probe, PSF.

***Steady State Emission Study.*** At the saturation level of interaction of PSF with DMPC, addition of $\beta$-CD results in an increase in the fluorescence intensity associated with a small blue shift from 584 nm (in 1 mM DMPC) to 580 nm (Figure 6a). On the contrary, addition of $\beta$-CD to the PSF−DMPG

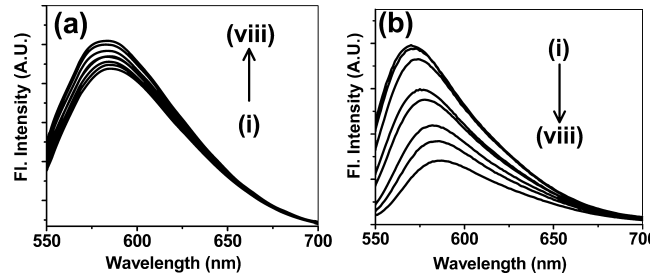



**Figure 6.** Emission spectra of PSF with added $\beta$-CD ($\lambda_{ex}$ = 520 nm) in (a) 1 mM DMPC and (b) 0.6 mM DMPG. Curves i−viii correspond to 0, 1, 3, 5, 6, 8, 9, and 10 mM $\beta$-CD.

complex results in a remarkable decrease in the fluorescence intensity with a large red shift from 572 nm (in 0.6 mM DMPG) to 585 nm (Figure 6b). The observation indicates that there is a differential effect of the addition of $\beta$-CD to the PSF−lipid systems. This makes the problem more complex and interesting. There are two possibilities. The first one refers to a selective binding interaction of the lipid with the added $\beta$-CD, leading to the release of the probe to the bulk aqueous phase. The second one points to a disruption of the PSF−lipid complex resulting in a preferential binding of the PSF with the added $\beta$-CD. We have tried to decipher the picture for each of the lipids, following a series of intricate steady state and time-resolved studies described in the upcoming sections.

Possibly, PSF undergoes a competitive binding interaction between DMPC and $\beta$-CD, choosing the latter for preferential complexation in the presence of both. This seems plausible considering the stronger interaction of PSF with $\beta$-CD compared to that with DMPC as revealed from the percentage binding of the probe with the two hosts. Thus, upon addition of $\beta$-CD in the PSF−DMPC system, PSF is released from DMPC and preferentially binds with $\beta$-CD, resulting in the observed enhancement of fluorescence intensity and the small blue shift. The drastic modification in the fluorescence properties of PSF upon addition of $\beta$-CD to a solution of DMPG containing the probe reveals that there is a disruption of the PSF−DMPG complex. $\beta$-CD can then act on either PSF or DMPG depending on the specific characteristics of the latter two and their binding affinities with $\beta$-CD. A higher binding constant value of PSF with DMPG compared to that with $\beta$-CD indicates that the fluorophore does not prefer $\beta$-CD over DMPG as a host. Hence, the experimental observation in the emission characteristics of PSF upon addition of $\beta$-CD in the DMPG medium, whereby the emission maximum of PSF comes almost back to that in aqueous buffer solution, can only be rationalized by proposing a selective complexation of DMPG with $\beta$-CD leading to the release of PSF into the bulk water. We have substantiated the proposition from various studies described in the upcoming sections.

***Bromide Ion Induced Fluorescence Quenching Study.*** Fluorescence quenching study provides information about the accessibility of the entrapped fluorophore toward the quencher, and thus, an assessment about the location of the probe in different microheterogeneous environments like micelles, reverse micelles, cyclodextrins, lipids, and proteins can be made.[16,17,22,26] In the present work, quenching study using $Br^-$ quencher offers valuable information regarding the probable site of the fluorophore in the lipid environments in the absence and in the presence of $\beta$-CD.[7,8] It is known that bromide ion is preferentially available in the polar region, i.e., in the water−bilayer interface and in the bulk aqueous phase. It is not supposed to be available in the hydrophobic interior of the vesicles.[16] Hence,

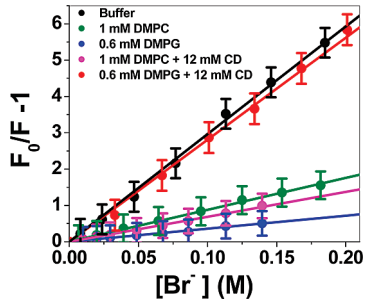



**Figure 7.** Stern−Volmer plots for the fluorescence quenching of PSF by $Br^-$ ions in different environments. The different environments are shown in the legends. (The quenching plot in $\beta$-CD is omitted to avoid clumsiness of the figure.)

**TABLE 1: $K_{SV}$ Values of PSF in Different Environments**

| environment | $K_{SV}$ ($M^{-1}$) |
|---|---|
| buffer | 31.9 ± 1.5 |
| DMPC (1.0 mM) | 9.8 ± 0.5 |
| DMPG (0.6 mM) | 3.6 ± 0.2 |
| $\beta$-CD (12 mM) | 8.2 ± 0.4 |
| DMPC (1.0 mM) + $\beta$-CD (12 mM) | 6.9 ± 0.4 |
| DMPG (0.6 mM) + $\beta$-CD (12 mM) | 29.1 ± 1.5 |

had the fluorophore been penetrated deeper in the hydrophobic region of the bilayer, there would have been inappreciable fluorescence quenching due to the lack of availability of the probe toward the quencher. On the other hand, if the probe is located in the water−bilayer interface, it is expected to be more accessible than the aforesaid situation. At the saturation level of interaction with the lipid and cyclodextrin, fluorescence quenching studies have been performed. Quenching of the fluorescence of PSF with the addition of quencher ($Br^-$) is found to follow the Stern−Volmer relation:

$$F_0/F = 1 + K_{SV}[Q] \quad (7)$$

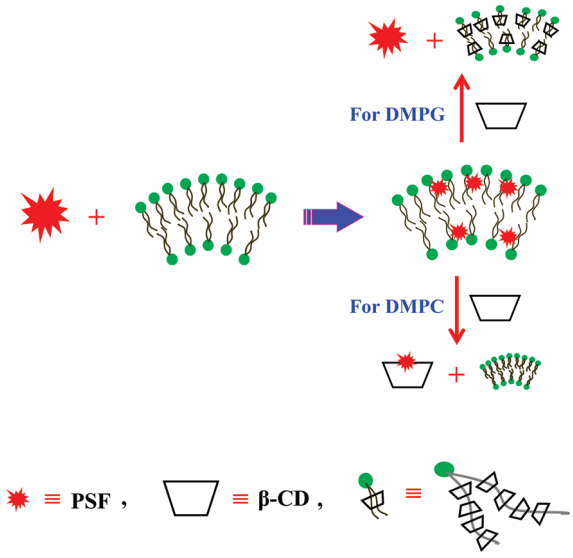
where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the molar concentration of the quencher, and $K_{SV}$ is the Stern−Volmer quenching constant. Figure 7 depicts the Stern−Volmer plots as a function of the bulk concentrations of $Br^-$ in aqueous buffer, lipid, $\beta$-CD, and lipid−$\beta$-CD environments. The slope of each plot gives the Stern−Volmer quenching constant ($K_{sv}$). The estimated $K_{SV}$ values are collected in Table 1.

Figure 7 and Table 1 reveal that the fluorescence quenching of PSF in lipid membranes and $\beta$-CD environments is appreciably lower than that in the aqueous buffer solution. This therefore indicates that the fluorophore is buried both in the CD and in the lipid environments to varying extents as compared to the situation in water. The degree of exposure of the probe toward the ionic quencher is appreciably reduced inside these organized assemblies, making it inaccessible to the quencher, resulting in a drop in the $K_{SV}$ values. However, the $K_{SV}$ values are different in different environments. The $K_{SV}$ value in DMPG is much less than that in DMPC, indicating a stronger binding interaction of the cationic probe with the anionic DMPG vesicle as compared to the zwitterionic DMPC vesicle. On addition of $\beta$-CD to DMPC, the $K_{SV}$ value further decreases, although slightly, and it approaches the $K_{SV}$ value observed in $\beta$-CD. This corroborates our proposition of a preferential complexation of PSF with the $\beta$-CD leaving behind DMPC. A greater extent of binding of PSF with $\beta$-CD as compared to DMPC as evident from the partition and binding studies is thus substantiated. On the contrary, the $K_{SV}$ value of PSF increases and reaches close to that obtained in water upon addition of $\beta$-CD in the presence of DMPG. This indicates that addition of $\beta$-CD to a solution of PSF containing DMPG disrupts the complex between PSF and DMPG, resulting in the release of PSF into the bulk aqueous medium. This further suggests that DMPG prefers to be engaged in the formation of a stable DMPG−$\beta$-CD complex, resulting in the release of the fluorophore into the aqueous phase. Such complexes between the phospholipid chains and the added CD molecules are already available in the literature.[40,49,50] Attachment of $\beta$-CD molecules to the phospholipid chains presumably leads to the formation of a rotaxene-like string.[40,49] An independent experiment was conducted to find the relative extent of solubilization of DMPC and DMPG by $\beta$-CD. The experiment revealed that $\beta$-CD solubilizes DMPG remarkably more than DMPC and reinforces the preferential interaction of $\beta$-CD with DMPG over DMPC.

On the basis of the above discussion, we infer that the differential interaction of $\beta$-CD on the two PSF-bound lipids of varying surface charges follows the depicted cartoon and the scheme (Scheme 2) given below. The idea of the stringing of the $\beta$-CD onto the phospholipid molecules in the case of DMPG has been adopted from the literature.[49]

***Dynamic Light Scattering Study.*** In order to substantiate the scheme depicting the interaction of $\beta$-CD with two PSF-bound lipids, DLS study has been carried out on the lipids in the absence as well as in the presence of $\beta$-CD. DLS experiments on both DMPC and DMPG reveal a fairly monomodal distribution with an average diameter of 30 ± 3 nm for DMPC and 13 ± 2 nm for DMPG (*vide* Figure 1). The DLS spectra of 5 and 8 mM solutions of $\beta$-CD also show monomodal patterns. The dimensions of $\beta$-CD aggregates were found to be 45 ± 5 nm in the former solution and 60 ± 5 nm in the latter case. The DLS spectrum of the 8 mM $\beta$-CD solution was much broadened relative to the other. The experimental observations are consistent with the literature reports and corroborate the poor solubility of $\beta$-CD in water compared to the other members of the series, namely, α- and $\gamma$-CDs. The literature also reports that, while α- and $\gamma$-CDs form only spherical aggregates in a water medium, $\beta$-CD forms an elongated suprastructure.[51,52] Since an increase in the $\beta$-CD concentration leads to a wider distribution of the particle size, we preferred to perform the DLS experiment with a 5 mM $\beta$-CD solution. Addition of 5 mM $\beta$-CD on PSF-bound lipids results in a broad band for DMPC and a broad band together with a small finger for DMPG. Resolution of these experimental spectra is done following the method described in the Experimental Section. Resolution of the DLS spectrum for DMPC yields two overlapping bands, one peaking at 33 ± 2 nm and the other at 52 ± 3 nm. The first one corresponds to the uncomplexed or free DMPC lipid and the second one to the PSF encapsulated $\beta$-CD. This is in support with Scheme 2, which indicates a preferential complexation of PSF with $\beta$-CD over DMPC. Partial encapsulation of PSF in $\beta$-CD results in a slightly enhanced hydrodynamic radius which is reflected in a small shift toward the higher dimension in the case of complexed $\beta$-CD as compared to the free $\beta$-CD. A similar resolution of the DLS spectrum in the case of DMPG (in the presence of 5 mM $\beta$-CD) yields three bands with peak positions at 12 ± 2, 34 ± 2, and 49 ± 5 nm. The smallest one is ascribed to the uncomplexed DMPG lipid while the biggest one to the free $\beta$-CD aggregates (see above). The new species corresponding to a diameter of ∼34 nm is assigned to the DMPG−$\beta$-CD complex (Scheme 2). Stringing of the $\beta$-CD onto the phospholipid chains results in a swelling of the lipid

**SCHEME 2: Cartoon and Schematic Representation of the Interaction of $\beta$-CD with Two PSF-Bound Lipids**




PSF + DMPC → [PSF — DMPC]

[PSF — DMPC] + β-CD → [PSF — β-CD] + DMPC

PSF + DMPG → [PSF — DMPG]

[PSF — DMPG] + β-CD → [DMPG — β-CD] + PSF

signatured by an increase in the diameter of the DMPG−$\beta$-CD complex compared to the free DMPG lipid. Figure 8 depicts a representative DLS spectrum for the PSF-bound DMPG upon addition of 5 mM $\beta$-CD.

***Steady State Fluorescence Anisotropy Study.*** Measurement of fluorescence anisotropy provides significant information about the physical characteristics and the nature of the environment around the fluorescent probes.[16] An increase in the rigidity of the environment surrounding the fluorophore results in an increase in the fluorescence anisotropy. It thus serves as an indicator of the degree of rigidity imposed on the probe in different microheterogeneous media. Figure 9 shows the variation of fluorescence anisotropy of PSF with increasing concentrations of the lipids and $\beta$-CD.

As is obvious from the figure, the steady state fluorescence anisotropy increases in both the lipids and in $\beta$-CD from that in aqueous buffer solution, and finally reaches their corresponding saturation values. This indicates that the fluorophore is trapped in motionally restricted regions within $\beta$-CD as well as the lipids individually compared to that in the buffer medium. In the case of $\beta$-CD, this implies that the probe is getting encapsulated (at least partially) within the CD cavity. In the case of the two lipids, a higher value of anisotropy at the saturation concentrations of DMPG (~0.19) compared to DMPC (~0.12) indicates that the probe is experiencing a more rigid environment in DMPG than DMPC. This is a signature of the fact that the probe is more tightly held in DMPG than in DMPC as inferred from the other experiments discussed above. A stronger binding of the cationic probe with anionic DMPG compared to zwitterionic DMPC is thus substantiated again.

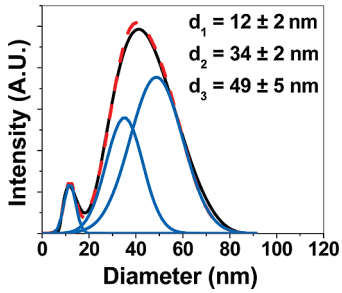



**Figure 8.** DLS size distribution graph for 0.6 mM DMPG containing $7 \times 10^{-6}$ M PSF in the presence of 5 mM $\beta$-CD in aqueous buffer solution at pH 7.4. The black line denotes the experimental spectrum, blue lines denote the resolved bands, and the red dashed line denotes the curve convoluted from the resolved bands.

When $\beta$-CD is added to the probe in the DMPC environment, the fluorescence anisotropy value is found to decrease slightly. However, the anisotropy value even at the saturation level of CD interaction is quite higher than that observed in bulk aqueous solution, indicating that PSF is still experiencing a motionally restricted environment compared to the bulk phase. This is an indication toward the fact that PSF is not released into the bulk aqueous medium upon addition of $\beta$-CD. The decrease in the anisotropy value presumably results from the disruption of the PSF−DMPC complex. The saturation value of the anisotropy of PSF with added $\beta$-CD, much higher than the same in water, indicates the onset of the formation of a preferential PSF−$\beta$-CD complex. Addition of $\beta$-CD to a solution of DMPG, however, results in a sharp decrease in the anisotropy value of PSF from 0.19 to ~0.045 at 12 mM $\beta$-CD concentration. The final value is close to that observed in an aqueous environment, indicating that the probe is released into the bulk water phase. The steady state fluorescence anisotropy thus reveals a differential effect of addition of $\beta$-CD on the lipid-bound PSF in zwitterionic DMPC and anionic DMPG.

***Fluorescence Decay Study.*** Being sensitive to the environment and excited state interactions, the fluorescence lifetime often serves as an indicator to explore the environment around a fluorophore.[16,26,53−55] It also provides valuable information relating to the binding of the probe with the vesicles.[7,8] For the same reason that the emission spectra of the fluorophores are sensitive to the local environment, their fluorescence lifetimes often reflect intermolecular interactions. In order to see how the fluorescence lifetime of PSF is affected as one moves from the bulk aqueous phase to the lipid and CD environments, we have performed the fluorescence decay measurements in all of the environments studied. In buffer medium, PSF shows a monoexponential decay with a short lifetime of ~1 ns. Single exponential fluorescence decay indicates that the fluorescent species exists in a single environment or that there is a rapid exchange between the multiple environments over the time resolution of the measurement.[56,57] The decay, however, becomes biexponential in both the lipids and CD environments. Deviations from the single exponential nature of the decay behavior indicate that either the fluorescent species exists in multistable environments where the exchange rate is slow or simultaneous relaxation from the multiple excited electronic states occurs. Such behavior is often attributed to the distribution of the probe in different regions of the biomimetic environments.[56,57] The lifetime data of PSF in different media are tabulated in Table 2.

The lifetime of PSF increases inside the $\beta$-CD cavity and is consistent with the steady state fluorometric results. The biexponential decays are characterized by a shorter component with lifetime ($\tau_1$) and a relatively longer decay component. The shorter component is close to the lifetime value of the probe in

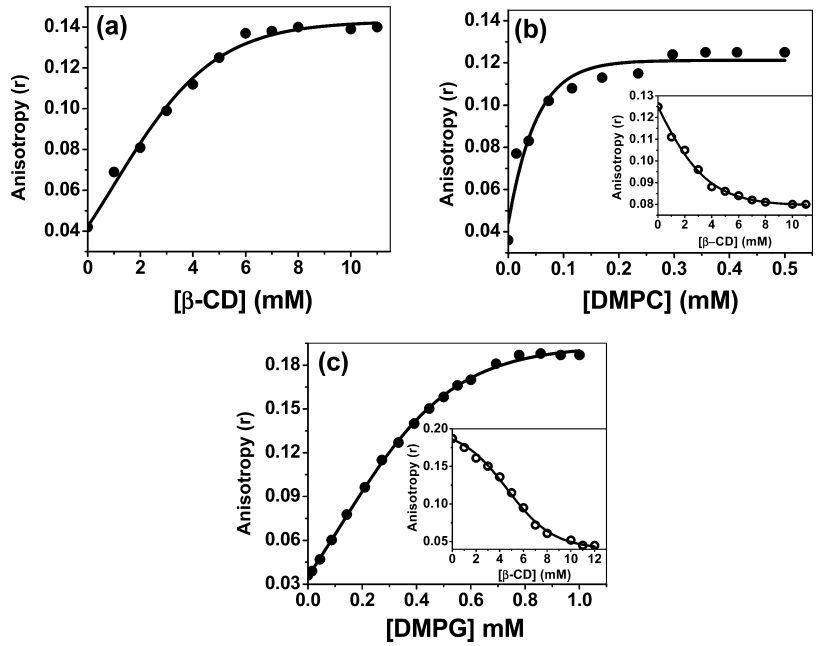



**Figure 9.** Variation of the fluorescence anisotropy of PSF as a function of increasing concentration of (a) $\beta$-CD, (b) DMPC, and (c) DMPG. The insets of parts b and c show the variation of the fluorescence anisotropy of PSF bound to the lipids as a function of increasing concentration of $\beta$-CD.

**TABLE 2: Fluorescence Lifetimes of PSF in Different Environments**

| environment | $a_1$ | $\tau_1$ (ns) | $a_2$ | $\tau_2$ (ns) | $\chi^2$ |
|---|---|---|---|---|---|
| buffer | 1.0 | $1.03 \pm 0.08$ | | | 1.07 |
| DMPC (1 mM) | 0.93 | $0.95 \pm 0.10$ | 0.07 | $3.11 \pm 0.2$ | 0.95 |
| DMPG (0.05 mM) | 0.97 | $0.97 \pm 0.10$ | 0.03 | $3.87 \pm 0.3$ | 1.09 |
| DMPG (0.1 mM) | 0.92 | $0.92 \pm 0.09$ | 0.08 | $3.82 \pm 0.3$ | 1.03 |
| DMPG (0.2 mM) | 0.86 | $0.92 \pm 0.09$ | 0.14 | $3.21 \pm 0.2$ | 1.11 |
| DMPG (0.4 mM) | 0.86 | $1.22 \pm 0.12$ | 0.14 | $3.27 \pm 0.2$ | 1.15 |
| DMPG (0.6 mM) | 0.83 | $1.36 \pm 0.14$ | 0.17 | $3.25 \pm 0.2$ | 1.16 |
| $\beta$-CD (12 mM) | 0.94 | $1.05 \pm 0.10$ | 0.06 | $3.61 \pm 0.3$ | 1.04 |
| 1 mM DMPC + 12 mM $\beta$-CD | 0.92 | $1.06 \pm 0.11$ | 0.08 | $3.49 \pm 0.2$ | 1.13 |
| 0.6 mM DMPG + 12 mM $\beta$-CD | 0.89 | $0.91 \pm 0.09$ | 0.11 | $2.30 \pm 0.1$ | 1.11 |

the bulk aqueous medium, while the longer component is significantly different. In the microheterogeneous environments, observation of two distinctly different time constants in the fluorescence decays implies the existence of two dynamical processes that occur on different time scales. In the $\beta$-CD environment, the obvious possibility is that the short and the long component in the fluorescence decay arises from the distribution of the dye in bulk water and in the CD cavity, respectively. The closeness of the shorter component with that in water strengthens this proposition. Also, the relative contribution of the shorter lifetime component is quite larger than that of the longer one, i.e., $a_1 \gg a_2$. This indicates that the encapsulation of the probe within the CD is not remarkably high, due to which most of the probe molecules experience a bulk aqueous environment.

In both of the lipid media, the decays are biexponential in nature. The shorter lifetime component ($\sim$1 ns) of PSF in the 1 mM DMPC medium is ascribed to the free probe in bulk aqueous medium. The longer component signifies the percentage of PSF interacting with the DMPC vesicles. At lower concentrations of DMPG, the decay consists of a shorter lifetime component, closer to the value of the lifetime of PSF in aqueous buffer solution ($\sim$1 ns), and a longer lifetime component ($\sim$3 ns). As before, the shorter lifetime component is assigned to the free probe in bulk aqueous solution, the relative contribution of which decreases progressively with an increase in DMPG concentration (Table 2). The longer lifetime component is ascribed to the probe−lipid complex. The lifetime of this component decreases gradually, and its relative contribution to the average lifetime increases with increasing DMPG concentration. This indicates a decrease in polarity of the microenvironment around the fluorophore and a stronger binding of the cationic probe with the anionic lipid vesicle. At higher concentrations of DMPG, it is seen that the longer lifetime component remains virtually constant, indicating that the binding interaction of the probe with the lipid is saturated. Again, at higher concentrations, DMPG vesicles lead to an increase in the viscosity of the bulk medium.[40] Therefore, an increase in the shorter lifetime component, ascribed to the lifetime of the free probe in bulk aqueous medium, is rationalized in light of an increase in the overall bulk viscosity of the environment at higher DMPG concentrations. For both of the lipids, the relative contribution of the shorter lifetime component ($a_2$) is remarkably higher. This indicates that, in spite of binding with the lipids, the majority of the PSF molecules remain free. However, $a_2$ values reflect that the amount of the lipid-bound probe is more in DMPG than DMPC, indicating that the binding of PSF with the former is stronger. This differential affinity has already been attributed to the electrostatic interaction between the cationic PSF and the anionic DMPG. The $a_2$ values in the lifetime analyses in both DMPC and DMPG agree well with the fraction of probe bound to each of the lipids as obtained from the partition and binding studies and discussed in the later part of section 3.1.

Addition of 12 mM $\beta$-CD into a 1 mM DMPC solution containing PSF results in a slight increase in the lifetime of the longer component ($\tau_2$). The lifetime of this component is close to that of PSF encapsulated within the CD cavity. This gives a sound support to the fact that PSF preferably forms a complex with $\beta$-CD over DMPC when both are present in the system. On the other hand, addition of 12 mM $\beta$-CD to the PSF−DMPG complex results in a decrease in the lifetime of the probe. The lifetime of the shorter component becomes close to that of PSF

in bulk aqueous medium, with an increase of its relative contribution. Also the longer lifetime component decreases appreciably from that in 0.6 mM DMPG. The cumulative results obtained from the time-resolved studies indicate that addition of $\beta$-CD results in a preferential complexation of $\beta$-CD with DMPG, leading to release of PSF into the bulk aqueous environment. This corroborates the inferences drawn from the steady state studies described above. The above discussion provides an explanation for the solubilization of the DMPG vesicles and/or their protection from UV light as reported in the literature.[25,40,41]

## Conclusion

The present study reports the interaction of a cationic phenazinium dye (PSF) with the two lipids (DMPC and DMPG) of varying surface charge characteristics. The effect of a neutral cyclic oligosachharide $\beta$-cyclodextrin on the stability of these dye-bound lipid bilayers has further been investigated. Fluorometric behavior, fluorescence anisotropy, and quenching studies reveal that the probe binds to a greater extent with the anionic DMPG compared to the zwitterionic DMPC, due to favorable electrostatic interaction with the former. Addition of $\beta$-CD leads to a differential interaction on the two probe-bound lipids. Addition of $\beta$-CD to a solution of PSF in DMPC results in a preferential binding of PSF with $\beta$-CD leaving aside the lipid, whereas, in the case of DMPG, PSF is released into the bulk aqueous phase because of a preferential DMPG$-\beta$-CD complexation. The essence of the work is depicted in Scheme 2. Since $\beta$-CD has no self-surface activity and is nontoxic and nonintrusive in nature, the method has the potential to be applied effectively for cholesterol desorption in membranes and cells.

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