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Diffusion of Chlorin-p_6 Across Phosphatidyl Choline Liposome Bilayer Probed by Second Harmonic Generation

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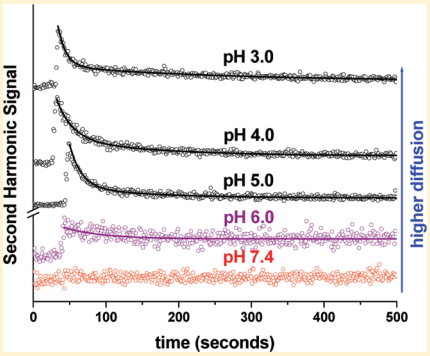
Supporting Information

ABSTRACT: We have investigated the diffusion of the photosensitizer Chlorin-p_6 (Cp_6) across a egg lecithin lipid bilayer at different pH by the Second Harmonic Generation (SHG) method. Cp_6 has three ionizable carboxylic acid groups, and consequently, neutral and several ionic forms of Cp_6 are expected to be present in the pH range 3−8. The absorption spectra of Cp_6 get considerably modified in the presence of liposomes as the pH is decreased indicating that the drug liposome binding is pH dependent. The first pK_a of interconversion (D−C) has been identified at pH ∼7.0 by fluorescence measurement in an earlier work. In this work, the second pK_a of interconversion (C−B) has been identified at pH ∼4.8 by the hyper-Rayleigh scattering method. At acidic pH (3, 4, and 5), where species A, B, and C are dominant, the addition of liposomes to a Cp_6 solution generates an instantaneous rise (less than 1 s) in the second harmonic (SH) signal followed by decays whose time constants ranged from ten to hundreds of seconds. The instantaneous rise is attributed to the adsorption of Cp_6 to the outer lipid bilayer, and the decay is attributed to the diffusion of the neutral and charged (A and B) species of the drug. The observed fast and slow time constants for diffusion in the pH range 3−5 are attributed to the neutral (A) and ionic form (B) of Cp_6, respectively. At pH 6, the intensity of the generated SH signals on the addition of liposome reduced, and at physiological pH, it was too weak to be detected. These results are consistent with previous studies that show that the interaction between Cp_6 and egg−PC liposomes is pH dependent. At lower pH due to the presence of the hydrophobic species (A and B) of Cp_6, its interaction with liposomes is strong, and at higher pH, the abundance of the negatively charged hydrophilic species (C and D) decreases the interaction with the like charged liposomes. We have also studied the effect of increasing the bilayer rigidity by decreasing the temperature of the medium or by incorporating 50 mol % cholesterol in the lipid bilayer and observed that lowering of temperature has more profound effect on the diffusion rates. The characteristics of the SH signal changed significantly when liposomes incorporating 50 mol % cholesterol were used at a low (3 °C) temperature. Under these conditions, the SH signal consisted of an instantaneous (<1s) followed by a slower rise (10−90s), and then, it decayed on a much longer time scale. This slow rise of the SH signal at pH 3 and 4 may be attributed to the temperature dependent adsorption of the anionic species (B) of Cp_6 with the liposomes. Further investigations are required in order to understand clearly the pH dependent diffusion of this drug across lipid bilayers.



INTRODUCTION

Chlorin-p_6 (Cp_6), a porphyrin based photosensitizer (Scheme 1), has three ionizable carboxylic acid side chains whose protonation and deprotonation play an important role in controlling the hydrophobic and hydrophilic species of the drug at different pH.[1] It has been previously shown that a change in pH from physiological to acidic results in the formation of Cp_6 species with less charge and higher lipophilicity due to protonation of the carboxylic side chains.[1] Cp_6 has three ionizable carboxylic acid groups, and consequently, neutral and several ionic forms (A−D, Scheme 1) of Cp_6 generated by acid−base equilibrium are expected to be present in the pH range 3−8 as shown in Scheme 1. This property is believed to play an important role in the higher uptake of Cp_6 in tumors where extra-cellular pH can be slightly acidic.[2−4] Indeed, it has been observed that lowering the pH of the medium from physiological to slightly acidic leads to an increase in the cellular uptake of Cp_6 in certain cancer cells.[5] It has also been demonstrated that the binding of Cp_6 to the lipid bilayer depends on the pH of the medium.[6] Thus, knowledge of the factors governing the dynamics of the diffusion of Cp_6 across membranes may be useful for a better understanding of the cellular uptake of the drug.

To study the adsorption and transport of molecules across the lipid bilayers, a variety of methods like NMR,[7−11] EPR,[12] absorption,[13,14] and fluorescence spectroscopy[15−23] have been used. For example, by the use of fluorescence spectroscopy, it could be shown that where the diffusion of deuteroporphyrin (a dicarboxylic porphyrin) is rapid,[19−21] a disulfonated phthalocyanine is retained in the outer monolayer only.[22] Recently, the

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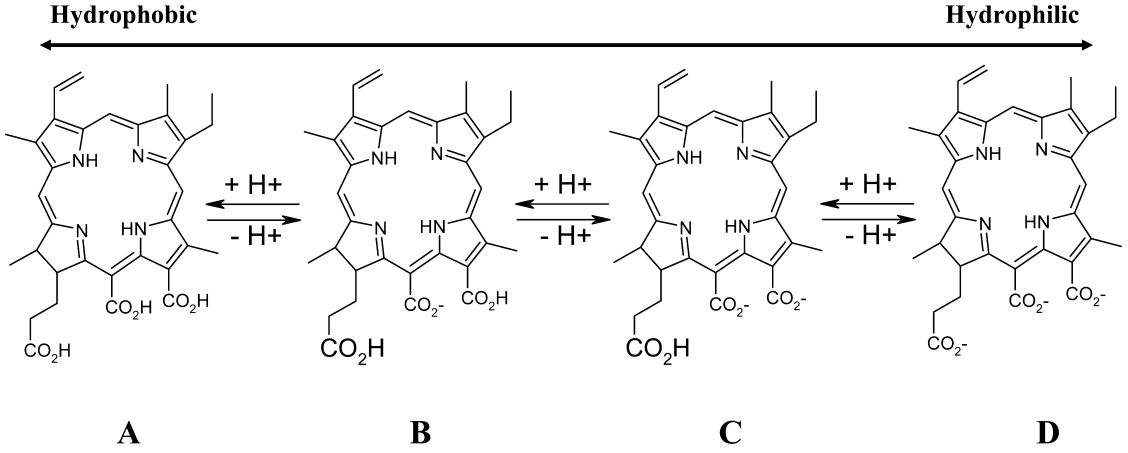
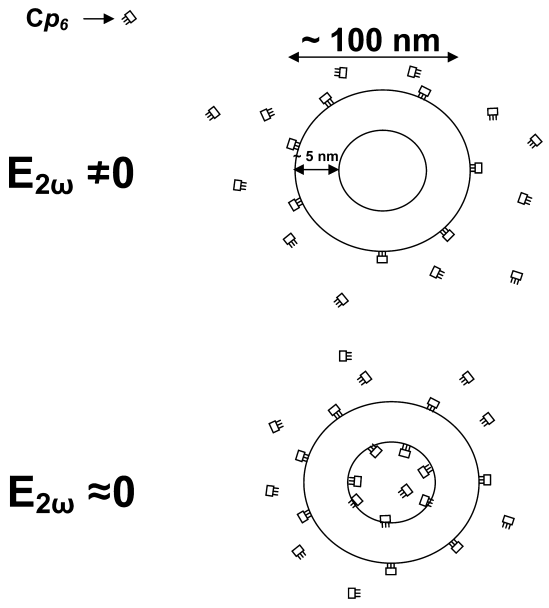
Scheme 1. Different Structures of Chlorine-p_6 Present in the pH Range of 3−8



membrane diffusion properties of different chlorine derivatives were also monitored by ^{1}H NMR spectroscopy.[11] However NMR investigation of kinetics is restricted to observation of slow processes (100s of hours) only, and the amount of drug used for these studies were a concern. It is pertinent to note that the success of these spectroscopic techniques requires that the molecules adsorbed on the surface of the liposomes should show different signature than those in bulk solution.

The adsorption and transport kinetics of dye molecules and ions across a lipid bilayer can also be monitored by the second harmonic generation (SHG) technique.[24−30] If the size of the liposomes is of the order of the wavelength of the fundamental (~800 nm) radiation, the SH field generated from the dye molecules adsorbed on the outer lipid bilayer can add coherently to generate a nonzero SH field (Scheme 2). In contrast, dye molecules in the bulk water will not generate SHG because, due to their random orientation, they are expected to behave as being centrosymmetric. However, they can generate SH by the hyper-Rayleigh scattering (HRS)[35] mechanism. When dye molecules diffuse across the lipid bilayer and get absorbed to the inner surface of the liposome, the adsorbed molecules on the inner and outer surface of the lipid bilayer are oppositely oriented. Since the oppositely oriented molecules are separated by the bilayer thickness (~5 nm), which is expected to be much less than the coherence length of the SHG process, the SHG generated from the oppositely oriented molecules will cancel each other. Thus the resulting SH field generated from the molecules adsorbed on the liposome surface will be proportional to the population difference between the outer and inner surface. Therefore, the diffusion process can be monitored in real time by monitoring the SHG signal subsequent to the addition of molecules to the liposomes. It is important to note here that for the adsorption and subsequent diffusion across a negatively charged lipid bilayer, it is necessary that the molecule should have some net positive charge. Therefore, diffusion of Malachite green (MG), a positively charged organic dye, which could easily get adsorbed on negatively charged liposomes, could be monitored by the SH signal generated.[25−30] When the molecule of interest and liposome have similar charge, adsorption and, subsequently, transport across the lipid bilayer is more difficult. However, if by changing some external stimuli, like pH, the lipophilic character of the molecule is enhanced, it is expected to bind to the liposome irrespective of the charge, and then, the transport process may be measured by the SHG method. We have therefore explored the use of the SHG method to investigate the transport of the drug Cp_6 across liposomes made from egg lecithin at different pH. Further, the effect of temperature and cholesterol in this diffusion process has also been investigated.

Scheme 2. SHG Generated from Molecular Adsorbates (e.g., Cp_6) on Liposome[a]



[a]Initially when molecules are adsorbed at the outer surface, the SH signal increases rapidly. As the molecules diffuse through the lipid bilayer and adsorb onto the inner surface, the SHG signal decreases. Adapted from refs 26 and 27. See text for more details.

EXPERIMENTAL SECTION

Cp_6 was prepared and purified according to the procedure described in ref 1. The presence of an absorption band at ~400 nm[1], which is in resonance with the second harmonic frequency of the fundamental laser input (800 nm) leads to a resonance enhancement of the SH signal. L-α-phosphatidyl choline (the major phospholipid present in egg lecithin) from dried egg yolk (Sigma; Product No. P-5394) and cholesterol (Titan Biotech, India; Product No. 305) were used as received. Unilamellar liposomes with and without 50 mol % cholesterol were prepared by the method described in ref 31. Liposomes were

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prepared in 100 mM buffer solutions having pH values 3, 4, 5, 6, and 7.4. For acidic pH (3, 4 and 5) solutions, sodium citrate buffer was used, and for other pH, phosphate buffer was used. The size and the zeta potential of the liposomes were measured by a Brookhaven Instrument (90 plus size and zeta potential analyzer). The average size and the zeta potential of the prepared liposomes are given in the Supporting Information.

HRS and SH measurements were performed using the 800 nm quasi-CW output of a Ti-Saphhire (Coherent Mira) laser pumped by a green (532 nm, Coherent Verdi 5W) laser. The average laser power used in the experiments was 500 mW, and the pulse width at this wavelength was ~150 fs. The polarization of the laser was fixed in vertical plane by using a quarter wave plate. The laser beam was focused into the sample by a convex lens having a focal length of 10 cm. The generated HRS/SH light was detected using a Edinburgh Instruments LifeSpec single photon counting system. A band-pass filter was placed before the monochromator to reject the fundamental. The wavelength resolution of the monochromator was 2 nm. The HRS/SH light (400 nm) was detected at right angles with respect to the fundamental (800 nm) by a PMT using single photon counting technique.

For HRS and SH experiments, the signal was averaged for two and one seconds, respectively. We have observed that at neutral pH (where Cp_6 exists as monomer), the HRS signal of 10 μM Cp_6 is measurable with our experimental conditions. Therefore, for HRS measurements, the concentration of Cp_6 was kept at 10 μM, and the signal was corrected from the HRS response of the buffer and the changes in absorption of Cp_6 at 400 nm due to a change in the pH. It is important to mention here that Cp_6 does not fluoresce at 400 nm.[1] The sample in the cuvette was constantly stirred during the measurement using a magnetic stirrer. Sample temperature was controlled by a Neslab circulating water chiller. SH experiments were done as follows: first, the signal from 2 mL of buffer was recorded followed by the addition of 3 μM Cp_6 (unless otherwise specified), and finally, 50 μL (0.2 mM) of liposome solution was added. The SH signal from the 3 μM Cp_6 solution alone (which arises due to the HRS of water and Cp_6) is insignificant, and the signal on the addition of 50 μL of liposome to 2 mL of buffer also was insignificant. The final ratio of Cp_6/lipid was 0.6. The SH signals were fitted exponentially to extract the decay constants.

RESULTS

Figure 1 shows the absorption spectra of Cp_6 at different pH (7.4, 5.0 and 3.0) in the presence and in the absence of egg−PC liposomes. The absorption spectra of Cp_6 exhibit a strong Soret band near 400 nm and a weaker Q-band between 600 and 700 nm, which is similar to other porphyrin derivatives.[32,33] On decreasing the pH, the Soret band undergoes a red shift from 401 nm at pH 7.4 to 407 nm at pH 3.0. A broadening in the Q-band, accompanied by the development of a new band around 674 nm, is observed. This band becomes most prominent at pH 3.0. At this pH, the absorption spectra show two prominent peaks at 640 and 675 nm. These results are consistent with previous studies.[1,6] The addition of egg−PC liposome at pH 5.0 and 3.0 increases the intensity of the soret band significantly, and the broad Q-band becomes a single band at 672 nm. In comparison, the effect of the addition of liposome at pH 7.4 on the absorption spectra of the drug is insignificant.

The pK_a of interconversion between two of the anionic forms C and D (Scheme 1) of the drug has been estimated to be around pH ~7.0 by fluorescence measurements.[1] However, the determination of the other pK_a values of the carboxylic groups is difficult by fluorescence measurements as aggregation of drug occurs at acidic pH, and consequently, the fluorescence intensity decreases significantly. It is interesting to note that the aggregates of Cp_6 formed at pH 3.0 are shown to be noncentrosymmetric in nature.[34] Since we have observed a measurable HRS signal from 10 μM Cp_6 at pH 7.4, it might be possible to monitor the pH dependent aggregation of Cp_6 by the hyper-Rayleigh scattering (HRS) method.[35] HRS is a

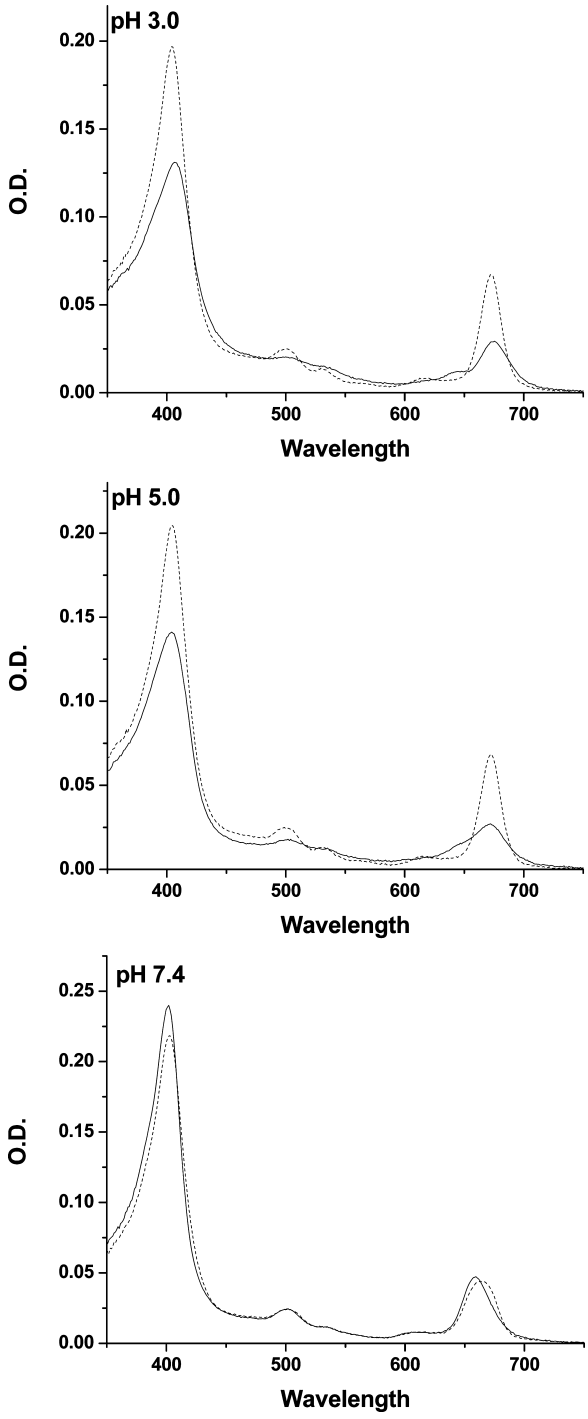

Figure 1. Absorption spectra of 3 μM Cp_6 at pH 3.0 (top), 5.0 (middle), and 7.4 (bottom) in the absence (solid line) and presence (dashed line) of PC liposomes.

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technique that has been used to monitor the aggregation processes of organic molecules in solution.[37] The change in the HRS intensity of Cp_6 with pH is shown in Figure 2. The HRS

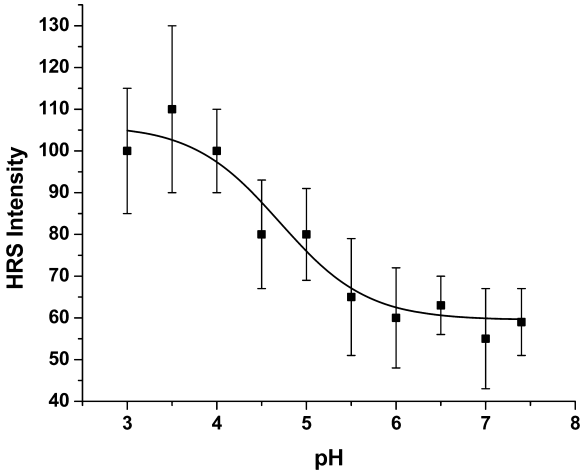

Figure 2. Changes in the HRS intensity of 10 μM Cp_6 at different pH The inflection point is at 4.8 ± 0.4. Laser wavelength, 800 nm; average power, 500 mW; detection wavelength, 400 ± 1 nm; signal integration time, two seconds. See experimental section for further details.

intensity[36] increases with a decrease in pH, and this is attributed to the formation of noncentrosymmetric aggregates of Cp_6. The sigmoidal increase of the HRS intensity has a point of inflection at a pH value of 4.8 ± 0.4.

Figure 3 shows the time profiles of the second harmonic (SH) signals obtained on irradiation of a buffer solution

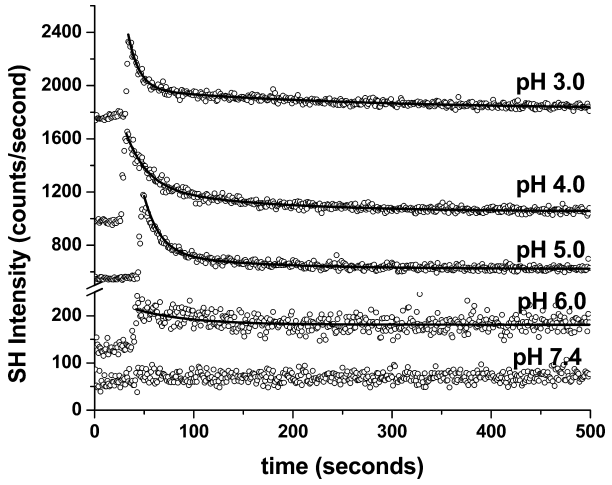

Figure 3. Decay curves at different pH of SHG signal upon the addition of PC liposome solution (50 μL in 2 mL) into 3 μM Cp_6 solution. Laser wavelength, 800 nm; average power, 500 mW; detection wavelength, 400 ± 1 nm; signal integration time, one second. The curves are shifted vertically for clarity.

containing Cp_6 and egg−PC liposomes at different pH using an 800 nm femtosecond laser. No significant SH signal was observed from buffer alone as well as buffer containing Cp_6 (3 μM). On addition of 50 μL of egg−PC liposome (drug/lipid ratio 0.6), the intensity of the SH signal increased considerably for lower pH values. The initial increase in the SH signal (which corresponds to the adsorption of the drug to the outer lipid bilayer[25]) is completed within the time resolution of our experiment (~1 s). Subsequently, the intensity of the SH signal decreased with increasing time, and for time greater than 500 s, it was comparable to the SH intensity obtained before the addition of the liposomes. The maximum rise in the intensity of the SH signal (on addition of liposomes to the Cp_6 solution) occurred for a pH range of 3−5. For the pH 6, the increase in the intensity of the SH signal on the addition of egg−PC liposome was significantly lower, and at physiological pH, it was almost negligible. The decay curves at pH 3, 4, 5, and 6 were fitted exponentially, and the time constants obtained are listed in Table 1. At lower pH, the decay of SH intensity could be

Table 1. Diffusion Time (in seconds) of Chlorine-p_6 Across PC Lipid Bilayer under Various Conditions[a]

25 °C	25 °C + 50 mol % cholesterol	3 °C	3 °C + 50 mol % cholesterol
		pH 3	
11 ± 1 (30%), 90 ± 8	30 ± 5 (60%), 250 ± 40	30 ± 5 (10%), 190 ± 40	10 ± 2 (growth), 55 ± 5 (38%), 500 (62%)[b]
		pH 4	
19 ± 3 (25%), 120 ± 20	39 ± 7 (34%), 250 ± 30	55 ± 8 (9%), 300 ± 40	20 ± 5 (growth), 430 ± 40[b]
		pH 5	
17 ± 4 (30%), 110 ± 11	29 ± 5 (10%), 130 ± 30	30 ± 10 (growth), 184, ∞[b]	90 ± 20(growth), 350, ∞[b]
		pH 6	
50 ± 10			

[a]The error bars represent standard deviation obtained from three different experiments. All time constants are in seconds. The values in parentheses denote the pre-exponential factors. [b]Decays are taken on a 1000 s time window to extract the time constants.

fitted satisfactorily to a biexponential function. The faster time constants were observed to be around tens of seconds. Its value was relatively constant for pH 3, 4, and 5 (11, 19, and 17 s, respectively) but increased to ~50 s for pH 6. For pH 3, 4, and 5, in addition to the fast component of 10−20 s, the SH decay had a slower component whose relative contribution was higher than that of the faster component. The time constant for this slower component was observed to be of the order of hundreds of seconds (Table 1). At pH 6.0, the signal level does not decay to baseline during the 500 s time window, and thus, the relative contribution of the slower time constant could not be ascertained.

The transport of Cp_6 is also expected to depend upon the fluidity of the lipid bilayer, which in turn depends upon the amount of cholesterol present in the bilayer and/or the temperature of the system. Therefore, we have investigated the dependence of the SHG signal on temperature and on the cholesterol content in egg−PC liposomes. Figures 4−6 show the measured changes in the SH intensity upon the addition of egg−PC liposomes as a function of temperature and cholesterol content at pH 3, 4, and 5.0. For other pH values (6 and 7.4), the SH signal was too low to be analyzed with the present setup.

It was observed that at room temperature, the addition of 50 mol % cholesterol increases both the time constants for the slower and faster decay of Cp_6 by a factor of 2 to 3 at pH 3 and 4. In addition, the relative contribution of the faster decay increases 2-fold at pH 3. For pH 5, although, the time constant

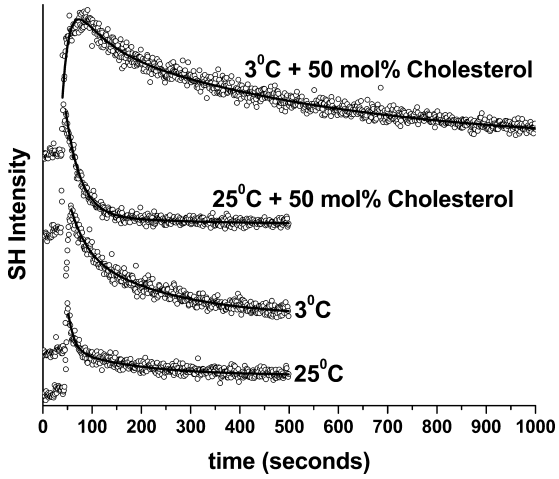

Figure 4. Decay curves at room and low temperature (with and without cholesterol) of the SHG signal at pH 3. Experimental conditions are similar to those in Figure 3. The curves are shifted vertically for clarity.

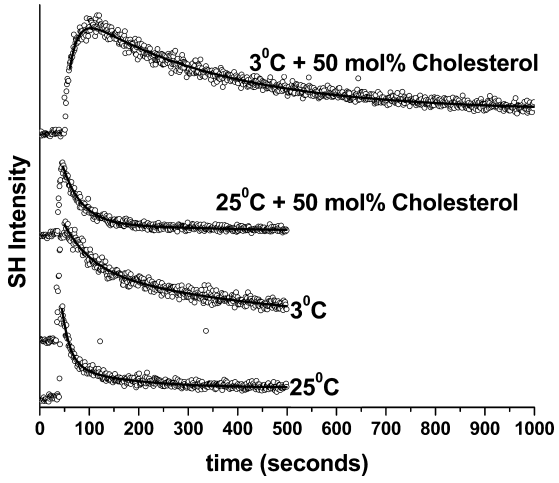

Figure 5. Decay curves at room and low temperature (with and without cholesterol) of the SHG signal at pH 4. Experimental conditions are similar to those in Figure 3. The curves are shifted vertically for clarity.

of the faster decay increases by a factor of 2; the time constant for the slower decay remains more or less unchanged, but its relative amplitude increases.

A decrease in the temperature from 25 to 3 °C, causes the decay of the SH signal at pH 3 and 4 to be considerably slower. However, at pH 5, the lowering of temperature results in an instantaneous rise, which is then followed by a further slower rise, followed by a slow decay of the SH signal. In order to estimate the time constants from the decay of the SH signal, it was recorded in a longer (1000 s) time window. The SH signal could be fitted with a rise time of ∼30 s followed by a biexponential decay of ∼180 s and a long component whose time constant can not be correctly estimated at this time window.

In addition to the instantaneous rise and decay, the SH time profiles of cholesterol containing egg−PC liposomes measured at 3 °C shows the appearance of a growth at pH 3.0−5.0. All the decays are recorded at a 1000 s time window to estimate the time constants as accurately as possible. While the rise time observed at pH 3 and 4 is of the order of tens of seconds, at pH

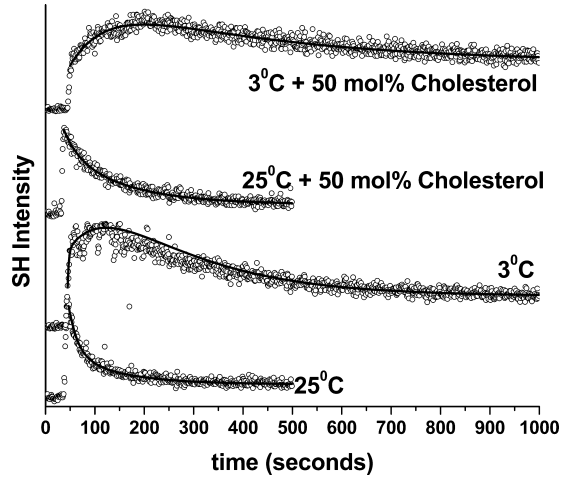

Figure 6. Decay curves at room and low temperature (with and without cholesterol) of the SHG signal at pH 5. Experimental conditions are similar to those of Figure 3. The curves are shifted vertically for clarity.

5, this increases to ∼90 s. In addition, the decay times also increase considerably, being multiexponential in nature. Especially for pH 5, even at the 1000 s time window, the accurate estimation of the slowest decay component is not possible. The details of the time constants along with their respective contributions are listed in Table 1.

DISCUSSION

The structural changes involved in the pH dependent acid−base equilibrium of Cp_6 are shown in Scheme 1. As the pH is lowered, protonation of the carboxyl groups leads to an increase in the hydrophobic character of the molecule (i.e., formation of species C−A). In absorption spectra, this is reflected by a red shift concomitant with a decrease in the intensity of the soret band and broadening of the Q-band (Figure 1). To help interpret the interaction that occurs between Cp_6 and liposomes at different pH, it is essential to know the pK_a values of interconversion between the various (A−D) species of the drug. The pK_a for interconversion between C and D has been reported to be ∼7.0,[1] which is similar to the value observed for the pK_a of the side chain carboxylic groups of other porphyrins.[32,33] Therefore, at physiological pH, the ∼50:50 mixture of species C and D is expected to be hydrophilic as they have two and three negative charges, respectively. Negatively charged liposomes used in this study would not be expected to significantly interact with the drug at physiological pH. This is consistent with the fact that the addition of liposomes at pH 7.4 does not produce significant changes in the absorption spectra of the drug (Figure 1). However, as the pH is lowered from 7.4 to 3.0, the absorption spectra of the drug gets considerably modified in the presence of liposomes (which is still negatively charged, Supporting Information) suggesting that successive protonation of the carboxyl groups increases the hydrophobic character of the drug. Since a decrease in pH is shown to be conducive to the formation of noncentrosymmetric aggregates,[34] HRS may be a suitable method to detect the formation of aggregates, which in turn may give an idea about the pK_a values of interconversion between the species A, B, and C. With a decrease in pH, the HRS signal from 10 μM Cp_6 was observed to increase in a sigmoidal fashion having an inflection point at

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pH = ∼4.8. It is pertinent to note that in an earlier study, it was shown that at pH 3.0, Cp_6 interacts with the positively charged surfactant CTAB at CTAB concentration significantly lower than its CMC.[34] This has been attributed to electrostatic interaction between Cp_6 and CTAB, which suggests that at pH 3.0, the drug is not completely protonated and remains probably as a mixture of A and B. Further, we have observed that at pH 3.0, Cp_6 can interact electrostatically with the positively charged polymer poly-L-lysine.[38] Therefore, we attribute this inflection point to the pK_a for interconversion between the species C and B. Our results thus suggest that at pH 5.0, species B and C are dominant. A further decrease in pH will reduce the population of species C and increase the population of species A. We note, however, that the lack of knowledge of the third pK_a prevents us at this stage to quantitatively assess the relative population of species A and B in the pH range 3.0−4.0.

The generation of an appreciable SH signal in the pH range of 3−5 (Figure 3) on the addition of liposomes thus may be assumed to originate from species A, B, and C. The intensity of SH signal was less at pH 6, and as the pH is increased to physiological, no distinct SH signal was observed, which is consistent with the fact that interaction between the liposomes and drug (species C and D) will be a minimum around physiological pH because both are negatively charged and the drug is hydrophilic at this pH. In the pH range of 3−5, the room temperature decay of the SH signal can be best fitted with two exponentials, which may be assumed to be dominated by the diffusion of species A and B. The neutral form of the drug (species A) is expected to diffuse through the outer lipid layer to the inner layer rapidly leading to shorter decay time and the singly deprotonated anionic species (B) of the drug are expected to diffuse slowly through a hydrophobic barrier. The observed biexponential decays at acidic pH thus represent the ensemble average of the diffusion rates of the two species A and B of the drug. The SH signal level does not decay to baseline at pH 6 suggesting that, at this time scale, Cp_6 has not distributed symmetrically between the inner and outer leaflet of the lipid bilayer. This is expected because at pH 6.0, where species C is dominant, the adsorption of the drug to the outer bilayer will decrease, and its diffusion across the bilayer will be slower. However, at this point, it is not clear why C, a dianionic hydrophilic species, would adsorb spontaneously to the negatively charged surface of the lipid vesicle. The origin of the observed ∼50 s decay constant at pH 6.0 is therefore currently not understood.

Recent ^{1}H NMR experiments have shown that the rate constants for the transfer of different chlorin derivatives across a phosphatidyl choline membrane correlate strongly with the pH of the surrounding medium.[11] In particular, in acidic solution, transfer across the membrane was strongly accelerated. The protonation of ionizable groups of the chlorin derivatives is suggested to be a major determinant for the transfer rates of these molecules across the bilayer. The kinetic profiles measured at acidic pH indicated the presence of a faster (less than an hour) and a slower (100 s of hours) component.

The diffusion of molecules across a lipid bilayer is also expected to be affected by the rigidity of the bilayer. Since the gel-crystalline phase transition temperature of the egg−PC liposomes is at −4 °C, it is not feasible to attain this in aqueous medium. Therefore, we used lowering of the temperature of the medium and/or the addition of cholesterol to the lipid bilayer to enhance the rigidity. As expected, adding cholesterol in the lipid bilayer led to an increase of the overall diffusion time. For example, the addition of 50 mol % cholesterol increases both the decay times by 2−3-fold at pH 3, 4, and 5. Since the adsorption and diffusion of molecules across a lipid barrier is a thermal process, a lowering of the temperature of the medium should lead to an increase in the diffusion time. Accordingly, at pH 3 and 4, the faster decay times show a ∼3-fold increase, and the slower decay times show a ∼2-fold increase. Further, the amplitude of the longer decay time was found to increase significantly. However, at pH 5, a dramatic change in the SH signal was observed. The SH signal now consisted of three parts: an instantaneous increase, followed by a slower increase of the SH intensity, which is then followed by a slower decay. Since at this pH, species B and C are expected to be present in roughly equal amount, the instantaneous increase in the SH signal may be attributed to the adsorption of species B to the outer lipid surface. However, the cause of the observed growth in the SH signal is currently unclear. The long decay of the SH signal (whose time constant could not be correctly estimated within the 1000 s time window) at this pH shows that the diffusion of species B has been considerably slowed down due to temperature-induced increased rigidity of the bilayer. It is thus clear that temperature has a pronounced effect on the SH decay profiles and, consequently, the diffusion of the different species of Cp_6 across the PC lipid bilayer. As expected, at low temperature, incorporation of 50 mol % cholesterol in the lipid bilayer further slows down the diffusion of Cp_6. In the 1000 s time window, all the decays now show a rising part of the time constant, which remains similar (10−20 s) at pH 3.0 and 4.0 and increases to ∼90 s at pH 5.0. The observed rise times at pH 3.0−4.0 may be attributed to the temperature dependent interaction of the anionic species B of Cp_6 with the lipid bilayer. In addition, the decays were observed to slow down further the effect of which is most pronounced at pH 5. All these data thus show that the dual effect of temperature and cholesterol has a profound effect on the diffusion of the various ionic species of Cp_6 across the egg−PC lipid bilayer.

We note that some of the results obtained in this study, especially the origin of the 50s decay observed at pH 6 and the origin of the slow growth in SH signal observed at pH 5, are not yet fully understood. To understand these aspects, further investigations on the relative amounts of forms A, B, and C present at different pH and the relationship between the diffusion of Cp_6 and the zeta potential of liposomes are needed.

In conclusion, we have investigated the diffusion of the photosensitizer Cp_6 across the egg PC lipid bilayer at different pH by the SHG method. The absorption spectra of Cp_6 gets modified considerably in the presence of liposomes as the pH is decreased indicating that the interaction between the drug and liposome is dependent on the pH. The HRS intensity of Cp_6 increases in a sigmoidal fashion as the pH is decreased from 7.0 to 3.0 with the inflection point at pH ∼4.8. This has been identified to the second pK_a of the drug, the first pK_a being ∼7.0 as observed in a previous study. The addition of PC liposomes to a Cp_6 solution generates an instantaneous increase of the SH signal (within 1 s), which then decays over a longer time (500−1000 s) scale. The decay of the SH signal at acidic pH (3−5) is observed to be biphasic. The faster time constant, which is of the order of tens of seconds, is attributed to the diffusion of the neutral species (A, Scheme 1) of Cp_6, and the longer time constant is attributed to the diffusion of the charged species (B) of the drug. At pH 6, the intensity of the SH signal decreases appreciably, and it is almost nonobservable

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at physiological pH. This is attributed to dominance of the negatively charged species (C and D) of the drug at this pH range, which does not interact significantly with the negatively charged PC liposomes, and as a consequence, the SHG signal intensity is decreased considerably. We have also studied the effect of increasing the bilayer rigidity by decreasing the temperature of the system and by incorporating 50 mol % cholesterol in the lipid bilayer. We have observed that the lowering of the temperature has a more profound effect on the diffusion rates. The dual effect of lowering temperature and incorporating 50 mol % cholesterol on the SH signal results, in addition to the instantaneous rise, in a slow rise of the signal with time followed by a slower decay. The slow rise in SH signal at pH 3.0−4.0 may be attributed to the slow interaction of the ionic species B of Cp_6 with the liposomes. Further studies are needed in order to understand the pH dependent diffusion process of the drug across lipid bilayers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table containing the size and zeta potential values of PC liposomes over the pH range 3.0 to 7.4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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$$I(2\omega) = G(N_1\langle\beta_1^2\rangle + N_2\langle\beta_2^2\rangle)I^2(\omega)e^{-N_2\alpha_2 l}$$

where $I(\omega)$ is the incident light intensity; $I(2\omega)$ is the intensity of generated HRS signal; G is a constant that depends upon the experimental conditions; β denotes the first order hyperpolarizability; N denotes the number density; and the exponential term accounts for the loss of HRS signal at the second harmonic wavelength. The subscripts 1 and 2 refer to the solute and solvent, respectively.
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