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Molecular Spectroscopic Study on the Interaction between Heparin and Neutral Red

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Abstract: The interaction between heparin and neutral red was investigated by molecular spectroscopic methods. The change of all spectra suggested that positively charged neutral red had interacted with negatively charged heparin. The study of influence factors indicated that electrostatic force and hydrophobic bond might be involved in the interaction. The total binding number per disaccharide unit and intrinsic binding constant were obtained using Scatchard model.

Keywords: Spectroscopic, interaction, heparin, neutral red.

Heparin (Hep) is one of the members in glycosaminoglycans (GAGs) family, and serves as anticoagulant, antithrombus, and antiatherosclerosis in clinic 1,2. This biopolymer consists of linear, negatively charged sugar chains with repetitive disaccharide units. As a cationic dye, neutral red (NR) has been reported to aggregate on nucleic acids 3,4.

Though the binding of dye molecules to GAGs has received more and more attention in recent years, the mechanism of the interaction is still obscure. Further theoretical study on the reaction of GAGs and spectroscopic probes is of great importance, not only to improve the analytical methods of GAGs, but also to help understand the interaction of GAGs and organic micromolecules in organisms. In this paper, the interaction between neutral red and heparin was investigated.

Experimental

A Hitachi UV-3010 spectrophotometer (Tokyo) was used for recording absorption spectra (UV-Vis) and measuring the absorbance, a Hitachi FL-4500 spectrofluorometer (Tokyo) for recording the resonance light scattering spectra (RRS) and measuring the intensity at a given wavelength (PMT voltage, 400V; Slit (EX / EM), 5.0 nm / 5.0 nm). Circular dichroism (CD) spectra were measured at 20 ℃ using CD-6 (JOBIN-YUON) spectropolarimeter equipped with a quartz cell having a path length of 1 cm. A digital 821 model (Zhongshan University, China) pH meter for pH adjustment.

Heparin (Sodium Salt, Jingke Com., Beijing) was used without further purification. The stock solution was prepared by dissolving the reagents in doubly deionized water and

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kept in the refrigerator at 4°C. In order to eliminate the influence of the polydispersity in molecular weight, one mole of Hep was defined as the mass of one repeating disaccharide unit of Hep (wt. 665). Neutral red (biological reagent) was obtained from Beijing (China) and the aqueous solution was prepared by dissolving the dye in doubly deionized water and kept in the dark. Britton-Robinson buffer was used to adjust the acidity of the solution. All other reagents used were of analytical reagent grade and without further purification, and doubly deionized water was used throughout.

A certain volume of buffer and NR solutions were transferred into each test tube, and Hep solutions were added in different amounts. The mixture was diluted to the scale with doubly deionized water. Then spectra or absorbance or intensity were measured.

The RRS spectra were recorded by synchronous scanning at $\lambda_{ex} = \lambda_{em}$ (**Figure 2**). The scattering intensity was very low for both the dye and Hep. When NR was mixed with Hep, there were two RRS peaks, appearing at 330 nm and 614 nm. The enhancement of intensity should be ascribed to the enhancement of coplanarity and rigidity, as well as the aggregation of the chromophores of the dye on Hep[2].

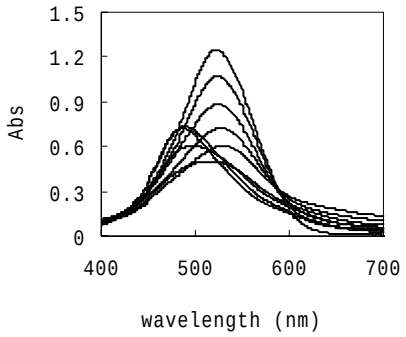
The CD spectra were also recorded (**Figure 3**) to investigate the conformational change of Hep after interaction with NR. No signal was obtained for NR and Hep,

Results and Discussion

Spectral characteristics

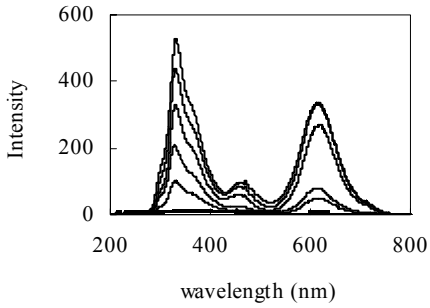
UV-Vis, RRS and CD spectra were investigated to characterize the interaction between NR and Hep. As indicated in **Figure 1**, NR itself has an absorption band at 522 nm. As Hep was added to NR solution, the absorbance of this band decreased gradually. At the same time, a new band appeared at 488 nm. The absorption spectral change suggested that there was an interaction occurring between NR and Hep.

Figure 1 The absorption spectra



Concentration of Hep (from top to bottom at 664 nm): 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 1.8, 2.0, 2.5×10^{-5} mol / L, NR: 5.0×10^{-5} mol / L pH 3.0

Figure 2 The RRS spectra

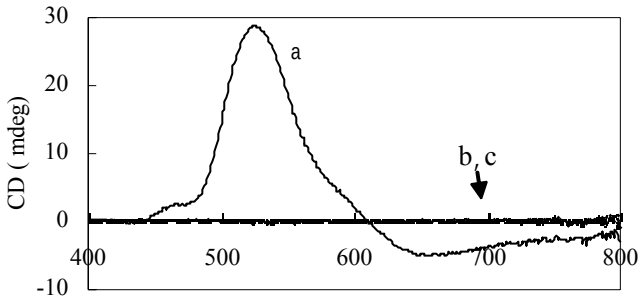


NR (1.0×10^{-4} mol / L), Hep (1.0×10^{-5} mol / L), and the mixture (from top to bottom Hep) 12.5, 10.0, 7.5, 5.0, 2.5×10^{-6} mol / L pH 4.25

respectively. However, the intense CD signal at 525 nm was induced when NR was mixed with Hep, which suggested a long-range organization of dye molecules. The conformation of GAGs was induced to change from random coil form to helical one by binding to NR. It was suggested that this long-range assembly involved a helical arrangement of the achiral dye molecules on the helical backbone of GAGs, which led to the chirality of NR aggregates and induced strong CD signal of NR[1].

The spectral observations suggested that positively charged NR have interacted with negatively charged Hep through aggregation of NR on Hep molecular surface, and primarily, electrostatic force might be the driving force.

Figure 3 The CD spectra of NR (5.0×10^{-5} mol / L), Hep (5.0×10^{-6} mol / L) and their mixture (from bottom to top) at pH 3.0



a: Spectrum of mixed NR and Hep b, c: Spectrum of NR and Hep, respectively.

Factors affecting the interaction by RRS technique

The scattering intensity was affected greatly by pH and the maximum appeared at pH 4.25. Also the scattering intensity at 330 nm and 614 nm was found to decrease greatly upon increase of ionic strength, suggesting that the extent of aggregation was reduced as ionic strength was increased to compete with the interaction[5]. Similar results were obtained upon addition of ethanol to the system. The scattering intensity at 330 nm and 614 nm decreased gradually. In this case, the addition of ethanol might affect the hydrophobic bond between dye molecules, which led to the change of spectra[6].

Binding stoichiometry at different pH

NR had a pKa value about 6.7. In the pH range studied, NR would be all positively charged and the number of charge is not likely to change with pH. There were five sulfate groups and two carboxyl groups per tetrasaccharide unit in heparin. The binding stoichiometry was expected to change with pH. At pH 2.0, the proton of carboxyl group was not dissociated and – COOH was not likely to interact with NR. The sulfate groups might be the only source of charge on Hep at this stage. With pH value at higher than that the pKa of –COOH group, both sulfate and carboxyl groups might participate in the interaction with NR. In this study, we attempted to apply Scatchard equation[7] into

investigating the interaction of dye with GAGs to verify the relation of binding number with pH.

Defining [L] and [L′] as the total concentration of free and bound NR, C_L as the analytical concentration of NR,

$$C_L = [L] + [L'] \quad (1)$$

The absorbance of the NR – Hep mixtures was:

$$A = A_L + A_{L'} = \varepsilon[L] + \varepsilon'[L'] = \varepsilon C_L + (\varepsilon' - \varepsilon)[L'] \quad (2)$$

$$[L'] = (\varepsilon C_L - A) / (\varepsilon - \varepsilon'); [L] = (A - \varepsilon' C_L) / (\varepsilon - \varepsilon') \quad (3; 4)$$

where ε and ε' stood for molar absorptivities of free and bound dye, respectively, which were constants when pH and measuring wavelength were fixed.

Let: $\Delta\varepsilon = \varepsilon - \varepsilon'$,

$$[L'] = (\varepsilon C_L - A) / \Delta\varepsilon; [L] = (A - \varepsilon' C_L) / \Delta\varepsilon \quad (5; 6)$$

Defining C_x as the concentration of Hep added, n and N as average and maximum number of binding sites per disaccharide unit, respectively, K as the intrinsic binding constant. At the very beginning of the reaction, there was only free NR molecules existing in the solution; when the interaction was over, all the dye molecules have transferred into bound species, so at this time,

$$A_{max} = \varepsilon C_L, \quad A_{min} = \varepsilon' C_L; [L'] = (A_{max} - A) / \Delta\varepsilon \quad (7; 8)$$

$$[L] = (A - A_{min}) / \Delta\varepsilon; n = [L'] / C_x = (A_{max} - A) / \Delta\varepsilon C_x \quad (9; 10)$$

$$n / [L] = (A_{max} - A) / (A - A_{min}) C_x \quad (11)$$

As Scatchard equation illustrated,

$$n / [L] = NK - Kn \quad (12)$$

The plot of (n / [L]) vs. n yielded a linear line, and K, N could be obtained from the slope (-K) and intercept (NK) of the regression line (Listed in **Table 1**). As expected, Scatchard model gave the results that the binding number at pH 2.0 was less than that at pH 5.0, and binding ratio was close to the charge ratio of Hep at these two pH values. All our results suggested that Scatchard model was suitable for this kind of study.

Table 1 The influence of pH on the parameters at 522 nm by UV-Vis method

pH	N[a]	K[b] ($\times 10^5$)	R^{2c}
2.0	3.59	5.04	0.986
5.0	5.34	3.24	0.906

[a] N represents total number of binding sites per disaccharide unit;
[b] K represents the intrinsic binding constant; [c] R^2 represents regression coefficient

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