INTRODUCTION

Photophysical studies on molecular aggregation have been extensively investigated by many research groups since it was first reported by Jelley.1 It is known that many chromophores exist as dimer and higher aggregates along with the monomeric form in solution at higher concentration or in viscous medium. This restricts free movement of the dye with far-reaching consequences. Self-aggregation of dyes has found numerous applications in photography,2 solar cells,3,4 nonlinear optical devices,5 semiconductors,6 and organic photovoltaic cells.7

Among the various natures of aggregation, two are most important: J- and H-aggregates.8,9 These two types of aggregates can be distinguished by their characteristic absorption spectra. H-aggregates are formed by head-to-head stacking of dye molecules and exhibit absorption at a lower wavelength. On the other hand, J-aggregates are comprised of edge-to-edge alignment with a bathochromic shift in the absorption spectrum with respect to the monomer. Characteristically, J-aggregates exhibit strong fluorescence often surpassing that of the monomeric dyes,8,11 which is not observed in thiazole orange (TO). The type and probability of aggregation is controlled by factors such as the structure of dye molecules,12 nature of the solvent,13,14 temperature, and environment.14,15

On the basis of this concept, several investigations have been made to modify the dye aggregation in micelles,16 DNA,17 lipid bilayer,18 polymers,19,20 and other media, such as, surfactants,24 cyclodextrins,25 etc. That the cyanine dyes can be forced to aggregate by changing the ionic strength of the medium to form higher order aggregates was reported by Mooi et al.26 The asymmetric cyanine dye, TO, is under attention of many workers due to its special properties. TO has very low quantum yield in water (0.0002) due to efficient nonradiative photoisomerization and free rotation of the benzothiazole and quinoline heterocycles.27–29 Interestingly, the fluorescence from TO increases appreciably on binding to nucleic acids,27,30 macrocycles,28,34 micelles,35 etc. and thus acts as an excellent sensor for designing molecular logic gates36 and DNA G-quadruplexes.37

Herein, we report the distribution and hence modification of the TO aggregates in different forms of negatively charged giant unilamellar vesicles (GUVs) of 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG) lipids. The giant lipid vesicles are convenient systems to study the biological pathways in membranes and widely used for experiments under microscopes, as they are large enough to see (>1 μm).38–40 We have demonstrated the formation of H-aggregates and dimers of TO up to a certain concentration of DMPG, followed by breaking into monomers at higher concentrations of lipid. The dynamics of aggregation were studied using steady-state spectroscopy with support from fluorescence lifetime imaging microscopy (FLIM). We selected different positions of a single GUV using a confocal microscope to collect FLIM data that

ABSTRACT: Thiazole orange (TO) exists mainly as a monomer in aqueous medium, where its fluorescence is negligibly small due to intramolecular movements. In the present study, it has been shown that in presence of giant unilamellar vesicles, produced from anionic lipid molecules, TO prefers to form H-dimer and H-aggregates at low lipid concentrations. The nonfluorescent form of TO (monomer) starts fluorescing in the aggregated or dimeric forms. At higher 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) concentration, the TO aggregates disintegrate to the monomeric variants. This is principally due to generation of more surface of residence for the TO molecules. The dye molecules/aggregates reside on the outer surface as well as percolate inside the lipid vesicles toward the inner water pool due to the presence of anionic charges at the interface. We adopted fluorescence lifetime imaging to find out the heterogeneity in photophysics of the different forms of TO inside the lipid vesicles supported by fluorescence correlation spectroscopy to characterize the formation or disintegration of the TO aggregates.
suggests superficial cleaving of TO aggregates to monomer in presence of higher number of GUVs in solution. The increase in diffusion time at low lipid concentrations, as studied by fluorescence correlation spectroscopy (FCS), clearly supports the above-mentioned phenomenon. To our knowledge, this is the first report for the study of the aggregation pattern of TO in giant lipid vesicles using a confocal microscope for detailed visual description of the dynamics.

RESULTS AND DISCUSSION

Photophysics of TO in DMPG GUV. The absorption spectrum (Figure 1A) of TO (∼3.8 μM) in water shows a maximum at 500 nm, corresponding to the monomer, and a shoulder at ∼476 nm and peak at ∼420 nm appear due to the H-dimers and higher H-aggregates, respectively.27,28 The absorbance for H-aggregates (420 nm) increases at the expense of the monomer and dimer on addition of DMPG for up to 10 μM. Introduction of more GUVs (above 10 μM DMPG) leads to increase in the dimer and monomer absorptions and decrease in higher H-aggregates (420 nm). Hence, the results show that up to a certain concentration of GUVs (below 10 μM DMPG), TO prefers to form the higher H-aggregates. However, on addition of more GUVs in solution, the probability of formation of monomer and dimer increases. Figure 1B provides the emission spectra of TO with increase in DMPG concentration exciting the sample at 465 nm. TO is negligibly fluorescent in aqueous medium due to free rotation of the benzothiazole and quinoline groups, as mentioned earlier. A gradual enhancement in fluorescence yield occurs as the lipid concentration increases up to 40 μM, with maximum at 640 nm, followed by quenching with a concomitant blue shift of ∼6 nm.

Because the peak at 640 nm is assigned to the TO dimers and H-aggregates, the band at 530 nm is due to the TO
monomer that fluoresces weakly in water at this wavelength.\textsuperscript{35} The fluorescence intensity increases at 635 nm with addition of the GUVs up to a certain concentration (\textasciitilde40 \muM DMPG). This indicates increase in TO H-aggregates (up to 0\textendash10 \muM DMPG) followed by the TO dimers (10\textendash40 \muM DMPG) on the vesicle surface. Disintegration into TO monomers initiates on addition of the GUVs having DMPG concentration above 40 \muM. The above observations are confirmed by Figure 2C that presents a broad understanding on the behavior of the TO molecules at different ranges of the DMPG concentrations. The plot of the relative intensities at 635 nm shows breaks at \textasciitilde10 and \textasciitilde40 \muM DMPG concentration. The H-aggregates of TO rapidly break to dimers above 10 \muM DMPG, followed by further breaking above \textasciitilde40 \muM DMPG to TO monomers having small fluorescence.

The excitation of TO at 490 nm (maximum absorption by the monomer) in aqueous medium shows initial existence of the monomeric form. On addition of the GUVs, TO prefers to adopt H-aggregation up to a certain concentration until when the monomer does not show much increase. Beyond 30\textendash40 \muM DMPG, TO monomers start to build up comparatively faster than the other aggregates (Figure 2A). The absorption and emission spectra of TO in glycerol\textendashwater mixtures confirm that the band at 530 nm is indeed for the TO monomer (Figure 1A), multiple equilibria are present in the dye\textendashlipid vesicle interaction. Hence, the lower lipid concentrations were considered in the plot. Varying temperature to encompass the gel\textendashfluid transition condition of the lipid vesicles showed that the change-over from TO H-aggregate to H-dimer followed by monomer occurs at around 30 \muM DMPG concentration, at 28 and 45 °C and at 40 \muM at 15 °C (Figure 3B). This indicates that the aggregation of TO molecules in presence of DMPG GUVs is somewhat dependent on the gel\textendashfluid transition of the lipid vesicles.

\textbf{FLIM and FCS Results on TO Aggregation in Lipid Vesicles.} In a previous report on aggregation of TO in sodium dodecyl sulfate/water and aerosol-OT (AOT)/heptanes systems, Choudhury et al. suggested that TO initially exists as a monomer in aqueous solution.\textsuperscript{35} However, we found that all forms of TO (monomer, dimer, and H-aggregate) exist in water. TO forms H-aggregate/dimer at low surfactant concentrations as there are premicellar aggregates.\textsuperscript{35} In contrast, at lower concentrations of DMPG (0\textendash10 \muM), we found mainly H-aggregates of TO. Disaggregation of the dye occurs beyond the critical micellar concentration.\textsuperscript{35} Similar to this was the observation with the GUVs, where the H-dimers of TO developed up to a certain lipid concentration followed by dominance of monomers. Intermediate disaggregation of the dye was obtained in the AOT/heptane system with enhanced AOT concentration forming H-dimers due to the electrostatic interactions between the dye and the AOT head groups.\textsuperscript{35} The increase in water content in the AOT reverse micelles favored disintegration of the H-dimers to the monomer form. This effect was stated to be due to hydration of the dye molecules at the aqueous interface.\textsuperscript{35} The lipid vesicles have two aqueous interfaces separated by a hydrophobic kernel. Hence, following the above reasoning, TO should be distributed in the lipid vesicle and the photophysics, thus, will be heterogeneous.

In our results, the emission due to the TO H-dimers and H-aggregates is quite high compared to that of the monomer. This is due to the rigidity of the two rotating chromophoric units after aggregation. The average binding constant (K) of the dye with DMPG vesicles was determined from the steady-state fluorescence intensity increases at 635 nm with addition of the GUVs up to a certain concentration (\textasciitilde40 \muM DMPG). This indicates increase in TO H-aggregates (up to 0\textendash10 \muM DMPG) followed by the TO dimers (10\textendash40 \muM DMPG) on the vesicle surface. Disintegration into TO monomers initiates on addition of the GUVs having DMPG concentration above 40 \muM. The above observations are confirmed by Figure 2C that presents a broad understanding on the behavior of the TO molecules at different ranges of the DMPG concentrations. The plot of the relative intensities at 635 nm shows breaks at \textasciitilde10 and \textasciitilde40 \muM DMPG concentration. The H-aggregates of TO rapidly break to dimers above 10 \muM DMPG, followed by further breaking above \textasciitilde40 \muM DMPG to TO monomers having small fluorescence.

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\begin{equation}
\frac{1}{\Delta F} = \frac{1}{\Delta F_0} + \frac{1}{K \Delta F_{\text{max}}} \frac{1}{[L]}
\end{equation}

where $\Delta F = F - F_0$ and $\Delta F_{\text{max}} = F_\alpha - F_0$. $F_\alpha$, and $F_0$ indicate the fluorescence intensities of the dye in absence, at an intermediate concentration, and at complete interaction with DMPG, respectively. $K$ is the binding constant that is calculated from the slope of the $1/\Delta F$ versus $1/[L]$ plot as $3.8 \times 10^5 \text{M}^{-1}$. As reflected by the absorption spectra (Figure 1A), multiple equilibria are present in the dye\textendashlipid vesicle interaction. Hence, the lower lipid concentrations were considered in the plot. Varying temperature to encompass the gel\textendashfluid transition condition of the lipid vesicles showed that the change-over from TO H-aggregate to H-dimer followed by monomer occurs at around 30 \muM DMPG concentration, at 28 and 45 °C and at 40 \muM at 15 °C (Figure 3B). This indicates that the aggregation of TO molecules in presence of DMPG GUVs is somewhat dependent on the gel\textendashfluid transition of the lipid vesicles.

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To explore the heterogeneity in properties, we performed FLIM on TO in a single GUV excited at 488 nm and

Figure 3. (A) Benesi\textendashHildebrand plot for the determination of the binding constant of TO with DMPG, (B) temperature-dependent relative emission intensities of TO with varying concentrations of DMPG. The 635 nm emission was monitored in both the cases.
monitored at two emission wavelengths, 550 and 650 nm. Two TO-containing stock solutions with vesicles constituted by 10 and 60 μM lipid concentrations were used. The size of the GUVs was in the range of 40−50 μm and the size of the water pool had a diameter of ∼30 μm. A drop of the sample solution was spread and dried on the cover slip. A GUV was identified through the microscope for the FLIM experiment. Figure 5 shows the images of the GUV recovered from the 10 μM stock to represent the distribution of TO. The lifetime data was calculated from the marked positions in the vesicle in Figure 5 and shown in Tables 1 and 2. The color code shows that TO has a greater fluorescence lifetime toward the water pool. Three decay components were mostly found for each position, represented by τ1, τ2, and τ3. Hence, it is apparent that all three types of species (monomer, H-dimer, and H-aggregate) exist under all circumstances inside the lipid vesicles. However, their decay times vary due to heterogeneity in surroundings. Noticeably, the lifetime of the monomer is much slower than that reported, as it is entrapped into the lipid vesicle. The fastest component with higher contribution at 550 nm is assigned to the monomer as the contribution increases at higher DMPG concentration. The longer lifetime components are due to the H-dimer and H-aggregate. The TO monomer fluoresces at 530 nm and is the major contributor while monitoring at 550 nm. As discussed above, on addition of the GUVs in higher concentration, the proportion of the TO monomers increases. This is also reflected in the time-resolved data at 60 μM DMPG concentration. We have picked the decay times at three regions (a−c) as marked in Figure 4. The monomer contribution reduces on penetration into the lipid vesicles. The H-dimer and H-aggregates fluoresce at 650 nm, and hence monitoring at this wavelength yields major contributions from the aggregated forms of TO that considerably decrease on addition of higher concentration of the GUVs. Moreover, we could hardly observe any contribution from the monomers in the inner aqueous core probably due to very low quantum yield of the TO monomeric forms. The observations show that the species present on the surface of the GUVs are most affected due to increase in concentration of the lipid vesicles. A schematic representation is shown in Figure 5 to visualize the effect of increased concentration of GUVs on TO.

| [DMPG] | 10 μM | 60 μM |
|--------|-------|-------|
|        | τ1 (ps) | τ2 (ps) | τ3 (ps) | χ² | τ1 (ps) | τ2 (ps) | τ3 (ps) | χ² |
| positions |       |       |       |     |       |       |       |     |
| outer (a) | 422 (66) | 1062 (25) | 4168 (9) | 1.09 | 578 (78) | 1237 (12) | 1647 (10) | 1.03 |
| middle (b) | 538 (62) | 1249 (27) | 5770 (11) | 1.02 | 638 (70) | 1913 (19) | 2454 (11) | 1.35 |
| inner (c) | 498 (60) | 1333 (28) | 4450 (12) | 1.13 | 623 (58) | 1587 (29) | 4460 (12) | 1.18 |
| outer (a) | 680 (17) | 1033 (70) | 2883 (13) | 1.06 | 589 (56) | 1308 (34) | 2930 (10) | 1.10 |
| middle (b) | 681 (34) | 1294 (54) | 2935 (12) | 1.18 | 498 (48) | 1361 (33) | 3885 (13) | 1.04 |
| inner (c) | 1221 (86) | 2566 (14) | 1.05 | 1623 (76) | 2173(24) | 2173(24) | 1.15 |

Table 2. FCS Data for TO at Various Concentrations of DMPG

| [DMPG] (nM) | τD1 (ms) | % contribution | τD2 (ms) | % contribution |
|-------------|----------|----------------|----------|----------------|
| 1           | 3.30     | 100            |          |                |
| 5           | 2.76     | 80             | 47.41    | 20             |
| 25          | 1.96     | 70             | 26.90    | 30             |
| 50          | 1.13     | 100            |          |                |
| 100         | 0.27     | 100            |          |                |

Figure 4. Confocal images of distribution of TO in DMPG GUV monitored at (A) 550 nm and (B) 650 nm.
Because it is shown that the TO molecules distribute themselves in different forms on the surfaces of the GUVs heterogeneously, we checked the diffusion of the lipid-bound TO molecules (∼2 nM) at various concentrations of DMPG (Figure 6). The excitation beam (488 nm) was focused above the surface of the cover slip containing the experimental solution to capture free diffusion. All FCS data were taken from samples in solution and not from stationary lipid vesicles used for FLIM. The measured diffusion times (τD) indicate the translational motion of TO adsorbed on the GUVs. Because TO is practically nonfluorescent in aqueous medium due to intramolecular rotation and nonradiative decay, FCS data due to diffusion of free TO could not be recorded. However, the fluorescence yield increases considerably on attachment of TO to the lipid vesicles due to the imposition of a restricted environment and hence FCS data could be obtained. The concentration of the DMPG was maintained in the nanomolar range to correlate the dye–GUV concentration ratio as used in the steady-state experiments.

The virtually single molecule resolution of the FCS technique helped to disclose the real heterogeneity of the different TO species at various concentrations of DMPG and hence of GUVs’. At very low lipid concentration (1 nM), fit to the raw data yielded a single diffusion time (3.30 ms) presumably corresponding to lower aggregates of TO. This was not clear from the steady-state measurements. On adding more lipids (5 nM) and hence enhancing the GUV population in solution, we obtained a two-component fit corresponding to two different species, one of which was diffusing considerably slowly (∼47 ms). This species could be the higher H-aggregates of TO that coexist with the lower aggregates of TO. The increase in GUV population (25 nM) in solution leads to cleavage of the higher aggregates, and hence the TO on the lipid vesicles diffuse faster (∼27 ms). More increase in lipid concentration leads to further breakdown of the aggregates, leading to formation of TO dimers that diffuse with the vesicle rafts in 1.13 ms. Much higher concentration of DMPG (100 nM) and hence of GUVs provide TO monomers on the GUV surface that diffuse the fastest (0.27 ms).

Figure 5. Schematic representation showing that TO resides as a dimer-aggregate at lower DMPG concentration, whereas at higher lipid concentration the monomers dominate.

Figure 6. Raw data and fits to the FCS data obtained for TO (∼2 nM) at various concentrations of DMPG.

CONCLUSIONS

In this work, we have shown that the DNA-binding dye, TO, exists mainly as monomer in aqueous medium, where its fluorescence is negligibly small due to intramolecular rotation and nonradiative decay. It forms H-dimer and H-aggregates in stages on interaction with DMPG GUVs. Because TO is a cationic dye molecule, the anionic lipid DMPG has been chosen to generate GUVs. The photophysics of TO changes considerably on aggregation and starts fluorescing due to reduction of the nonradiative decay processes. It was shown before that TO prefers aggregation on the micellar surface at low micelle concentrations in solution but disintegrates to monomers with increase in micelle concentration. We found through our experiments that this is true with the GUVs also and quantified the process of formation of H-aggregates, followed by disintegration to monomers. Because lipid vesicles have an inner water pool, it is expected that TO should move toward the interior of the lipid vesicles. Interestingly, we found that the TOs spread on both the aqueous interfaces of the GUVs and also exist in the hydrophobic kernel. We adopted FLIM to find out the heterogeneity in photophysics of the different forms of TO inside the lipid vesicles. The data are...
supported by FCS studied at single molecule resolution, where we found variations in diffusion of the dye molecules in different forms depending on the concentration of the GUVs.

**EXPERIMENTAL SECTION**

**Materials.** The dye TO and lipid DMPG (Figure 7A,B) were purchased from Sigma and were used as obtained. The stock solution of the dye was prepared in methanol, and its concentration was calculated from the molar extinction coefficient ($e = 63,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 500 nm). The GUVs were prepared following a previously reported protocol. Briefly, a weighed amount of DMPG was dissolved in chloroform to obtain a 0.1 M solution and 20 μL of it was added to a mixture of 980 μL of chloroform and 100–200 μL of methanol. The aqueous phase (5 mL of Tris buffer) was then carefully added along the wall of the flask. The organic solvent was removed by a rotary evaporator under reduced pressure (final pressure 60 mm Hg) at 40 °C and 40 rpm. Because of different boiling points of chloroform (61 °C) and methanol (64 °C), we observed two major boiling events. After evaporation for about 2 min, an opalescent solution was obtained containing a high concentration of GUV. The vesicles obtained by this procedure were exclusively GUVs, as observed under the microscope, and the size was in the range of 40–50 μm.

The microscope cover glasses and slides (Blue Star, India) were cleaned thoroughly by sequential washing in an ultrasonic bath with water, alcohol, acid, and alkali and dried properly. A 50 μL sample solution was placed on the slide, dried and covered by a cover glass, and used for the FLIM studies. The lipid vesicles were adsorbed on the surface of the cover slide, as confirmed by the repeated surface scan. For FCS, a 20 μL droplet of the 2 nM sample solution was put over a 0.1 mm cover slip. The measurement time is 30 s per sample. The signal was processed by DCC-100 single photon counting card, and the autocorrelation function $G(\tau)$ was generated. The correlation function $G(\tau)$ of the fluorescence intensities is given by eq 2:

$$G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

where $\delta F(t+\tau)$ is the fluctuation in intensity at a delay $\tau$ around the mean value, that is, $\delta F(t) = F(t) - \langle F(t) \rangle$, and $\langle F(t) \rangle$ is the average intensity.

A system containing $M$ diffusing species with comparable triplet decay times and $Y_i (\sum Y_i = 1)$ being their fractions, the general autocorrelation function is given by eq 3:

$$G_{M,T}(\tau) = \frac{1}{N} \sum_{i=1}^{M} \frac{Y_i}{1 + \tau/\tau_i}$$

where $T$ is the fraction of molecules in the triplet state with relaxation time $\tau_m$. $\tau_i$ denotes the diffusion time of a dye molecule in the confocal volume, $t$ is the delay time, $N$ is the average number of molecules in the excitation volume, and $r/z$ is the structure parameter ($r$ and $z$ being the radius of the volume element in $xy$ and $z$ direction). The dimension of the volume element was determined by using Rh6G of the known diffusion coefficient ($D_i = 426 \mu \text{m}^2 \text{s}^{-1}$). The estimated radius in $xy$ direction was 34 μm, with an effective volume of 3.3 fL. The diffusion constant was calculated from the following eq 4:

$$\tau_D = \frac{r^2}{4D_i}$$

where $\tau_D$ is the diffusion time in the focal volume. All FCS measurements were performed at room temperature.

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**Notes**

The authors declare no competing financial interest.

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