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Spectral Characteristics of the Aggregation of α,β,γ,δ-Tetrakis(p-sulfophenyl)porphyrin in the Presence of Proteins

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With the aid of absorption, fluorescence, and resonance light-scattering measurements, the aggregation of protonated water-soluble porphyrin, α,β,γ,δ-tetrakis(p-sulfophenyl)porphyrin, $[H_4tpps_4]^{2-}$, in the presence of proteins has been characterized. At pH 1.86, proteins dissolved in water can form a positively charged microphase, which can neutralize the negative charges of $[H_4tpps_4]^{2-}$ distributed in it, leading to the *J*- and *H*-aggregation of $[H_4tpps_4]^{2-}$. Exciton splitting signals, which are generally associated with the aggregation of porphyrins, as described by molecular-exciton theory, are observed at 436.1 nm on the resonance light-scattering spectra, supporting the porphyrin–porphyrin interactions in the positively charged microphase of proteins. It has been proved that positively charged microphase can protect aggregates of $[H_4tpps_4]^{2-}$ from water dilution and high temperature.

The molecular structure of the porphyrins is fairly rigid and has extensive electron delocalization in the nucleus. These properties must be essentially involved in the chemical activity and biological function of the macromolecule in which they are found.[1] The native porphyrins can bind to special proteins (chemoprotein, for instance) and perform an important function in some biophysical procedures, such as the transferring of O_2 and CO_2 to sustain respiration and electron transfer to sustain biological oxidation. However, the procedures are very complicated to study. Water-soluble synthetic porphyrins, since they are expected to display the function of native porphyrin derivatives in the physiological state, and their structural simplicities, in comparison to their native analogs, allow for an easier interpretation of structure-biological activity relations. Those water-soluble synthetic porphyrins may be present as dimers or higher aggregates in aqueous solution.[2—4] Since the formation of aggregates may have a strong effect on their physicochemical properties, a thorough understanding of the conditions that promote aggregation is of importance.

The aggregation of water-soluble porphyrin, α,β,γ,δ-tetrakis(p-sulfophenyl)porphyrin, $[H_4tpps_4]^{2-}$, has been well-studied.[1—8] The porphyrin-porphyrin interaction, including the *J*- and *H*-modes, results in a complete lifting of the degeneracy of component planar oscillators. The *J*- and *H*-type aggregates are in "sandwich-type", and the pyrrolic rings in the porphyrinato macrocycle are alternately ruffled up and down.[3,4] However, the *J*-aggregate can be viewed as forming a linear staggering card-deck structure, with the opposite peripheral phenyl groups overlapping adjacent porphyrinato macrocycles.[5] It is caused by a hydrophobic attraction due to an intermolecular charge–resonance interaction between the filled and vacant frontier orbitals of neighboring porphyrins in the ground state.[2] The *H*-aggregate, however, can be viewed as being a stacking card-deck arrangement with phenyl and macrocycle groups nearly directly above and below one another.[5] The stacking card-deck structure would result in a repulsion effect between adjacent N–N groups, and decrease the subject interaction distance, leading to a blue-shifted absorption band at 422 nm.[4] In addition, the observed *J*-band is sharp and intense while the *H*-band is rather weak.

Recently, we found that some water-soluble synthetic porphyrins, such as $[H_4tpps_4]^{2-}$,[9—11] α,β,γ,δ-tetrakis(p-carboxyphenyl)porphyrin, $[H_2tcpp]^{4-}$,[12] can interact with proteins. It is expected that those interactions of the porphyrins with proteins can supply good models for studies of the physiological process of native porphyrins. Here, we discuss the spectroscopic features of the absorption, fluorescence and resonance light scattering for the interactions of $[H_4tpps_4]^{2-}$ with proteins under an appropriate acidity and ionic strength. Considering the similarities of the spectroscopic features of the interactions between $[H_4tpps_4]^{2-}$ and proteins to those of the aggregation of $[H_4tpps_4]^{2-}$ in appropriate acidity and ionic strength of the aqueous solution,[2] and in the presence of cation-crown,[8] we suppose that the interactions of $[H_4tpps_4]^{2-}$ with proteins are due to the aggregation of $[H_4tpps_4]^{2-}$ in the positively charged microphase formed by proteins.

Resonance light-scattering spectroscopy, which can be obtained by using a common spectrofluorimeter, can provide sensitive and informative signals.[13—20] Analogous

to resonance-enhanced Raman scattering for aggregated molecules,[21,22] the basic theory of the spectroscopy is resonance-enhanced Rayleigh scattering in an absorption medium in which aggregate molecules exist.[13] Our studies,[17—20] together with those of other researchers,[13—16] show that resonance light-scattering has extensive applications. For example, the exciton splitting signals due to the aggregation of porphyrin can be observed by resonance light-scattering spectroscopy.[17] With enhanced resonance light scattering we could monitor the formation of suprahelical helixes of nucleic acids induced by 5,10,15,20-tetrakis-[4-(trimethyammonio)phenyl]porphyrin, $[H_2tapp]^{4+}$,[17] and determine nucleic acids at the nanogram levels.[18,19] We have also reported on a determination method of proteins at nanogram levels based on their enhancement effect on the resonance light-scattering of $[H_4tpps_4]^{2-}$.[20]

Experimental

Apparatus. Absorption spectra were obtained using a Shimadzu UV-265 spectrophotometer (Kyoto, Japan), while the fluorescence spectra and resonance light-scattering spectra were recorded with a Shimadzu RF-540 spectrofluorimeter (Kyoto, Japan). The scanning procedures of the resonance light-scattering spectra were the same as described in our previous reports.[17—20] A WH-861 vortex mixer (Jiangsu Instruments, Inc., China) was used to blend a mixture in a volumetric flask, and an 821 pH meter (Zhongshan University, China) was used to measure the pH value of a solution.

Reagents. Stock solutions were prepared by dissolving commercial bovine serum albumin (BSA, Sigma, USA), γ-globulin (γ-IgG, Serva, Germany), trypsin, pepsin, protamine sulfate (all were purchased from Shanghai Institute of Biochemistry, Chinese Academy of Sciences, China), chymotrypsin, lysozyme (both were purchased from Sino-American Biotech, Beijing, China) in doubly deionized water, except for γ-IgG, which was dissolved with the aid of a few milliliters of a 0.1 M NaCl solution (1 M = 1 mol dm^{-3}). All of the working concentrations of proteins were 8.5 $\mu g\,ml^{-1}$.

The water-soluble free-base porphyrin, $[H_2tpps_4]^{4-}$, which was synthesized in our laboratory according to the literature,[23] and identified by elemental analysis, IR and ^1HNMR, was dissolved in doubly deionized water. The concentration of the solution was determined according to the absorbance at 413.0 nm (the Soret maxima) by using $\varepsilon = 5.1\times10^5\ M^{-1}\,cm^{-1}$ at pH 6.0.[23]

Tris-HCl (pH 7.40) and HCl–NaCl (pH 1.86) buffer solutions were used to control the acidity, while a 1.0 M NaCl solution was used to adjust the ionic strength of the aqueous solution.

All of the reagents were of analytical grade and used without further purification. The water used throughout was doubly deionized.

Procedures. Into a 10 ml volumetric flask were added an appropriate working solutions of $[H_4tpps_4]^{2-}$ and some water until 5.0 ml. The solution was votexed, followed by the addition of a buffer solution. The mixture was at least diluted by doubly deionized water to 10 ml and thoroughly mixed. Then, the flask was put into a 50 °C water bath. Twenty min later the flask was taken out and cooled down by tap water. All of the absorption, fluorescence and resonance light-scattering measurements were made against a blank treated in the same way without proteins. The aggregate data of $[H_4tpps_4]^{2-}$ in the absence of proteins were obtained at a room temperature (20 °C) after saturation of the absorbance at 490 nm had been reached, which indicates the completion of aggregation.

Results and Discussion

Absorption Spectra. The porphyrin investigated in this study can have two chemical forms because of protonation of the two pyrrolic nitrogen atoms in the porphinato macrocycle (pK_a of $[H_4tpps_4]^{2-}$ is 4.8 at $I = 0.1$ and at 25 °C).[23] The two species, which are $[H_2tpps_4]^{4-}$ and $[H_4tpps_4]^{2-}$, depending on the pH value of the aqueous solution, exhibit a series of well-defined absorption bands in the visible region of the spectra. Figure 1 shows the absorption spectra of $[H_2tpps_4]^{4-}$ at pH 7.40 and $[H_4tpps_4]^{2-}$ at 1.86. At pH 7.40, $[H_2tpps_4]^{4-}$ exists as a free-base species, exhibits the absorption features of D_{2h} symmetry, and has absorption bands at 413.0, 515.6, 553.2, 577.4, and 632.6 nm. These absorption bands are assigned to a Soret band (namely the B(0,0) band), $Q_y(1,0)$, $Q_y(0,0)$, $Q_x(1,0)$, and $Q_x(0,0)$, respectively. However, at pH 1.86, it exhibite the features of D_{4h} symmetry. Its Soret band shifts from 413.0 to 434.0 nm, and its Q bands, Q(1,0) and Q(0,0), do not split, and are located at 594.0 and 645.0 nm, respectively.

As shown in Fig. 1, two new absorption bands appear simultaneously at 490.0 and 705.0 nm with increasing the concentration of $[H_4tpps_4]^{2-}$. The two new bands originate from the *J*-aggregation of $[H_4tpps_4]^{2-}$,[1—8] and are encouraged by increasing the acidity and ionic strength of the solution and the concentration of $[H_4tpps_4]^{2-}$. However, we found that the aggregation of $[H_4tpps_4]^{2-}$ decreases with increasing temperature, and that the aggregates were easily decomposed by the addition of water.

When protein solutions were added into a solution of $[H_4tpps_4]^{2-}$, which does not show an aggregation tendency, according to the absorption spectra, two new bands at 490.0 and 705.0 nm could be observed (Fig. 2a) with the Soret,

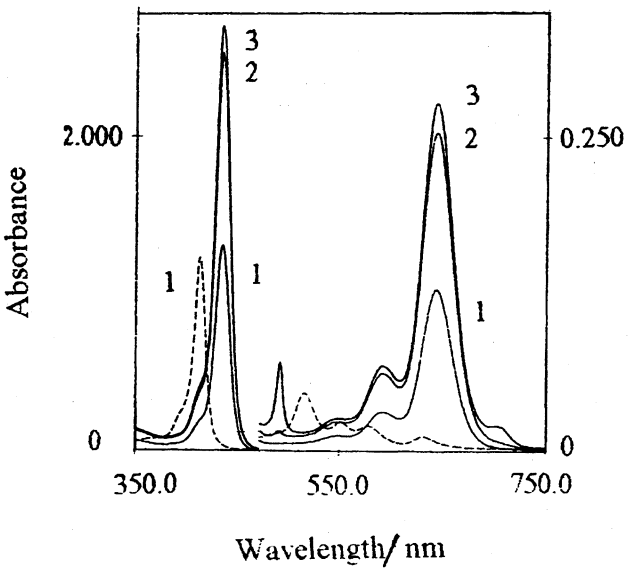

Fig. 1. Absorption spectra of $[H_4tpps_4]^{2-}$. The pH values for solid and broken lines are 1.86 and 7.40 respectively. Concentration ($\times10^{-6}$ M): 1, 5.0; 2, 10.0; 3, 11.0. Ionic strength, 0.05. 5.0 mm sample cell was used. Spectra from 470.0 to 750.0 nm were 8-fold enlarged (right scale).

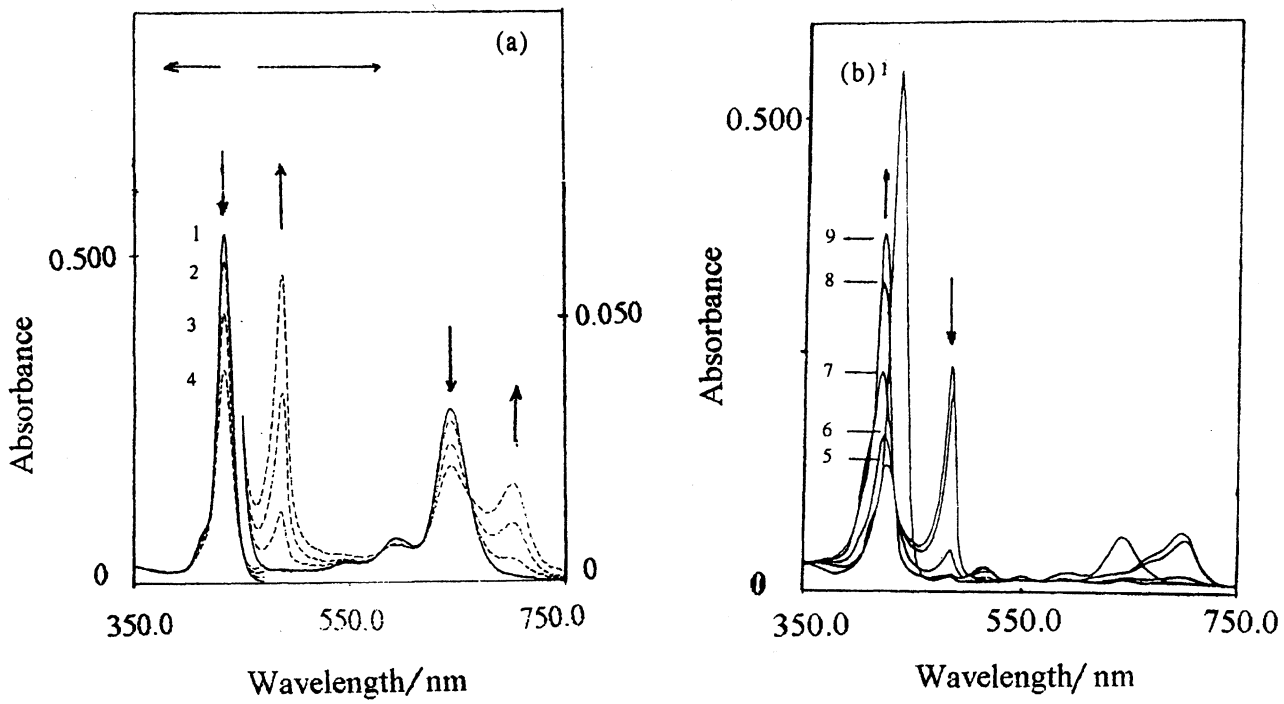

Fig. 2. Absorption spectra of the aggregates in the presence of BSA. pH 1.86. Concentrations: $[H_4tpps_4]^{2-}$, 1.0×10^{-6} M; BSA ($\mu g\,ml^{-1}$): 1, 0.0; 2, 0.34; 3, 0.68; 4, 1.02; 5, 6.63; 6, 11.4; 7, 16.56; 8, 22.08; 9, 33.13. 10 mm sample cell was used. Spectra from 470.0 to 750.0 nm were 8-fold enlarged (right scale).

Q(1,0) and Q(0,0) bands being reduced. The reducing Soret and Q bands became broad, and the Q bands even had a few nanometers' red-shift. The absorbance at 490.0 and 705.0 nm increased with increasing the concentration of proteins. Three isosbestic points were clearly observed at 451.8, 579.0, and 662.0 nm. By comparing the *J*-aggregation bands of $[H_4tpps_4]^{2-}$ in Fig. 1 with the 490.0 and 705.0 nm bands of $[H_4tpps_4]^{2-}$ in the presence of proteins (Fig. 2), it is easily understood that the species which has the 490.0 and 705.0 nm bands induced by proteins must be the aggregates of $[H_4tpps_4]^{2-}$. Therefore, the addition of proteins has a remarkable effect on the monomer-aggregate equilibrium of $[H_4tpps_4]^{2-}$. If excess proteins were added (Fig. 2b), a band at 422 nm appeared with decreasing the 490.0 and 705.0 nm band, and the 705.0 nm band tended to be hypsochromic. However, by increasing the concentration of $[H_4tpps_4]^{2-}$ (Fig. 3) while maintaining a constant protein concentration, it was found that the absorptions of the Soret and Q bands increased, but the 490.0 and 705.0 nm bands reduced with the 705.0 nm band being slightly red-shifted, and two isosbestic points at 494.0 and 718.0 nm were observed.

The effect of proteins can be elucidated by the monomer-aggregate equilibrium of $[H_4tpps_4]^{2-}$. The role of proteins played is possibly similar to the cation-crown ether complexes in $[H_4tpps_4]^{2-}$ aggregation, which drives the monomer-aggregate equilibrium towards the aggregate side.[8] Although the addition of proteins to $[H_4tpps_4]^{2-}$ at pH 1.86 does not increase the concentration of H^+, proteins can provide a microphase that is distinct from the aqueous solution in which they are dispersed.[24] In the microphase the negative charges of $[H_4tpps_4]^{2-}$ can be neutralized by

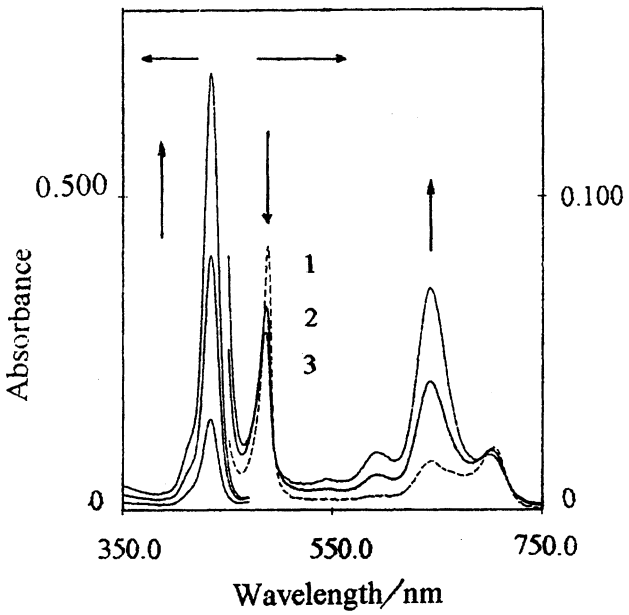

Fig. 3. Effect of the concentration of $[H_4tpps_4]^{2-}$ on the aggregation in the presence of BSA. pH 1.86. Concentrations: BSA, 0.68 μg ml; $[H_4tpps_4]^{2-}$ ($\times10^{-6}$ M): 1, 0.5, 2, 1.0; 3, 1.5. 10 mm sample cell was used. The spectra from 470.0 to 750.0 nm were 5-fold enlarged (right scale).

the positive charges of protein molecules. The $-NH_3^+$ group in protein molecules, for instance in acidic solution, can play the role of protons and counter the negative charges of $[H_4tpps_4]^{2-}$. It can thus be established that a positively charged microphase formed by proteins promotes the aggregation of $[H_4tpps_4]^{2-}$

Similarly, the 490.0 and 705.0 nm bands of $[H_4tpps_4]^{2-}$ produced in the presence of proteins should be ascribed to *J*-aggregation, which originates from linear oscillators polarized in the long axis of a rodlike aggregate.[2] Being different from the spatial arrangements of *J*-aggregation, *H*-aggregation produces an absorption band at a shorter wavelengths than the corresponding monomer band.[25] The *H*-band of the aggregated $[H_4tpps_4]^{2-}$ in the presence of excess proteins appears at 422 nm, which coincides with the absorption bands of a covalently linked complex formed by $[H_4tpps_4]^{2-}$ and proteins through $-SO_2-NH-$ bonds.[9] Thus, the aggregation of $[H_4tpps_4]^{2-}$ in the presence of proteins is likely to bind in the $-SO_3^-\text{–}^+NH_3-$ mode.[9]

Although the aggregates of $[H_4tpps_4]^{2-}$ in the absence of proteins can scarcely be formed at high temperature, and can be easily decomposed by the addition of water, those formed in the presence of proteins can stand up to a relatively high temperatures and are difficult to be decomposed by the addition of water. The optimal aggregation of $[H_4tpps_4]^{2-}$ in the presence of BSA and γ-IgG should advance at 50 °C. However, if the temperature is too high (80 °C, for instance) the aggregates would be decomposed. It can thus be supposed that proteins can form a protective microphase for the aggregates of $[H_4tpps_4]^{2-}$.

Fluorescence Spectra. Regardless of the inner nitrogen atoms of the pyrrolic ring on the porphyrin moiety being protonated, the porphyrin investigated in this study is a fluorescent molecule. At pH 7.40, $[H_4tpps_4]^{2-}$ exhibits fluorescence when the molecule is excited at 413.0 or 515.6 nm. At pH 1.86, because of the shift of the Soret band and Q bands, resulting from protonation of the nitrogen atoms of the pyrrolic ring, the emission wavelength shifts from 639.0 to 657.0 nm. Furthermore, fluorescence emission is observed at 650.0 and 755.0 nm with a shoulder peak at 672.8 nm upon excitation at the aggregate absorption maxima (490.0 nm) of $[H_4tpps_4]^{2-}$ (Fig. 4). It can be easily recognized from Fig. 4 that some aggregation of $[H_4tpps_4]^{2-}$ has taken place, even though no aggregation signals can be found according to absorption measurements. Figure 5 shows the case when $[H_4tpps_4]^{2-}$ interacts with proteins: The fluorescence excited at the Soret band is quenched, but that excited at the *J*-band is enhanced. This fact is another pieces of evidence that proteins accelerate the aggregation of $[H_4tpps_4]^{2-}$.

Resonance Light-Scattering Spectra. Figure 6 shows the resonance light-scattering spectra of $[H_4tpps_4]^{2-}$ in different concentrations. Analogous to other porphyrins,[15,17,18] because of the strong absorption of the Soret band, the light scattering in the Soret region is very small. With increasing the concentration of $[H_4tpps_4]^{2-}$, the resonance light scattering at 490.0 nm, where the *J*-band of the aggregate of $[H_4tpps_4]^{2-}$ is located, increases quickly. At the same time, a new peak at 625.5 nm appears in such a way that it increases initially with the concentration of $[H_4tpps_4]^{2-}$, and then decreases with an excess addition of $[H_4tpps_4]^{2-}$.

If proteins were added in to an $[H_4tpps_4]^{2-}$ solution, enhanced signals of resonance light-scattering, which are the same as the aggregation of $[H_4tpps_4]^{2-}$ in the absence of proteins, can be observed. The enhanced signals near to the Soret region increase with increasing the concentration of proteins (Fig. 7), while the resonance light-scattering at 652.8 nm varies in the same way as in Fig. 2: Increase initially, but decreases if excess proteins are added. In addition, Fig. 7 shows splitting signals at 436.1 nm; also, an equal

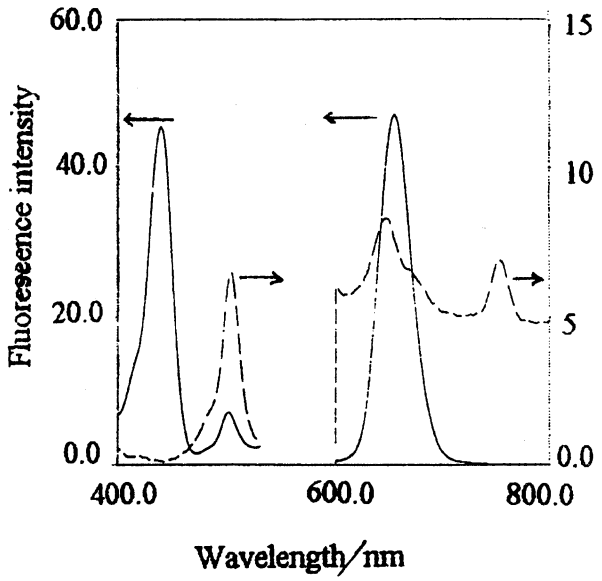

Fig. 4. Excitation and emission spectra of $[H_4tpps_4]^{2-}$. Concentration of $[H_4tpps_4]^{2-}$. 5.0×10^{-6} M. pH 1.86. Ionic strength, 0.25. Bandpass for excitation and emission: 10 nm. The broken line spectra, which stand for the fluorescence at both 650.0 and 755.0 nm excited at 490.0 nm, were 4-fold enlarged (right scale).

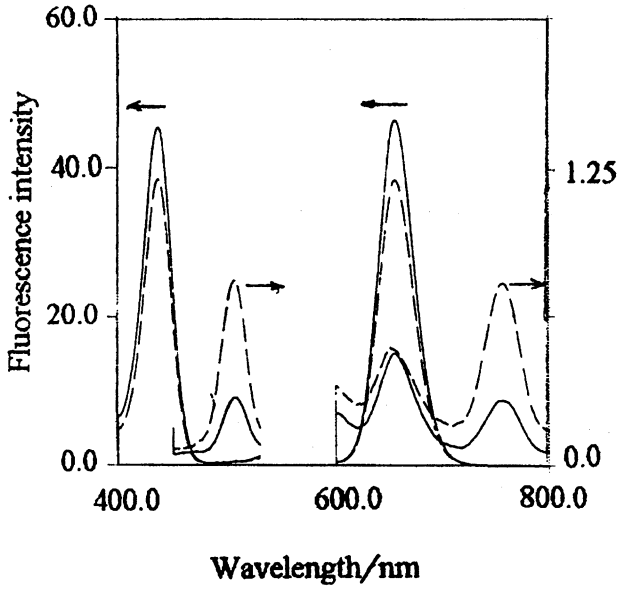

Fig. 5. Excitation and emission spectra of the aggregation of $[H_4tpps_4]^{2-}$ in the presence of BSA. Solid line, $[H_4tpps_4]^{2-}$; broken line, BSA–$[H_4tpps_4]^{2-}$. Concentrations: $[H_4tpps_4]^{2-}$, 1.0×10^{-6} M; BSA, 0.68 $\mu g\,ml^{-1}$. pH 1.86. Ionic strength, 0.05. Bandpass for excitation and emission, 20 nm. The spectra in broken line were 32-fold enlarged (right scale).

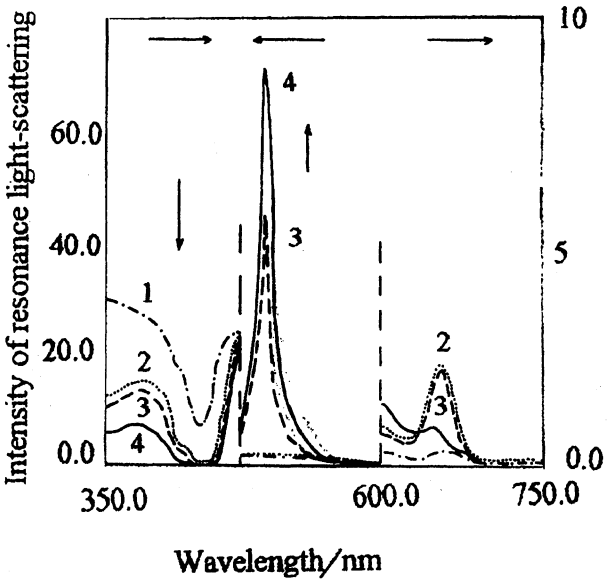

Fig. 6. Resonance light-scattering spectra of $[H_4tpps_4]^{2-}$. Concentrations of $[H_4tpps_4]^{2-}$ ($\times 10^{-6}$ M): 1, 1.0; 2, 8.0; 3, 10.0; 4, 10.4. pH, 1.86. Ionic strength, 0.05. Bandpass for the excitation and emission: 5.0 nm. The ordinate in the range from 350.0 to 470.0 nm and from 600.0 to 750.0 nm were 8-fold enlarged (right scale).

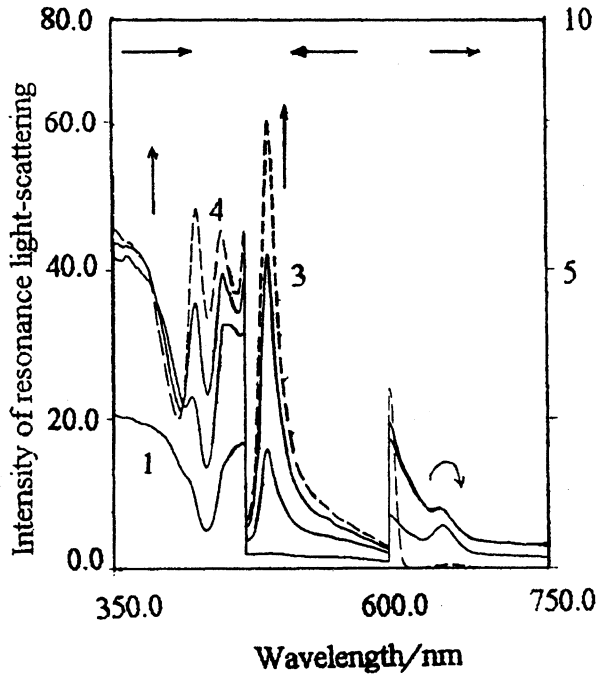

Fig. 7. Resonance light-scattering spectra of the aggregation of $[H_4tpps_4]^{2-}$ in the presence of BSA. Concentrations: $[H_4tpps_4]^{2-}$, 1.0×10^{-6} M; BSA ($\mu g\,ml^{-1}$): 1, 0.0; 2, 0.34, 3, 0.68; 4, 1.02. pH 1.86. Bandpass for excitation and emission: 5.0 nm. The ordinate in the range from 350.0 to 470.0 nm and from 600.0 to 750.0 nm were 8-fold enlarged (right scale).

resonance light-scattering point at 414.0 nm appears with increasing the concentration of proteins. One of the splitting peaks, matches the isosbestic point at 451.8 nm; the other one

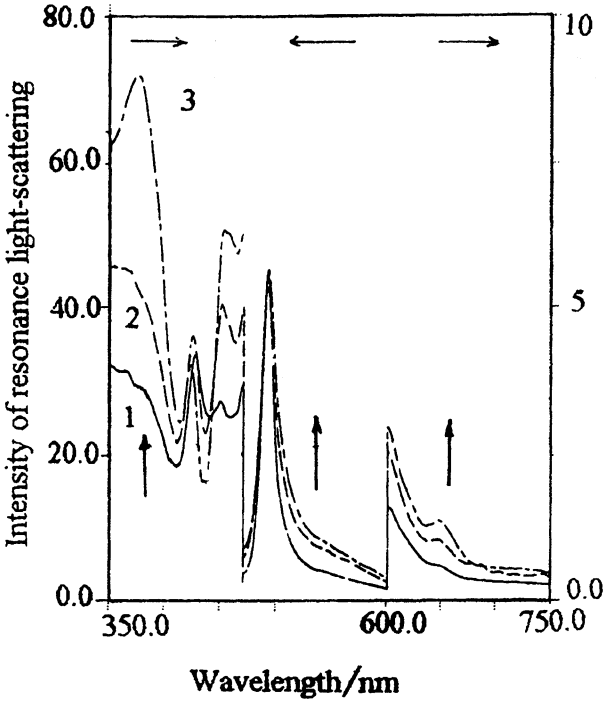

Fig. 8. Concentration effect of $[H_4tpps_4]^{2-}$ on the resonance light-scattering spectra of $[H_4tpps_4]^{2-}$ in the presence of BSA. Concentrations: BSA, 0.68 $\mu g\,ml^{-1}$; $[H_4tpps_4]^{2-}$ ($\times 10^{-6}$ M): 1, 0.5; 2, 1.0; 3, 1.5. Bandpass for the excitation and emission: 5.0 nm. The ordinate in the range from 350.0 to 470.0 nm and from 500.0 to 750.0 nm were 8-fold enlarged (right scale).

is 426.0 nm, which may be relative to the *H*-type aggregation of $[H_4tpps_4]^{2-}$. The splitting signals, which are generally associated with aggregation, as defined in molecular-exciton theory,[17,25] are indicative of the aggregation of $[H_4tpps_4]^{2-}$.

With increasing the concentration of $[H_4tpps_4]^{2-}$ while the concentration of proteins is held constant (Fig. 8), the resonance light scattering at 490.0 nm is scarcely changed, but the resonance light scattering at 652.8 nm increases all of the time, and the exciton splitting signals become significant even more.

Considering the splitting nature and red-shifted Q bands in the presence of proteins, it is possible that the aggregates of $[H_4tpps_4]^{2-}$ in the presence of proteins exist in a "sandwich-type", because that type always associates with the red-shifts of the Q-band, which are affected by the enhancement of exciton coupling.[5,8] Thus, the *J*- and *H*-aggregates of $[H_4tpps_4]^{2-}$ in the presence of proteins are similar to those in the absence of proteins. That is, proteins act as surroundings for encouraging aggregation, but do not change the linear staggering card-deck (*J*-aggregate) and stacking card-deck (*H*-aggregate) structures of the aggregates of $[H_4tpps_4]^{2-}$.

The 652.8 nm peak of the resonance light-scattering spectra for the protonated $[H_4tpps_4]^{2-}$ and its aggregate in proteins, analogous to resonance light-scattering of the protonated $[H_2tapp]^{4+}$,[17] matches the Q(0,0) band.

Aggregation of $[H_4tpps_4]^{2-}$ in the Presence of Different Proteins. Figure 9 shows the pH dependence of the inten-

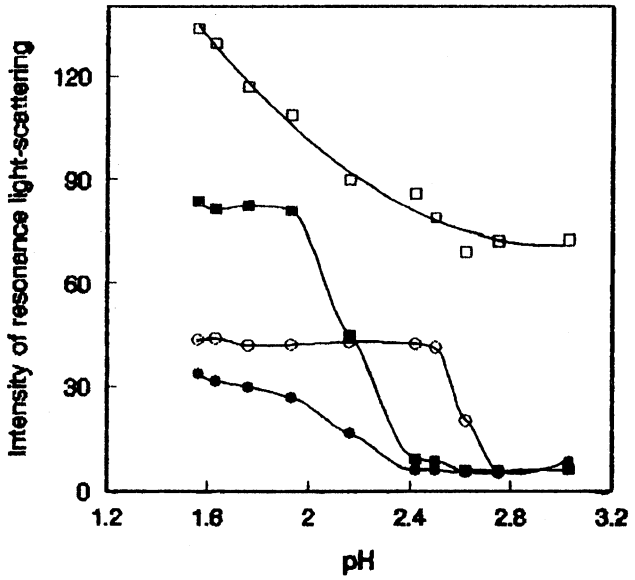

Fig. 9. pH dependence of the aggregation of $[H_4tpps_4]^{2-}$ in the presence of proteins. Concentrations: $[H_4tpps_4]^{2-}$, 1.0×10^{-6} M; proteins, 0.68 μg ml^{-1} (except γ-IgG, 1.02 μg ml^{-1}). Ionic strength, 0.05. (○), BSA; (●), γ-IgG; (□), protamine sulfate; (■), lysozyme. All the data were obtained at 490.0 nm.

sity of the resonance light-scattering measured at 490.0 nm; this figure reveals the aggregation features of $[H_4tpps_4]^{2-}$ in the presence of various kinds of proteins. Protamine sulfate shows the most strongly accelerative signal of the tested proteins, then lysozyme; however, trypsin and pepsin scarcely promote the aggregation of $[H_4tpps_4]^{2-}$ and fail to produce resonance light-scattering signals (not shown in Fig. 9). Even though, all of the resonance light scattering responses for different proteins share a common feature, which decreases with increasing pH and increases with increasing acidity for different kinds of proteins.

It is worth noting that the growth of the resonance light-scattering responses can reach saturated values with decreasing pH. Our studies show that protons participate in the aggregation of $[H_4tpps_4]^{2-}$, and that the proton number for the aggregation is about 2—3 and does not greatly change with the kind of proteins.[26] However, different proteins will lead to different numbers of aggregates per unit protein molecule; and since the number (namely, binding number) increases with the molecule weight of proteins. It can thus be easily understood that different proteins have different responses.

It is likely that the role of proteins in the aggregation of $[H_4tpps_4]^{2-}$ is relative to their denatural properties. The denaturation of proteins can expose more active groups, such as $-NH_2$, to the surface of the protein molecules, so that more positive charges can be formed.[27] Therefore, increasing the temperature, which induces a denaturation of proteins, promotes the aggregation $[H_4tpps_4]^{2-}$; also, it is possible that 50 °C is a suitable temperature for aggregation, considering that proteins can generally be denatured at 50—60 °C.[27] If the temperature is too high, 80 °C, for instance, proteins become excessively denatured and the protective role of the proteins will be lost. In this case, the aggregates of $[H_4tpps_4]^{2-}$ induced by proteins decompose.

Conclusion

Proteins dissolved in water can form a positively charged microphase that is significantly different from the water phase. The positive charges of the microphase can neutralize the negative charges of $[H_4tpps_4]^{2-}$, and can promote the aggregation of the anion porphyrin. The microphase is protective for the aggregates from water dilution and high temperature, which are not of benefit to the aggregation of $[H_4tpps_4]^{2-}$ in the absence of proteins. The accelerative effect of the microphase is affected by the charges of the protein molecules. The aggregation of porphyrin in the presence of proteins produces the same signals as does the self-aggregation of $[H_4tpps_4]^{2-}$ in the water surroundings at high acidity and ionic strength without proteins, and maintains the *J*- and *H*-aggregation features. Although both *J*- and *H*-aggregates of $[H_4tpps_4]^{2-}$ are of the "sandwich-type", the *J*-aggregates take on a linear staggering card-deck structure, while the *H*-aggregates take on a stacking card-deck structure, regardless of the presence of porteins.

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