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A resonance light scattering ratiometry applied for binding study of organic small molecules with biopolymer

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Abstract

Resonance light scattering (RLS) technique is a creative application of light scattering signals detected by using a common spectrofluorometer, but it has drawbacks such as the fluctuation of signals caused by poorly quantified or variable factors. Herein we develop a RLS ratiometry to overcome the drawbacks of the technique and apply to measure the binding nature of organic small molecules (OSM) with biopolymer using the binding of cation porphyrins with heparin (HP) as an example. In near neutral solution, cationic porphyrins *meso*-tetrakis [(trimethylammoniumyl) phenyl] porphyrin (TAPP) and *meso*-tetra (4-methylpyridy) porphyrin (TMPyP-4) interact with heparin, resulting in hypochromatic effect, and enhanced RLS signals. Linear relationship could be established between the ratio of enhanced RLS signals at two wavelengths, where the maximum and minimum are available in the ratio curve of UV–vis spectrum of porphyrin to that of heparin–porphyrin complex, and the logarithm of heparin concentration, and thus a wide dynamic range detection method of biopolymers could be developed. In comparison with RLS method, this RLS ratiometric one is less affected by environmental conditions such as pH, ionic strength. The mechanism of these interactions was investigated based on the charge density distribution of the two porphyrin molecules and it could be concluded that the enhanced RLS intensity is proportionally promoted by the charge capacity of components in the complex. Additionally, the binding number and binding constant were measured scientifically by Scatchard plot.

Keywords: Resonance light scattering ratiometry; Organic small molecules (OSM); Biopolymers

1. Introduction

Binding study of organic small molecules (OSM) with biopolymers is very important in elucidating the nature of drug-targeted biomolecules [1,2]. In order to obtain the binding information in terms of kinetics and binding affinities, techniques such as X-ray diffraction, NMR spectroscopy, circular dichromism, ultraviolet–vis (UV–vis) molecular absorption and fluorescence spectroscopy have been traditionally available [3–5]. It has proved that Resonance Raman (RR) spectroscopy with ultraviolet excitation radiation is a valuable method [4,5], and its increased sensitivity is a good example to show that light scattering signals could have been applied sensitively to tissue studies [1], in vivo cancer diagnosis [2], immunocytology application [3], and DNA hybridization [4]. Similar to the enhanced RR spectroscopy, resonance light scattering (RLS) technique is a newly developed tool by using a common spectrofluorometer to detect enhanced light scattering signals in the assemblies of π-stacking molecules [5,6]. This technique has the advantages of simple operation and high sensitivity, showing high promise in studies of biochemistry [7–11], pharmacology [12], and molecular biology [4]. In practical application, however, the RLS signals suffer from fluctuation caused by many poorly quantified or variable factors in solution such as the incident light intensity, reagent concentration, and environmental conditions in the medium including pH, ionic strength, temperature, polarity [13]. Thus, it is necessary to improve the technique to compensate for these defaults.

Here we develop a RLS ratiometry considering that ratiometry is a good method to solve the problems posed by single-wavelength measurement because the ratiometry could provide precise data by taking the intensity ratio at two suit-

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able wavelengths [14–17]. For example, fluorescent ratiometry has been commonly utilized in sensing physiological pH, oxygen and metal ion in cells [17–21]. We find that the RLS ratiometry could differentiate the binding difference of two cation porphyrins, *meso*-tetrakis [(trimethylammoniumyl) phenyl] porphyrin (TAPP) and *meso*-tetra (4-methylpyridy) porphyrin (TMPyP-4), with biopolymer, heparin (HP), a kind of sugaramic polysaccharide, which has widely applied to prevent thrombosis of extracorporeal circuit and to mediate activation of the hemostatic system during surgery [22,23]. Based on charge density distribution of the two porphyrin molecules, we try to discuss the binding dependence on molecular structure. Such knowledge then makes it possible to do systematic structural modifications of the drug molecule to optimize the binding interaction.

2. Experimental

2.1. Materials and apparatus

Heparin (HP) solution was prepared by dissolving heparin sodium (160 IU mg^{-1}, Shanghai Chemical Reagent Plant, Shanghai, China) in water. HP working solution is 10 $\mu g\,ml^{-1}$ (about 6.7×10^{-7} mol l^{-1} with an average molecular weight of 15,000). Commercially available *meso*-tetrakis [(trimethylammoniumyl) phenyl] porphyrin (TAPP) and *meso*-tetra (4-methylpyridy) porphyrin (TMPyP-4) were purchased from Aldrich (St. Lewis, WO, USA), and their working solutions were 2.1×10^{-5} and 1.0×10^{-4} mol l^{-1}, respectively. Britton–Robinson buffer was used in this experiment to control pH value. All other reagents were of analytical-reagent grade without further purification or pretreatment. Millipore purified water was used throughout.

Absorption spectra were measured on a Techcomp 8500 UV–vis spectrophotometer (Hong Kong, China). RLS spectra were measured on a Hitachi F-2500 spectrofluorometer (Tokyo, Japan) by scanning simultaneously the excitation and emission monochromators of the spectrofluorometer from violet to visible region. Both the excitation and emission slits were set at 5.0 nm. An S-10A digital pH meter (Xiaoshan Scientific Instruments Company, Zhejiang, China) was used to measure the pH values. The charge distributions of porphyrins are calculated using the Hyperchem Pro 7.0 (Hypercube, USA) software in AM1 mode.

2.2. General procedure

A 1.0 ml of porphyrin solution, 1.0 ml of Britton–Robinson buffer and a serial of heparin solution were added to a 10 ml volumetric flask successively. The mixture was diluted to 10.0 ml with water and shaken thoroughly. RLS spectra were then measured on the F-2500 spectrofluorometer by simultaneously scanning the excitation and emission monochromators with same starting wavelength and same scanning velocity. The RLS ratios were obtained by dividing the RLS intensity at 442 nm with 418 nm for HP/TAPP system and that at 448 nm with 418 nm for HP/TMPyP-4 system.

3. Results and discussion

3.1. Wavelength for ratiometric measurement

Titrated with HP, the molecular absorptions of both TAPP and TMPyP-4 undergo hypochromism without significant shift of maximum wavelength, simultaneously resulting in enhanced RLS signals characterized at 437 and 452 nm maximum and at 412 and 416 nm minimum, respectively (Fig. 1). The enhanced RLS signals result from the aggregations in an absorption medium when the excitation wavelength is close to the absorption band, and could be given as following equation [5,6]:

$$I(90) = \frac{16\pi^2 a^6 n_{\mathrm{med}}^4 I_0}{r^2 \lambda_0^4} \left| \frac{m^2 - 1}{m^2 + 2} \right| \quad m = \frac{(n_{\mathrm{real}} + i n_{\mathrm{im}})}{n_{\mathrm{med}}} \qquad (1)$$

Wherein I (90) is the light scattering intensity detected at the right angle to the incident light beam, a is the size of the aggregation species, n_{med} is the refractive index of the medium, I_0 is the intensity of the incident light beam, r is the distance between the aggregation species and the detector, λ_0 is the wavelength of the incident light beam, and m is a complex index involving in the molecular absorption and the medium environments. There always is a wavelength (λ) which is close to λ_0 that makes the denominator $m^2 + 2$ in Eq. (1) equals zero, and leads up to strong enhanced values of I (90).

It is well known that visible colors and absorption spectra of chromophores are the consequence of incident light through absorption and scattering [17], and enhanced RLS signals are strongly dependent on the absorption of aggregates [5,6]. Thus, it is necessary to simultaneously consider the molecular absorption and light scattering features in order to choose appropriate wavelengths for the light scattering ratiometric measurements. Our strategy is selecting the wavelengths at the peak and the valley of UV–vis spectral ratio curve, which could be obtained by dividing UV–vis spectrum of the chromophoric component with that in the presence of the additives. Experiments showed that $\lambda_{\max}$ and $\lambda_{\min}$ in UV–vis ratio spectra are 418 and 442 nm for HP/TAPP system, and 418 and 448 nm for HP/TMPyP-4 system, respectively (Fig. 2). The wavelength of $\lambda_{\max}$ 418 nm in HP/TAPP system corresponds to the 412 nm minimum region of RLS

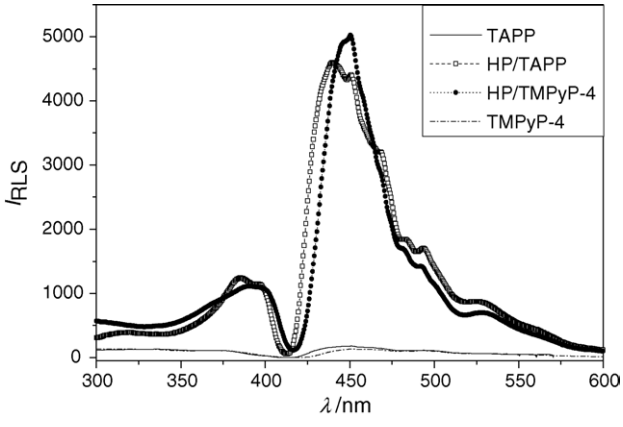

Fig. 1. Enhanced RLS signals of TAPP and TMPyP-4 by HP. c_{TAPP}, 4.2×10^{-6} mol l^{-1}; $c_{\mathrm{TMPyP\text{-}4}}$, 1.0×10^{-5} mol l^{-1}; HP, 1.5 mg ml^{-1}; pH, 7.2.

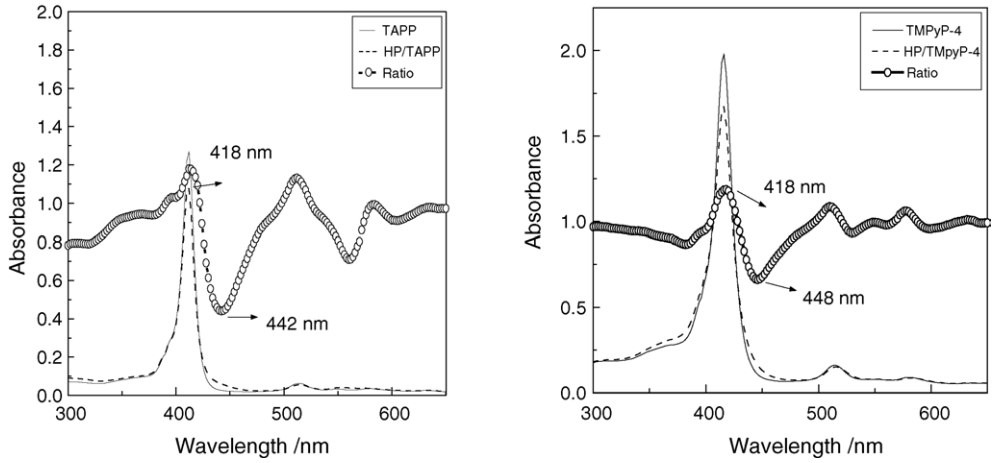

Fig. 2. Absorption spectra of TAPP, TMPyP-4 and their complexes with heparin. Dotted line with open circles represents the ratio of the absorption spectra of porphyrin to that of heparin–porphyrin complex. Concentrations: TAPP, 2.1×10^{-6} mol l^{-1}; TMPyP-4, 1.0×10^{-5} mol l^{-1}; Heparin, 0.2 μg ml^{-1}; pH, 7.2.

spectrum, while that of λ_{min} 442 nm does to the 437 nm peak in RLS spectrum (Fig. 1). Similar phenomenon could be found for HP/TMPyP-4 system. From those features, it could confirm that the λ_{max} and λ_{min} in UV–vis ratio curve are of paramount importance related to RLS signals. Thus we choose the RLS ratio of I (λ_{min})/I (λ_{max}) for RLS ratiometric measurements.

3.2. Performance of heparin detection

It was found that RLS ratios decrease exponentially with HP concentration and have a perfect linear relationship with the logarithm of HP concentration in a wide range (Fig. 3). This constitutes the law to determine the binding extent between HP and porphyrins. By controlling the pH of the solution, different detection concentration ranges may be obtained (Fig. 4). At pH 1.8, the best detection range is from 0.01 to 3.2 μg ml^{-1}. At pH 7.0 and 10.0, the detection range changes from 0.02 to 2.2 μg ml^{-1}, respectively. In lower pH values, the higher acidic medium will lead to the protonation of nitrogen atom of TAPP molecule and TAPP form H_2TAPP^{2+} [7], which magnifies the binding capability of TAPP to HP. As shown in the inset plot of Fig. 4, the UV–vis spectrum of TAPP in pH 1.8 occurs to a red

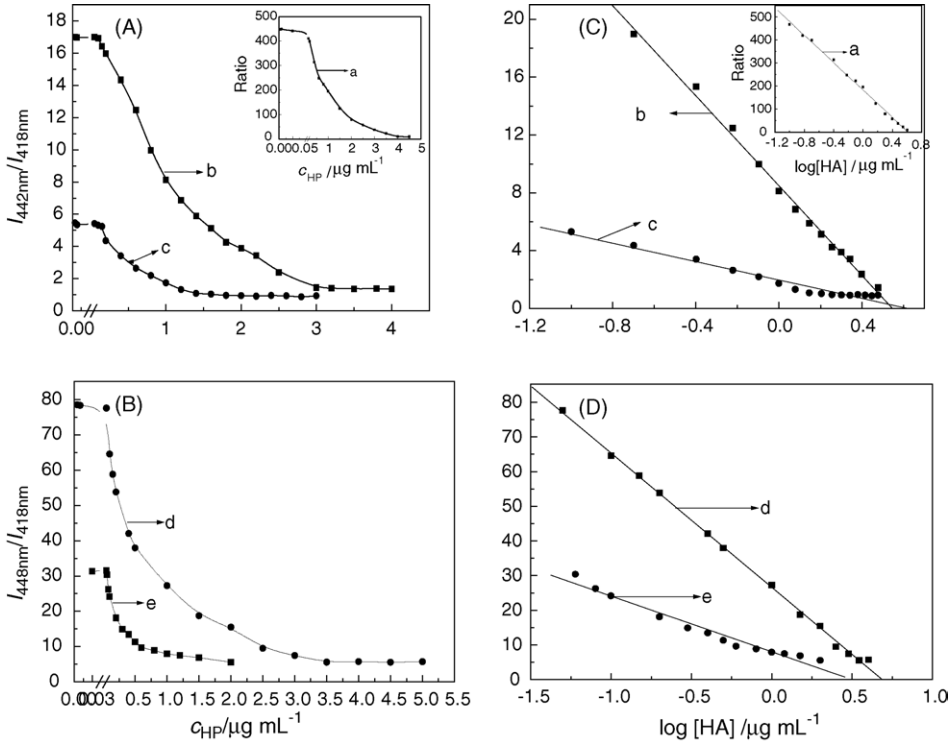

Fig. 3. Dependence of RLS ratios vs. HP concentration (A, B) and log[HP] (C, D) at various concentrations of TAPP (a, b and c in A and C) and TMPyP-4 (d and e in B and D). Concentrations: TAPP in curves a, b and c, 4.2, 2.1 and 1.0×10^{-6} mol l^{-1}, respectively; TMPyP-4 in curves d and e, 1.0 and 0.8×10^{-5} mol l^{-1}; pH, 7.2.

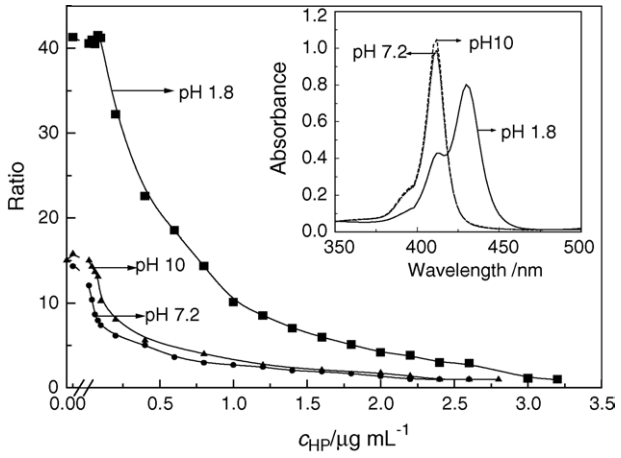

Fig. 4. Effects of pH on the dynamic ranges of HP/TAPP complex response to HP. Concentrations: TAPP, 2.1×10^{-6} mol l^{-1}; heparin, 1.0 μg ml^{-1}. Inset plots shows absorption spectra of TAPP in different pH-values.

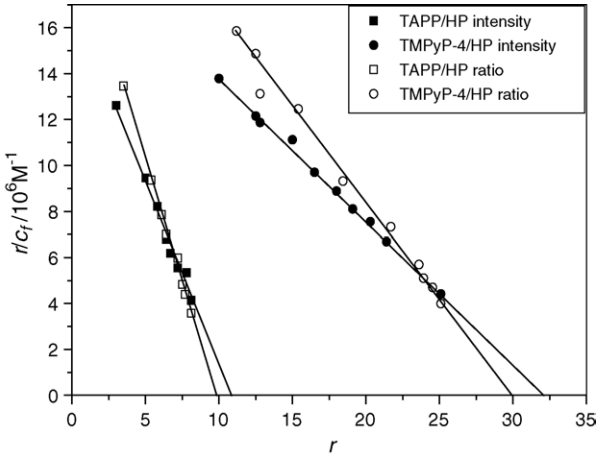

Fig. 5. Scatchard plots for the binding of TAPP and TMPyP-4 with heparin. Scatchard equations for HP/TAPP and HP/TMPyP-4 systems calculated by RLS intensity are $r/c_f = 1.6 \times 10^6 (11.7 - r)$ (solid square) and $r/c_f = 6.2 \times 10^5 (32.3 - r)$ (solid circle), while those calculated by RLS ratio are $r/c_f = 2.2 \times 10^6 (9.7 - r)$ (open square) and $r/c_f = 8.5 \times 10^5 (29.8 - r)$ (open circle). Titration of 2.67×10^{-7} mol l^{-1} HP solutions was made with 0.20 ml aliquots of 5.0×10^{-6} mol l^{-1} TAPP solution and aliquots of 1.0×10^{-4} mol l^{-1} TMPyP-4 solution, respectively; pH, 7.2.

Table 1
Results for the detection of heparin in the clinic injection solution by RLS ratiometric method

Sample number	Heparin specified (IU/2 ml)	Average (IU/2 ml) ($n = 5$)	R.S.D. (%) ($n = 5$)
011201[a]	12500	12712	1.73
010723[b]	12500	12657	1.45
020815[c]	12500	12469	1.54

Concentration: TAPP, 4.2×10^{-6} mol l^{-1}; pH, 7.2.
[a] Xuzhou Wanbang Biochemical Pharmaceutical Factory of China.
[b] Changzhou Qianhong Biochemical Pharmaceutical Co. Ltd.
[c] Shanghai Biochemical Pharmaceutical Factory of China.

shift and hypochromism and has a shoulder in Soret band. This feature indicates that a change occurs in the form of TAPP, which is the reason why the detection range in pH 1.8 is magnified.

The effects of ionic strength on RLS ratio and RLS intensity were investigated in the range of 0.003–0.203 mol l^{-1}. When the ionic strength gets increased from 0.003 to 0.113 mol l^{-1}, the RLS ratios for HP/TAPP system remain consistent, and the ratios get increased beyond this range with increasing ionic strength. On the contrary, the RLS intensity of the system only remains constant when the ionic strength is lower than 0.053 mol l^{-1}, and then decreases with increasing ionic strength. The same phenomenon was occurred in the HP/TMpyP-4 system. The constant and increasing RLS ratios with further ionic strength indicates that the ratiometric method has higher tolerance ability than RLS method has. On the other hand, the effect of ionic strength just on the complex means that the binding models of heparin and porphyrins are mainly ascribed to the electrostatic attraction since HP is highly negatively charged and the increasing Na^+ will compete with porphyrin to bind with HP [24,25].

To prove RLS ratiometric method feasible, clinic heparin sodium injections of three different trademarks were detected by the RLS ratiometric method. The clinic injections were just diluted without other pretreatments. The experimental results are listed in Table 1. The recoveries are in agreement with the results given by the manufactories at 95% confidence level. The R.S.D. for the three samples ($n = 5$) is between 1.45 and 1.73%.

3.3. Mechanism of the interactions

Both TAPP and TMPyP-4 have the tendency to form stacking-type aggregation on biopolymer templates such as DNA [6,25]. Heparin is a highly negatively charged oligosaccharide with an average molecular weight of about 15,000 and an average charge of −70 [22]. The high negative charge of HP leads to strong repellence among the disaccharide units, and make it exist as a linear anionic polyelectrolyte [22]. In addition, one HP molecule contains 42 monosaccharide units

Table 2
Average binding number of heparin with porphyrin calculated by RLS and RLS ratiometric method

Porphyrin	$c_{porphyrin}$ (10^{-6} mol l^{-1})	RLS method		RLS ratiometric method	
		Linear range ($\times 10^{-7}$ mol l^{-1})	n	Linear range ($\times 10^{-7}$ mol l^{-1})	n
TAPP	4.2	0–3.33	14	0–4.0	10.5
	2.1	0–1.66	12.6	0–2.14	9.8
TMPyP-4	10	0–2.0	50	0–3.0	33.3
	8	0–2.1	48	0–2.42	33.1

n is the maximum binding number of heparin to porphyrin; pH, 7.2.

and each tetrasaccharide unit has three *O*-sulfate groups, two *N*-sulfate groups, and two carboxyl groups [22], thus the total binding capacity per HP molecule is 73.5 in theory. Therefore, one HP molecule could theoretically bind with 18.4 porphyrin molecules in average through electrostatic attraction if taking the charges of one porphyrin molecule as +4.

Using mole ratio variation method, the binding number (*n*) of TAPP to per HP molecule could be roughly calculated as 13 and that of TMPyP-4 to HP as 49, respectively (Table 2). Using RLS ratiometric method, however, the *n*-values of TAPP and TMPyP-4 to HP are 10 and 33, respectively. Scatchard analysis [26] could further prove the binding difference between HP and porphyrins. When using RLS radiometric data for the Scatchard analysis (Fig. 5), the binding constants of TAPP and TMPyP-4 with HP are $2.2 \times 10^6\ M^{-1}$ and $8.5 \times 10^5\ M^{-1}$, respectively, with a binding affinity difference factor of 2.6, and the maximum *n*-values of TAPP and TMPyP-4 binding to per HP molecule are 10 and 30, respectively, which are much identical to the reports of Refs. [27], demonstrating that RLS ratiometry is much reliable. The *n*-value of 30 for TMPyP-4 binding with one HP molecule is much higher than the theoretical binding capacity of 18.4, indicating that self-aggregation of TMPyP-4 induced by HP occurs,

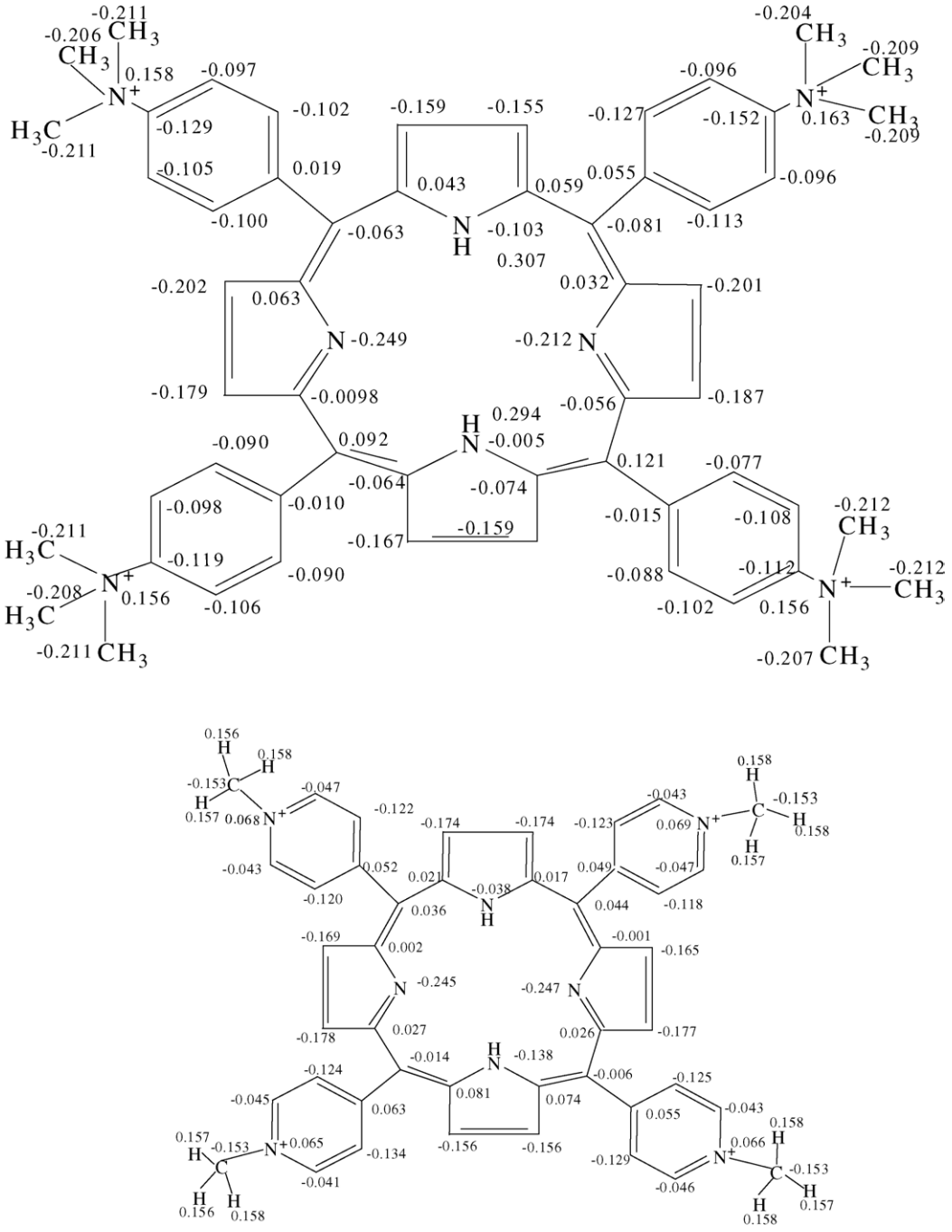

Fig. 6. Charge distribution of TAPP (top) and TMPyP-4 (bottom) calculated by the Hyperchem Pro 7.0 software in AM1 method.

and TMPyP-4 is highly stacked along the HP molecule. Thus, it is reasonable to deduce that hydrophobic interaction between TMPyP-4 molecules could exist in the presence of HP.

As improved above, HP–porphyrin involves electrostatic attraction and hydrophobic affinity. When TAPP and TMPyP-4 of same concentration were mixed with heparin, the RLS intensity of HP/TAPP was much greater than that of HP/TMPyP-4. Therefore, TAPP has higher affinity than TMPyP-4 toward HP. This difference can be referred to the less net charge of TMPyP-4 than that of TAPP. Though two porphyrins have same cationic charge of four ammonium ions, the cationic charge of ammonium ions in TMpyP-4 is greatly lessened by the conjugative effect of pyridine. To enforce the conclusion, the charge distributions of TAPP and TMPyP-4 were calculated in AM1 mode using Hyperchem Pro 7.0 software (Fig. 6). The results show that the net charge of nitrogen atom in ammonium ions in TAPP molecule is +0.158 in average, while that in ammonium ions in TMPyP-4 molecule is +0.067 in average since the charge of ammonium ions in TMPyP-4 is greatly lessened by the conjugative effect of pyridine. The 2.4-fold net charge difference between the nitrogen atoms in ammonium ions of the two porphyrins is much close to the binding affinity difference factor of 2.6. Thus, it could deduce that the charge difference mainly decides the electrostatic attraction and hydrophobic affinity, which decides the size, shape of the HP–porphyrin complexes, and the HP–porphyrin complexes display enhanced RLS spectra.

Based on the measurements of photon correlation spectroscopy, the dynamic diameter of TAPP gets increased from 730 to 1075 nm in the range of 0.4–1.2 $\times 10^{-5}$ M, and that of TMPyP-4 is smaller than 3 nm. Experiments have shown that TAPP is easier to induce a superhelical structure of DNA than TMPyP-4 does in aqueous medium due to its aggregation tendency [7], thus the values of TAPP diameters in Table 3 are perhaps referred to the molecular associates rather than single molecules, while the small diameters of TMPyP-4 samples and their weak scattering are the single-molecular signatures. Therefore, their bindings with HP then result in larger dynamic diameter, for example, with an increasing factor of 1.3 for TAPP (Table 3). The dynamic diameters get increased with porphyrin concentrations, indicating that aggregation of porphyrin induced by HP really occurs. These data prove that the enhanced light scattering signals, which greatly dependent on the size and numbers of aggregate particles in medium [5,6], could differentiate the binding difference of porphyrins with biopolymers.

Table 3
Dynamic diameters of porphyrin and HP–porphyrin

$c_{porphyrin}$ (10^{-5} M)	TAPP (nm)	HP/TAPP (nm)	HP/TMPyP-4 (nm)
0.4	729	917	298
0.6	829	1034	356
0.8	968	1324	468
1.0	1068	1416	580
1.2	1075	1420	1003

HP, 1.0 μg ml^{-1}; pH, 7.20. Detection angle is 90.0° for TAPP and HP/TAPP, while that is 30° for TMPyP-4 and HP/TMPyP-4 due to their weak scattering signals at 90.0°. The dynamic diameters of TMPyP-4 in different concentration are lower than 3 nm, the lower limit of detection range of N5 Submicon particle size analyzer (Beckman Coulter, Miami).

4. Conclusion

Herein, we developed a RLS ratiometric method to study the drug-biopolymer binding. The newly assay method can circumvent the interferences from exoteric environment associated with single-intensity measurement. The method can provide more precise measurement with a larger linear range of analysis, which is a good dynamic range for biological affinity aggregation. From the measurement of binding number *n* and investigation of reaction mechanism, it can conclude that the RLS ratiometric method is a new tool of dynamics to measure the extent of association reactions. The mechanism of the interacting system, heparin and porphyrins, demonstrated that the electrostatic attraction and hydrophobic affinity plays a dominant role in HP–porphyrin interaction and the enhancement of RLS intensity is proportionally promoted by the charge capacity of components in complex.

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