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Conversion of purpurin 18 to chlorin P_6 in the presence of silica, liposome and polymeric nanoparticles: A spectroscopic study

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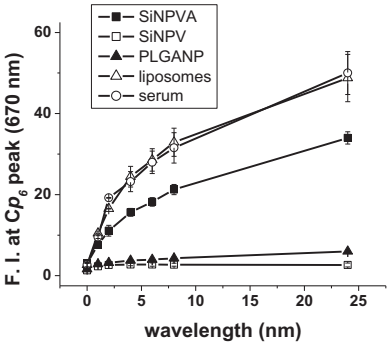
HIGHLIGHTS

- Suitability of SiNP as carrier of purpurin 18 (Pp18) was studied spectroscopically.
- Conversion of Pp18 to chlorin p_6 was compared among four different NP systems.
- Hydrolysis rate of Pp18 was found to be lowest in vinyl modified silica NP (SiNP-V).
- Our results suggest that SiNP-V could be used as the carrier for Pp18.

GRAPHICAL ABSTRACT

Effect of nanoparticle (NP) environment on the conversion of entrapped purpurin 18 (Pp18) to chlorine p_6 (Cp_6) was studied spectroscopically and compared among four NP systems: organically modified silica NP (SiNP) having vinyl and/or amino propyl groups, poly-lactic-co-glycolic acid (PLGA) NP and liposome. Our investigation suggests that SiNP having vinyl functional groups might be a better carrier of Pp18 at physiological pH.



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ABSTRACT

Effect of nanoparticle (NP) environment on the conversion of entrapped photosensitizer purpurin 18 (Pp18) to chlorin p_6 (Cp_6) was studied spectroscopically. This was investigated by monitoring the time dependent absorption and emission kinetics of the Pp18 entrapped in four different NPs suspended in buffer at physiological pH in the presence and absence of serum up to 24 h. These were organically modified silica NP (SiNP) having vinyl and/or amino propyl groups, poly-lactic-co-glycolic acid (PLGA) NP and liposome. The results of the spectroscopic studies reveal that Pp18 is least converted to Cp_6 (i.e., most stable) in the SiNP having vinyl groups whereas its conversion is fastest in PC liposome. In the presence of serum proteins, although the conversion is enhanced, the trend remains similar. Our investigation suggests that SiNP having vinyl functional groups might be a better carrier of Pp18 at physiological pH.

1. Introduction

Photodynamic therapy (PDT) has gained considerable clinical acceptance in various countries for the treatment of cancer [1,2]. The selective accumulation of a photosensitizer in tumor is an important factor in the effectiveness of photodynamic therapy. It is believed that tumor selectivity depends on the hydrophobic character of the photosensitizer since this enables its better uptake in cells via interaction with low density lipoproteins and the cellular membrane [3,4]. However, the hydrophobic photosensitizers generally tend to aggregate in aqueous medium that can result in poor uptake in cells. The ability of nanoparticles (NPs) to carry

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hydrophobic photosensitizers has attracted tremendous interest for their use in PDT of cancer. Different types of nanoparticles are being currently explored for the delivery of such drugs [5–10]. For drug delivery it is required that the NPs are biocompatible, stable in physiological conditions and able to carry the drug without any chemical alteration [11]. Among these, organically modified silica nanoparticles (SiNPs) prepared using triethoxyvinylsilane and/or 3-aminopropyl triethoxysilane have shown to satisfy these features [12–15]. Due to the presence of a hydrophobic core, these NPs can be loaded with hydrophobic drugs and since the surface is charged these can be easily solublized in aqueous medium [11–15,16]. Such NPs have been used for gene delivery, entrapping dyes/probes for imaging, and hydrophobic photosensitizers for PDT, etc. [17].

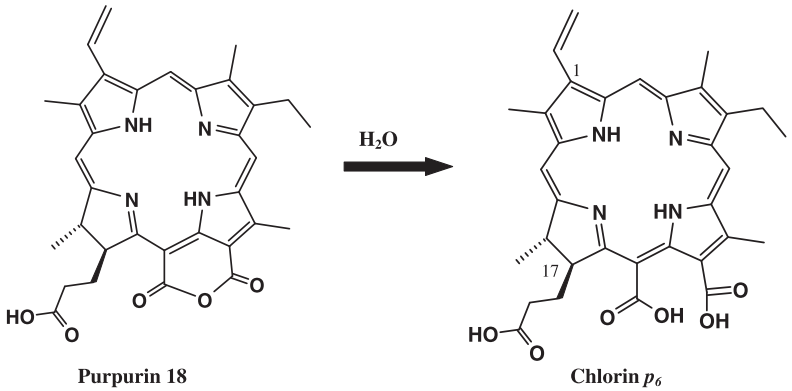
Earlier, it has been reported that purpurin 18 (Pp18), a hydrophobic chlorophyll derivative, can be incorporated in liposomes and the formulation was found to deliver the photosensitizer successfully in human colon cancer cells [18]. Pp18 shows promising optical properties for use in PDT such as an absorption band at $\sim$ 700 nm with extinction coefficient $\sim$41,800 M^{-1} cm^{-1}. However, since Pp18 contains an anhydride ring in the molecule, it undergoes rapid hydrolysis at physiological pH (shown in Scheme 1) which results in the formation of chlorin p_6 (Cp_6), a water soluble photosensitizer, having Q band absorption blue shifted to $\sim$656 nm with decreased extinction coefficient ($\sim$24,800 M^{-1} - cm^{-1}). Although it was possible to stabilize Pp18 in liposomes prepared at lower pH (6.0) and thus deliver Pp18 in cells [18], during incubation for time periods beyond 1 h significant conversion of Pp18 into Cp_6 was observed [18]. Other carriers such as Cremophor EL and Poly L lactic acid nanofibers have also been used to carry Pp18 [19,20]. Recently, Droget et al. reported synthesis of a stable and water soluble Pp18 in which the anhydride ring is chemically modified into cycloimide and the carboxylic group at '17' carbon position (IUPAC system) is attached to polyethylenimine, a water soluble polycationic molecule [21]. However, the singlet oxygen yield of this derivative was substantially compromised ($\sim$ 4 times lower) as compared to Pp18.

In this paper we investigated spectroscopically the suitability of SiNPs as carriers of Pp18 in comparison with liposomes and polymeric NPs, which are the conventionally used drug carriers. This was studied by monitoring the stability of Pp18 against its conversion to its hydrolytic product Cp_6 in aqueous buffer (at physiological pH). To mimic biological environment, the same study was done in the presence of 10% serum (as it forms the major part of a biological system). The conversion was monitored by measuring the absorption and fluorescence of the drug in the four NP systems at various time intervals, over the period of 24 h.

2. Materials and methods

All the reagents used in the experiment were of spectroscopic grade. These were mainly surfactant dioctyl sulfosuccinate (AOT, 99%), poly-lactic-co-glycolic acid (PLGA), phosphatidylcholine and polyvinyl alcohol (PVA) from Sigma, co-surfactant 1-butanol, acetone from Merck, triethoxyvinylsilane (VTES, 97%) and 3-aminopropyl triethoxysilane (APTS 99%) from Acros chemicals and Fetal bovine serum (serum, used as 10% by volume) from Himedia, Mumbai, India. All the reagents were used as received. SiNPs were synthesized in the non polar core of AOT/1-Butanol/water micellar system using VTES and APTS silica precursors as described in literature [12]. In short SiNPs were synthesized in the non polar core of AOT/1-Butanol/water micellar system. The micelles were prepared by dissolving 0.44 g AOT and 800 µl of 1-butanol in 20 ml of water by vigorous magnetic stirring. Neat VTES (200 µl) was added to the micellar system and the resulting solution was stirred until clear. After this20 µl of either ammonia solution (to make SiNP-V) or APTS (to make SiNP-VA) was added and stirred for about 20 h. The entire reaction was carried out at room temperature. At the end of the process, a bluish white translucency indicating the formation of nanoparticles was observed. The SiNPs were purified from AOT and butanol by dialyzing (using a membrane having a molecular cut-off 10 kDa) against neutral Millipore water for a period of $\sim$96 h. Nano-PLGA formulations were prepared by nano-precipitation technique with minor modifications [22]. In brief, 90 mg of PLGA was dissolved in 10 ml of HPLC grade acetone over a period of 3 h to get a uniform PLGA solution. This solution was added drop wise to 20 ml of aqueous solution containing 1.5% of PVA over a period of 10 min on a magnetic stir plate operated at 800 rpm. Within a few minutes, precipitation of nanoparticles was observed. This suspension was stirred at room temperature for 24 h to evaporate the acetone completely. Larger aggregates and free PLGA/PVA polymers were removed by centrifugation at 5000 rpm for 10 min. Then centrifuged at 18,000 rpm for 1 h and washed the sediment with water. Small unilamellar liposomes were prepared by ethanolic injection method. In short 100 µl ethanolic solution of phosphatidylcholine (20 mg ml^{-1}) was injected into 1.0 ml solution of 10 mM phosphate buffer at pH 7.4. Purpurin 18 and Cp_6 were prepared from dry spinach leaves following the reported procedure [23]. The stock was prepared in acetone.

The size and zeta potential of these nanoparticles was measured using a 90 Plus particle size analyzer from Brookhaven Instruments Corporation, USA. The average diameter of both SiNP particles was measured to be $\sim$ 32 nm using dynamic light scattering and the zeta potential of the SiNP-VA and SiNP-V was $\sim -36.0 \pm 3.0$ mV



Purpurin 18 Chlorin p_6

Scheme 1. Chemical structure of Pp18 and Cp_6 and the conversion of Pp18 to Cp_6, due to its hydrolysis in the presence of water.

and − 45.0 ± 3.0 mV, respectively. The size of the PLGA NP and liposomes was ∼30 nm and the zeta potential was ∼ − 8 ± 2 mV, −41 ± 4 mV respectively.

Absorption spectra were recorded, with 1 nm resolution, using Cintra 20 spectrophotometer (GBC, Australia) and emission spectra was recorded with 4 nm resolution using Fluorolog-2 spectrofluorometer (Spex, USA) using excitation wavelength as 405 nm. All the experiments were performed at pH 7.4 in 10 mM phosphate buffer and the concentration of Pp18 added to various preparations was 2 μM unless otherwise specified. The ratio of NP to drug was maintained similar in the experiments. The absorption and emission of Pp18 and Cp_6 was first studied in neat media: acetone (a non-polar medium), buffer and buffer containing serum to understand their spectral features and relate them with that in NP systems. For time dependent studies, the absorption and emission of Pp18 added to SiNP-V, SiNP-VA, PLGA NP and liposome suspended in buffer and buffer containing 10% serum was monitored at several time points up to 24 h. The time point immediately after the addition of Pp18 in the NP suspension was taken as $t = 0$ and thereafter the spectra were taken at 1, 2, 4, 6, 8, and 24 h.

3. Results and discussion

First we present the spectroscopic properties of the two drugs in different environments without NPs. Fig. 1 shows the absorption and fluorescence spectra of Pp18 and Cp_6 in three environments: hydrophobic (acetone), hydrophilic (buffer) and buffer containing serum. In all the three environments, as shown in Fig. 1a–c, while the Q band absorption peaks of the two molecules lie at different positions, their Soret band overlap at ∼405 nm. Thus, for emission spectroscopy, these were excited at 405 nm. In acetone (Fig. 1a), the Q band absorption peak for Pp18 and Cp_6 lies at 695 nm and 663 nm respectively, whereas the fluorescence is centered at ∼ 710 and ∼670 nm respectively (Fig. 1d). Both, the absorption and emission peak of Pp18 are sharp as well as intense indicating it to be in monomeric form. However, in buffer, shown in Fig. 1b, the absorption peaks of Pp18 are considerably reduced and broadened with respect to that in acetone with the appearance of an additional peak at 760 nm. This indicates its aggregation [18], which is also seen in the emission spectra (Fig. 1e, Pp18 intensity is multiplied by a factor of 100 for comparison) where the intensity is reduced by ∼ three orders of magnitude when compared to that in acetone. In the presence of serum, shown in Fig. 1c, the spectral features are intermediate, as compared to acetone or buffer. The aggregate peak disappears whereas monomer peak at 702 nm appears and the Soret band is sharpened with respect to buffer. The emission intensity also increases but is significantly less as compared to that in acetone (Fig. 1f). This indicates that in serum there is an equilibrium between the monomers and the aggregates of Pp18. Cp_6 on the other hand, (shown as red curves in Fig. 1) owing to the presence of three negatively charged carboxyl groups at physiological pH, is readily soluble in buffer showing sharp absorption and emission peaks [24]. Cp_6 and Pp18 show different spectroscopic signatures in the 600–750 nm region. The Q band absorption peak of Pp18 monomer is at 702 nm and that for Cp_6 is at ∼655 nm. The emission band of Pp18 is located at ∼ 712 nm whereas for Cp_6 it is at ∼ 670 nm [18]. Therefore, in this study, the conversion of Pp18 to Cp_6 was investigated by monitoring both absorption and emission in the 600–750 nm region in the four NP systems, suspended in buffer as well as in serum.

3.1. Conversion of Pp18 to Cp_6 in aqueous buffer in the presence of different NPs

Fig. 2 shows the absorption and fluorescence spectra of Pp18, taken at different time interval, in all the four NPs in the buffer medium. For clarity only two spectra, taken at 0 and 24 h, are shown. Spectra taken at all other time points are shown in Supporting information S1. In all the nanoparticulate systems, immediately after the addition of Pp18, i.e., at time $t = 0$, two prominent bands around 405 and 702 nm due to monomer are observed. Also, there is a peak at ∼ 760 nm due to aggregates, which is significantly higher in SiNP-V as compared to SiNP-VA and PLGA NP (where it is seen as a small hump) however in liposome it is not visible. With the increase in time, the peak intensity due to aggregates decreases in all systems whereas the monomer peak (at 702 nm) first increases and then decreases (Supporting information S1). Simultaneously, another peak appears at 655 nm due to the formation of Cp_6, which is prominently seen in SNP-VA, liposomes and serum. This suggests that the aggregates of Pp18

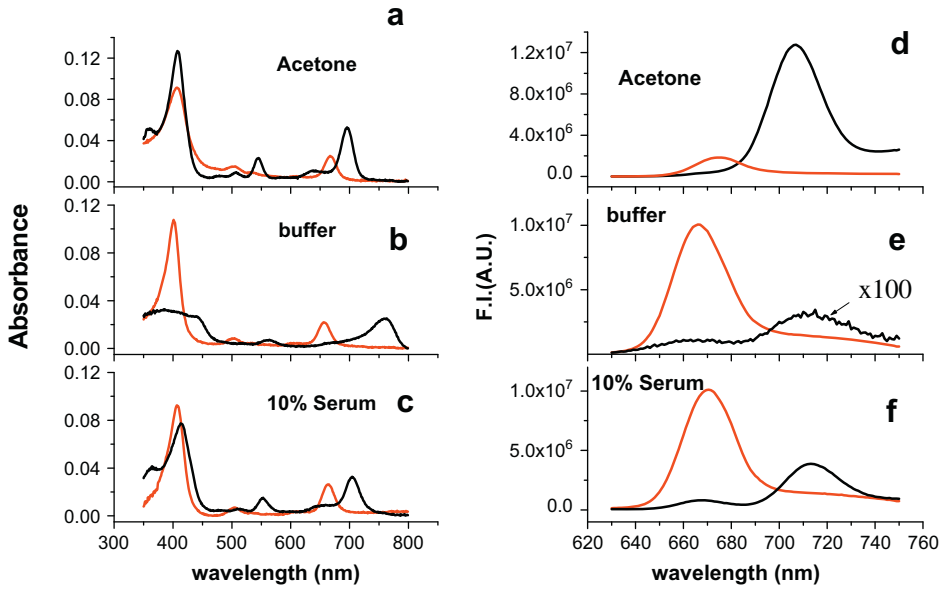


Fig. 1. Absorbance and fluorescence spectra of Pp18 (black) in acetone, buffer and serum at pH 7.4. The spectra for Cp_6 (red) are also shown for comparison. The concentration of dyes is 1 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

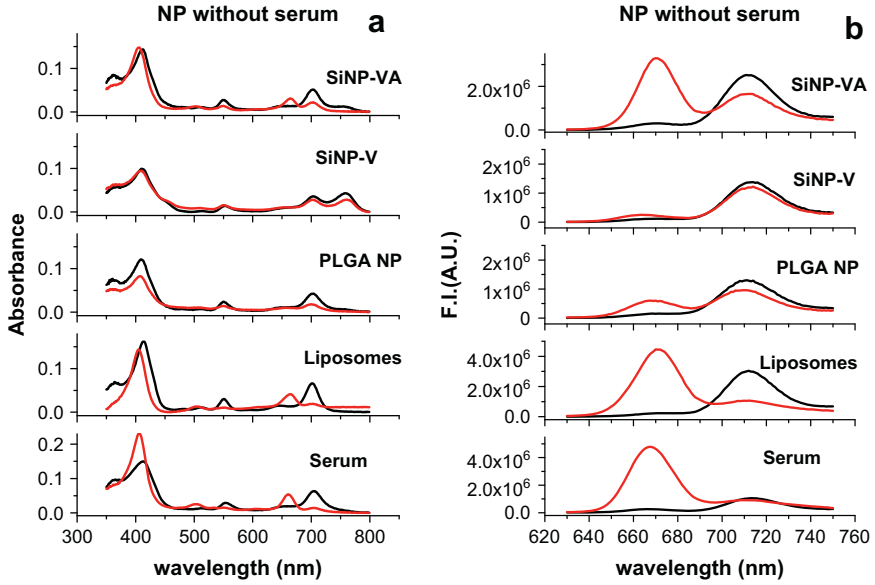


Fig. 2. The absorption (a) and fluorescence (b) spectra of Pp18 in SiNP-VA, SiNP-V, PLGA NP and liposome suspended in neat buffer at pH 7.4, monitored immediately after adding Pp18 time $t = 0$ (black) and after 24 h (red). The spectra in 10% serum are also given for reference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are getting dissociated with time (Supporting information S1), monomers are being formed which are also getting hydrolyzed to Cp_6. It is to be noted that in SiNP-V the aggregate peak remains significantly high and does not vanish even after 24 h. This suggests that aggregates are still attached to SiNP-V and their dissociation with time is a very slow process.

The corresponding time dependent fluorescence of Pp18 in these particulate systems was also monitored up to 24 h. The fluorescence spectra at time 0 h and 24 h are shown in Fig. 2b. Spectra taken at all other time points are shown in Supporting information S2. The spectra also show, in general, two peaks centered at 712 nm and 670 nm corresponding to Pp18 and Cp_6 fluorescence. With increase in time, Pp18 intensity decreases and Cp_6 increases. From Fig. 2 it is clear that the time dependent changes in intensities of these bands are different in all the four NPs. The change is largest in liposomes and SiNP-VA and smallest in SiNP-V. For comparison, the spectra of Pp18 in buffer containing only serum is also shown which also shows large decrease in Pp18 intensity.

3.2. Conversion of Pp18 to Cp_6 in aqueous buffer containing 10% serum in the presence of different NPs

To mimic the physiological condition the conversion was also monitored in the presence of 10% serum as it is a major constituent of blood and mammalian cell culture medium. Fig. 3 shows the absorption and emission spectra of Pp18, taken at time 0 and 24 h in the presence of all the four NPs suspended in buffer containing serum. Spectra taken at all other time points are shown in Supporting information S3 and S4 respectively. In all the NP systems the decrease of absorption peak at 702 nm and increase in the intensity at 655 nm is faster in the presence of serum (Fig. 3a) as compared to neat buffer (Fig. 2a). In SiNP-V while the 760 nm peak (at $t = 0$) disappears in few hrs, the peak at 702 nm is clearly seen even after 24 h whereas in the other NPs the 702 nm peak is not visible after 24 h. The emission spectra at time 0 h and 24 h (corresponding to the absorption spectra described above) are shown in Fig. 3b. Compared to neat buffer (Fig. 2b), in the presence of serum, the increase in the intensity at 670 nm is higher in all NP systems.

The relative change in the fluorescence intensities at 670 nm and at 712 nm, corresponding to Cp_6 and Pp18 monomer concentration in different NP systems, is plotted as a function of time and is shown in Figs. 4 and 5 respectively. It is clear from Fig. 4a that the intensity of Cp_6 is least for SiNP-V and polymeric NPs while it is highest for SiNP-VA and liposomes (~10 times compared to SiNP-V). Also the normalized fluorescence intensity of Pp18 up to 24 h, in Fig. 5a shows a fast decrease in liposome (~ 75%) whereas in SiNP-V the intensity is almost constant over the period of time (less than 15% decrease). This suggests that SiNP-V protect the drug from the environmental perturbations better as compared to liposome.

When the buffer medium is replaced with serum medium (Figs. 3 and 4b), the conversion to Cp_6 increases in all NP systems however the increase is significantly different among the particles. While in the presence of liposome and SiNP-VA the conversion is increased by ~1.5 times, in the presence of SiNP-V and polymeric NPs this conversion increases by about 10–14 times as compared to that without serum (Fig. 4a). Still the absolute intensity of Cp_6 in the serum media is least in SiNP-V among all the four NPs and Pp18 highest as shown in Fig. 5b.

From the results presented so far, in the presence of NPs, the time dependent changes in the Pp18 absorption (at 702 nm) as well as fluorescence (at 712 nm, shown in Fig. 2b) spectra are modulated by the two effects, namely, dissolution of Pp18 aggregates into monomers as well as monomer conversion to Cp_6. This can be represented as follows:

$$Pp18_{aggregates} \rightarrow Pp18_{monomers} \rightarrow Cp_6$$

The relative changes in Pp18 fluorescence will depend upon rate of dissolution of Pp18 aggregates and rate of conversion of Pp18 to Cp_6. As a result the changes in Pp18 fluorescence are not expected to correlate to that of Cp_6. Therefore, to get an idea about the conversion of Pp18 to Cp_6 by fluorescence technique, the changes in Cp_6 fluorescence only might be considered.

The reason of increase in the conversion to Cp_6 in the presence of serum, as shown in Fig. 3 might be due to two factors. (1) The presence of hydrophobic pockets in the serum proteins solubilizes

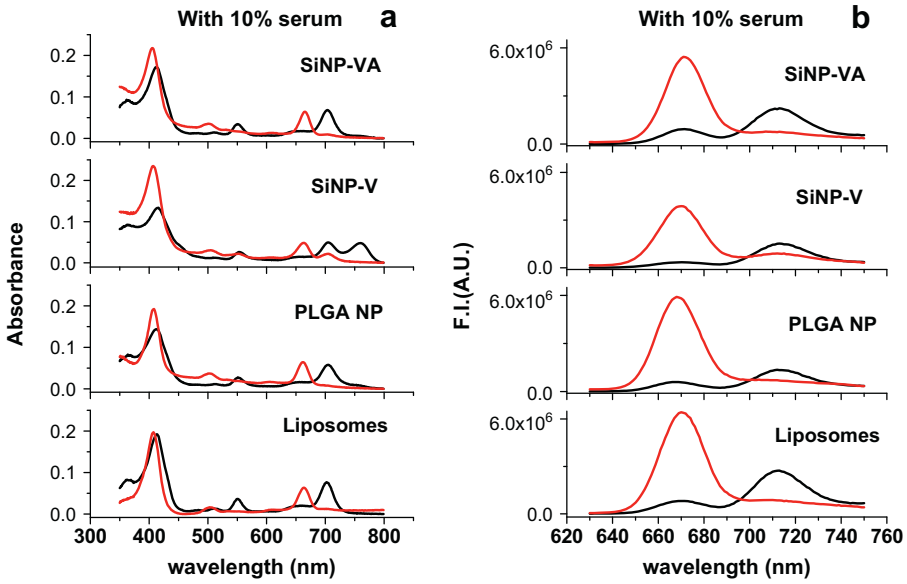


Fig. 3. The absorption (a) and fluorescence (b) spectra of Pp18 in SiNP-VA, SiNP-V, PLGA NP and liposome suspended in buffer at pH 7.4 in presence of 10% serum, monitored immediately after adding Pp18 time $t = 0$ (black) and after 24 h (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

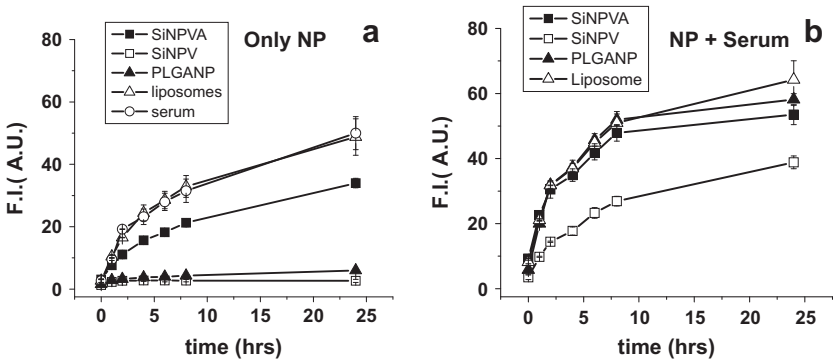


Fig. 4. The plot of fluorescence intensity at 670 nm (corresponding to Cp_6 emission), in all four NPs suspended in buffer at pH 7.4, as a function of time in the absence of serum (a) and in the presence of 10% serum (b).

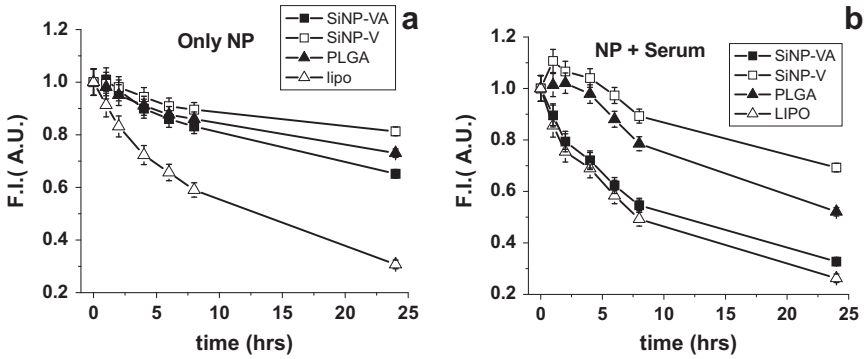


Fig. 5. The plot of normalized fluorescence intensity, at 712 nm (corresponding to Pp18 emission) in all four NPs suspended in buffer at pH 7.4, as a function of time, in the absence of serum (a) and in the presence of 10% serum (b).

Pp18 as monomer as seen in Fig. 1c and f [25]. Particularly in case of SiNP-V, in the presence of serum, a fast decrease in the aggregate peak is observed, as shown in the absorption spectra (Figs. 3a and S3). In Fig. 5b an initial increase in Pp18 monomer peak in SiNP-V also supports the fast monomerization of Pp18 aggregates. (2) Proteins contain amino groups which can catalyze the hydrolysis of the anhydride ring by working as base.

To compare among the two SiNPs, the conversion of Pp18 to Cp_6 is significantly large in SiNP-VA as compared to SiNP-V (Fig. 4a). Although the two SiNPs are similar, there are additional amino

groups present on the surface of SiNP-VA (due to the additional silica precursor, APTS, used in the synthesis having 3-amino propyl group) [16]. At physiological pH ~2.5% of these amino groups are still neutral (as pK_a of amino groups is ~9) which therefore can act as base and thus catalyze the hydrolysis of the anhydride ring. This can be attributed to the enhanced conversion of Pp18 to Cp_6 in SiNP-VA.

The conversion is larger in liposomes as compared to SiNP-VA. The reason is not clear, however it might be speculated that the polar head group of the lipid molecule in liposome might facilitate the hydrolysis of the monomeric Pp18 sitting in the lipid bilayer.

4. Conclusion

In this paper, the suitability of SiNPs as carrier of hydrophobic photosensitizer Pp18 was investigated spectroscopically by comparing the stability of Pp18 against its conversion to its hydrolytic product Cp_6 in four NP systems. The absorption and fluorescence of Pp18 entrapped in nanoparticle formulations such as SiNP-V, SiNP-VA, PLGA nanoparticles and PC liposome suspended in aqueous environment at pH 7.4 (buffer as well as in serum), monitored over the period of 24 h, was found to depend on the NP environment. The conversion was found to be lowest in SiNP-V and highest in liposome. The difference in the conversion is suggested to be the presence of the groups, which were able to catalyze the hydrolysis of the anhydride ring. Our results also suggest that SiNP-V could be used as the carrier for Pp18.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2013.12.019.

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