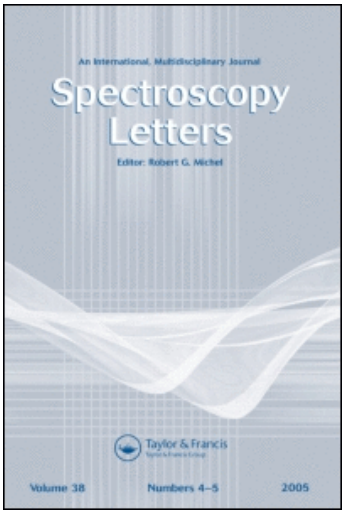

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THE INTERACTION OF POLYSACCHARIDES WITH A SPECTROSCOPIC PROBE: THE ANION EFFECT ON THE BINDING SITE OF HEPARIN

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THE INTERACTION OF POLYSACCHARIDES WITH A SPECTROSCOPIC PROBE: THE ANION EFFECT ON THE BINDING SITE OF HEPARIN

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ABSTRACT

Heparin (Hep) has a variety of biological activities, most of them due to Hep's high anionic groups. The average binding number (n) is proposed to express the density of anion's group (binding site) on Hep. To gain insight into the binding site of Hep under the interference of anions, we have developed a method that makes use of isosbestic points in the reaction system in which two reagents compete for the same dye ion, and one complex is colorless. It is also necessary to determine the molar absorptivities of the various species concerned.

Key Words: Anion; Binding site; Heparin; Azure A

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INTRODUCTION

A number of physiological effects have been ascribed to heparin (Hep) since its discovery almost 80 years ago, many of which are independent from its first-described and best-characterized activity as an anticoagulant. Hep is believed to possess many biological activities that include the ability to modulate embryonic development, neurite outgrowth, tissue homeostasis, wound healing, metastasis, cell differentiation, and cell proliferation and inflammation (1–9). The efficacy of Hep correlates with the gross sulfate content of the compound, reflecting a requirement for a level of sulfation not directly dependent upon the molecular weight or the anticoagulant activity. The binding sites of Hep were successfully investigated by the theoretical model proposed by Jiao and Liu (10), but this method could be interfered by anions (10–13). The present work is undertaken in an attempt to clarify the general principles of interference involved in Hep-spectroscopic probe interaction. For these purposes, Hep, cationic dye, and sodium chloride (NaCl) were selected as a relatively simple system to study and one of special importance because the natural environment of the Hep contains much NaCl.

We calculate from the experimental measurements, with the aid of simplifying assumptions, the average number of azure A molecules bound to the Hep molecule in solutions of varied composition of NaCl. We propose a new approach to characteristics the isosbestic points found as the system compositions vary.

MATERIALS AND METHODS

A Kontron Uvikon 860 spectrophotometer (Kontron, Switzerland) is used for recording absorption spectra, or measuring the absorbance at a given wavelength, using a 1-cm path length. A pH-HJ90B model portable acidity meter (Beijing Hangtian Computer, China) is used for the pH measurements.

Hep (structure shown in Fig. 1) and sodium salt (≥160 IU/mg) are obtained from Shanghai Reagent, China and used without further purification. The average molecular mass of commercial Hep preparations is 12,000 Da (14). The aqueous Hep solution (5.21×10^{-5} mol/L) is prepared by dissolving 50 mg Hep reagent in 80 mL deionized water. From this stock solution of Hep 2 mL is pipetted into

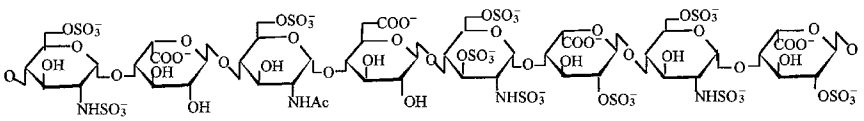

Figure 1. Structure of Hep octasaccharide sequence (15).

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100 mL volumetric flask, and then diluted with water to the mark. These stock solutions are stable for several weeks when kept in the dark at 4°C.

The azure A is purchased from Shanghai Reagent, China. The azure A stock solution (3.43×10^{-3} mol/L) is prepared by dissolving 0.5 g dye in 500 mL deionized water. The operating solution of azure A is prepared by diluting 5 mL stock solution with water, to get the total quantity of 30 mL. Dye operating solution should be used soon after preparation, although little difficulty is encountered with dye solutions that are several hours old.

All other reagents are of analytical or guaranteed reagent grade.

Azure A operating solution is transferred into a series of 12×100 mm test tubes, then Hep solution, and aliquots of NaCl are added in different amounts to each test tube. The mixtures are diluted to a certain volume with water and mixed either by inversion or vortexing. After 15 min and before 1 h, spectra or absorbances of these solutions are measured with reference to water. After 24 h a precipitate is not observed. Since the Hep–azure A complex has a tendency to bind to cuvettes, there is a decrease in absorbance after it stands for long in cuvettes. As a precaution the absorbance of samples should be read 1–5 min after the azure A–Hep mixtures are pipetted into cuvettes. This still gives ample time to read samples. The binding of the Hep–azure A complex has been observed only with quartz cuvettes and may be eliminated by using plastic cuvettes.

All runs are thermostated at room temperature and are performed in triplicate.

RESULTS AND DISCUSSION

A study using the combination of Hep with a cationic dye, ion has recently been described. A previous investigation of the Hep with cationic dye in solutions of NaCl showed that NaCl bound with cationic dye, gives a colorless reaction, but there were interferences in the Hep–dye assay, causing an underestimatation of the Hep concentration (11–13). However, no systematic investigation on the binding site of Hep under the interference of anions has been found in the literature. We, therefore, investigate the mechanism of activation of the interference.

Absorption Spectra of Hep-Dye Complex in the Salts Solution

Figure 2 shows the absorption spectrum of azure A–Hep system in the NaCl solution. It is obtained by keeping the pH, azure A and Hep concentrations constant, and changing the NaCl concentration. With the increase in NaCl concentration, the absorption peak at 620 nm increases strikingly, while the absorption peak at 510 nm decreases. One well defined isosbestic point is formed at 550 nm.

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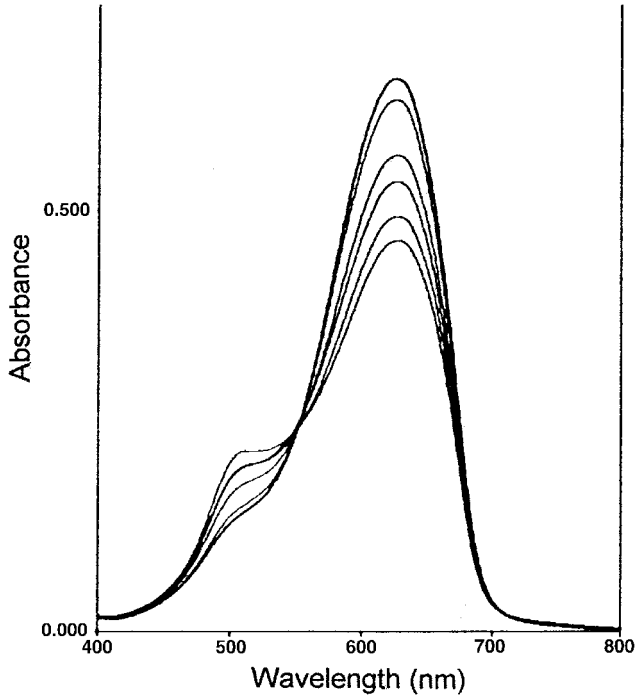

Figure 2. Absorption spectra of azure A–Hep complex in the salts solution: $D_T = 4.76 \times 10^{-5}$ mol/L, pH = 8.05, $C_H = 1.74 \times 10^{-7}$ mol/L. In order of decreasing peak absorbance at 510 nm, sodium chloride usage (C_{NaCl}) are a) 0; b) 1.14×10^{-2}; c) 2.28×10^{-2}; d) 3.42×10^{-2}; e) 4.56×10^{-2}; f) 5.70×10^{-2} mol/L in the total assay volume.

Figure 3 shows the curves of Hep–azure A complexes under a series of concentrations of NaCl aqueous solution. The concentrations of Hep and azure A are kept constant, the amount of azure A–Hep complex is dependent on the concentration of NaCl, when the concentration of NaCl salt is increased, the complex dissociates (16).

In view of the molecular structure of azure A, NaCl, and Hep, it is not possible to reach a conclusion that Hep and NaCl with a particular group combines preferentially with azure A to form a complex. A reasonable explanation of these molecular events is that azure A interacts with Hep and NaCl by nonspecific, electrostatic forces. Owing to the presence of the sulfate groups, the whole Hep molecules are negatively charged under the conditions of Figure 2. However, the azure A species, D^+, has a positive charge. Hep has more negative charge than NaCl, it takes priority in binding to dye. While with an increase in NaCl concentration (if its concentration is greater than

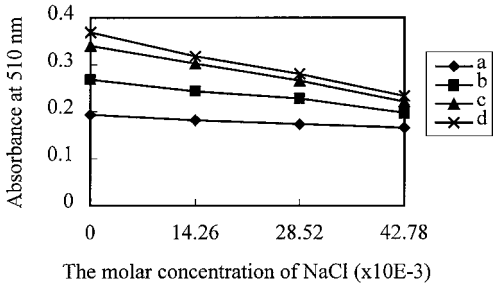

Figure 3. The effect of NaCl on Hep–dye complex: $D_T = 4.76 \times 10^{-5}$ mol/L, pH = 8.05, C_H are a) 8.68×10^{-8}; b) 17.36×10^{-8}; c) 26.03×10^{-8}; d) 34.72×10^{-8} mol/L in the total assay volume.

100 times that of the Hep (11,17)) will cause spectral changes as can be seen in Figure 2.

Figures 2 and 3 show that azure A combines with Hep in the presence of an inorganic salt. Suppose the reactions in which two reagents Hep and NaCl compete for the same cationic dye are as follows (omitting electrical charges):

$$\begin{aligned} S + D &\rightarrow DS \\ H &\rightarrow DH \end{aligned} \tag{1}$$

where D represents the azure A species, S refers to NaCl, H refers to Hep, DS represents NaCl–dye complex, and DH refers to the Hep–dye complex.

Molar Absorptivity Measurements

Suppose the reaction (D + H) is a colored reaction and the other reaction (D + S) is a colorless reaction in a competitive system. The absorption spectra of relevant species chosen in this study are shown in Figure 2. The absorbance of the solution is expressed as

$$A = \varepsilon_D[D] + \varepsilon_{DS}[D_S] + \varepsilon_{DH}[D_H] \tag{2}$$

where ε_D, ε_{DS} and ε_{DH} are the molar absorptivities of [D], [D_S], and [D_H], respectively. Where [D] is the total concentration of free dye, [D_S] is the total concentration of dye bound NaCl, and [D_H] is the total concentration of dye bound Hep, and D_T is the analytical concentration of dye.

$$D_T = [D] + [D_S] + [D_H] \tag{3}$$

In order to calculate the average binding number, the molar ε_D, ε_{DS} and ε_{DH} are required. Experiments are undertaken to determine these quantities. Figure 4

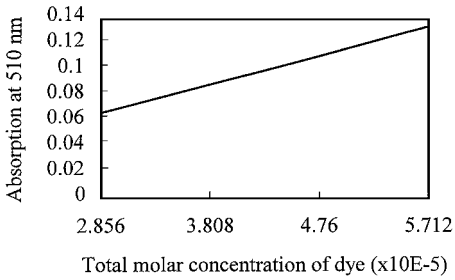

Figure 4. Beer's law plot for the free dye under the usual Hep assay conditions. Molar absorptivity from the slope equals 2.34×10^3 L mol^{-1}.

shows the measurement of the free dye absorptivity as the slope of a Beer's law plot.

Experiments are carried out to determine the molar absorptivity of the bound dye for NaCl. The results are shown in Table 1.

From Table 1 we can get a conclusion that the molar absorptivity of dye–NaCl is equal to that of free dye. For the dye bound NaCl, the dye activates NaCl by forming "1:1" binding complex (18), dye molecules could not come close enough to aggregate. Under such conditions, π-electrons (including chromophore and auxochrome) of the dye cannot interact with each other, so that a hypochromism and then a hypsochromism could not occur. So NaCl binding dye gives no extra color reaction, and the molar absorptivity of NaCl–dye complex is the same as that of free dye at the same wavelength measured.

$$\varepsilon_{DS} = \varepsilon_D \tag{4}$$

Experiments are also carried out to determine the molar absorptivity of the bound dye for Hep. These measurements are performed out under conditions of high Hep to dye ratios. Where this ratio is sufficiently large, all or almost all dye molecules will be bound to Hep, and the absorbance at 510 nm will therefore be

Table 1. The Molar Absorptivity of Sodium Chloride–Dye

	Absorbance at 510 nm (mol/L)				
C_{NaCl} (mol/L)	D_T: 2.86×10^{-5}	3.81×10^{-5}	4.76×10^{-5}	5.71×10^{-5} (mol/L)	ε (L mol^{-1})
0	0.067	0.089	0.112	0.134	2.34×10^3
14.26×10^{-3}	0.065	0.088	0.111	0.134	2.32×10^3
28.52×10^{-3}	0.067	0.089	0.112	0.133	2.33×10^3
42.78×10^{-3}	0.067	0.088	0.111	0.134	2.33×10^3

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completely due to Hep–dye complex. For Hep in less abundant supply, and for those cases where the amount of free dye is not extremely low, an extrapolation to infinite Hep concentration is carried out to find the maximum absorbance for a known small amount of dye, from which the molar absorptivity is calculated as A_{max}/D_T.

Explanation of the Formation of the Isosbestic Point

In the conditions of Figure 2, however, there are three color species in solution. The existing theory (19) cannot give an explanation in this case. In the following discussion, an attempt will be made to give an interpretation for the formation of the isosbestic point in Figure 2.

Substituting Equations (3) and (4) into Equation (2) yields

$$A = \varepsilon_D([D] + [D_S]) + \varepsilon_{DH}[D_H] = \varepsilon_D D_T + (\varepsilon_{DH} - \varepsilon_D)[D_H] \quad (5)$$

where $\varepsilon_D D_T$ is the absorbance of the dye blank solution. Figure 2 shows that the maximum values of ε_D and ε_{DH} are at 620 and 510 nm, respectively. We could deduce that there should be a wavelength between 510 and 620 nm, where ε_D equals ε_{DH}. As we can see from Figure 2, this particular wavelength is 550 nm. It is at this wavelength that $\varepsilon_D = \varepsilon_{DH}$, Equation (5) becomes $A = \varepsilon_D D_T$, which is a fixed value, unchanging with increase of salt's concentration, so the isosbestic point appears.

The above discussion gives an explanation to the appearance of the isosbestic point in Figure 2. Furthermore, based on the above discussion one model for the description of the average number of azure A binding Hep under the interference of NaCl could be developed, as follows.

The Average Binding Number

According to $n_B = [D_B]/C_H$ proposed by Atherton (20), D_B refers to bound dye. However, this model does not seem appropriate for describing the average binding number of azure A with Hep in the salt solution considered here, because azure A binds not only to Hep here, but also to NaCl. D_B will increase with NaCl concentration, accompanied by n_B elevated (10). The results are shown in Figure 5.

Rearranging Equation (5) yields:

$$[D_H] = A - \varepsilon_D D_T/(\varepsilon_{DH} - \varepsilon_D) \quad (6)$$

If ε_{DH} and ε_D are determined previously from the absorbance of solutions with an excess of Hep and without Hep and NaCl, In fact, the molar absorptivity difference $\varepsilon_{DH} - \varepsilon_D$ ($\Delta\varepsilon$) is a known quantity. Where A is the absorbance of dye–Hep mixtures

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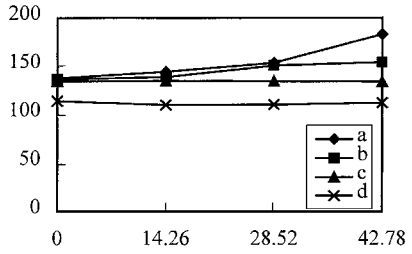

Figure 5. Effect of the NaCl concentration on n_B. $D_T = 4.76 \times 10^{-5}$ mol/L, pH = 8.05, C_H are a) 8.68×10^{-8}; b) 17.36×10^{-8}; c) 26.03×10^{-8}; d) 34.72×10^{-8} mol/L in the total assay volume.

in salt solution, and $\varepsilon_D D_T$ the absorbance of a zero-Hep solution. Both A and $\varepsilon_D D_T$ can be measured.

In the present work, an attempt is made to calculate the average binding number of azure A with Hep in salt solution. Define the average binding number of dye molecules per Hep molecule as:

$$n = [D_H]/C_H \tag{7}$$

where C_H represents the analytical concentration of Hep. This equation is derived on the assumption that all dye-binding sites are independent and identical.

A set of experiments are done, keeping the azure A concentration constant and increasing the NaCl and Hep concentrations. The results are shown in Table 2 and Figure 6, that an increase in salt concentration causes a significant decrease in n values, thus decreasing the sensitivity of the azure A–Hep binding assay (11,13). This effect may be explained as a competition between anion and Hep for the

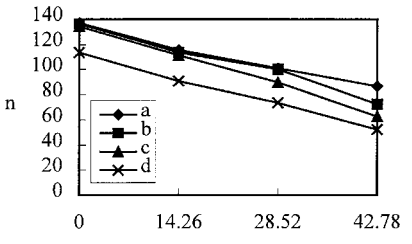

Figure 6. Effect of the concentration of NaCl on $n \cdot D_T = 4.76 \times 10^{-5}$ mol/L, pH = 8.05, C_H are a) 8.68×10^{-8}; b) 17.36×10^{-8}; c) 26.03×10^{-8}; d) 34.72×10^{-8} mol/L in the total assay volume.

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Table 2. Effect of NaCl Concentration on the Binding Site of Hep, pH = 8.05, $D_T = 4.76 \times 10^{-5}$ mol/L, $\Delta\varepsilon = 6.39 \times 10^3$ L/mol^{-1} at 510 nm

C_{NaCl} (mol/L)	C_H (mol/L)	A	$[D_H]$	n
0	8.68×10^{-8}	0.193	11.89×10^{-6}	136.98
	17.36×10^{-8}	0.268	23.63×10^{-6}	136.12
	26.03×10^{-8}	0.340	34.72×10^{-6}	134.08
	34.72×10^{-8}	0.369	39.44×10^{-6}	113.59
14.26×10^{-3}	8.68×10^{-8}	0.181	10.02×10^{-6}	115.44
	17.36×10^{-8}	0.243	19.72×10^{-6}	113.59
	26.03×10^{-8}	0.302	28.95×10^{-6}	111.22
	34.72×10^{-8}	0.318	31.46×10^{-6}	90.61
28.52×10^{-3}	8.68×10^{-8}	0.173	8.76×10^{-6}	100.92
	17.36×10^{-8}	0.228	17.37×10^{-6}	100.05
	26.03×10^{-8}	0.266	23.32×10^{-6}	89.59
	34.72×10^{-8}	0.280	25.51×10^{-6}	73.47
42.78×10^{-3}	8.68×10^{-8}	0.165	7.51×10^{-6}	86.52
	17.36×10^{-8}	0.197	12.52×10^{-6}	72.12
	26.03×10^{-8}	0.221	16.28×10^{-6}	62.54
	34.72×10^{-8}	0.233	18.15×10^{-6}	52.28

same binding sites on dye species. In this case the concentrations of the anions are 100–1,000 fold higher than that of the dye species (11,13), so the cationic dye species are actually surrounded by anions that prevent the azure A species from binding to Hep, thus decreasing assay response to Hep.

If the Hep molecule is saturated with dye, n becomes equal to N, the total number of binding sites. Most of Hep physiology is dependent on the gross number of anionic groups. The binding site (the density of anionic groups), n is decreased with NaCl according to Table 2. Because of the complex environment in biology, the physiology of Hep is interfered by many agents, so we set up a method to investigate the Hep–small ion interaction in salt's solution, which could partially show the real interaction of Hep–small ion or molecules in biology.

The experimental approach used here in probing the binding mechanism has been specifically to define the binding of Hep with dye in the salt environment. We hope that this more mechanistic understanding of the anion system will allow prediction of other possible interferences and promote a more effective use of the assay.

REFERENCES

1. Witt, D.P.; Lander, A.D. Curr. Biol. **1994**, *4*, 394.
2. Yoshida, T.; Toyama-Sorimachi, N.; Mivasaka, M.; Lee, Y.C. Biochem. Biophys. Res. Commum. **1994**, *204*, 969.

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3. Silvestro, L.; Viano, L.; Macorio, M.; Colangelo, D.; Montrucchio, G.; Panico, S.; Fantozzi, R. Semin. Thromb. Haemostasis **1994**, *20*, 254.
4. Shute, J. Clin. Exp. Allergy, **1994**, *24*, 203.
5. Matzner, Y.; Mary, G.; Drexler, R.; Eldor, A. Thromb. Haemostasis **1984**, *52*, 134.
6. Bazzoni, G. J. Lab. Clin. Med. **1993**, *121*, 268.
7. Cerletti, C.; Rajtar, G.; Marchi, E.; de Gaetano, G. Semin. Thromb. Haemostasis **1994**, *20*, 245.
8. Norgard-Sumnicht, K.E.; Varki, N.M.; Varki, A. Science **1993**, *261*, 480.
9. Tangelder, G.J.; Arfors, K.-E. Blood **1991**, *77*, 1565.
10. Jiao, Q.C.; Liu, Q.; Sun, C.; He, H. Talanta **1999**, *48*, 1095.
11. Jiao, Q.C.; Liu, Q. Anal. Lett. **1998**, *31*, 1311.
12. Jiao, Q.C.; Liu, Q. Spectro. Lett. **1998**, *31*, 1353.
13. Liu, Q.; Jiao, Q.C. Spectro. Lett. **1998**, *31*, 913.
14. Malsch, R.; Guerrini, G.; Torri, G.; Lohr, G.; Casu, B.; Harenberg, J. Anal. Biochem. **1994**, *217*, 255.
15. Lindahl, U.; Lidholt, K.; Spiltmann, D.; Kjellen, L. Thromb. Res. **1994**, *75*, 1.
16. Jiao, Q.C.; Liu, Q. Spectrochimica Acta **1999**, *55*, 1667.
17. Jaques, L.B.; Sandra, M.W.; Hiebert, L.M. J. Lab. Clin. Med. **1990**, *115*, 422.
18. Yutaka, T.; Hatsuo, A.; Naomichi, O. Thromb. Res. **1989**, *55*, 329.
19. McBryde, W.A.E. Talanta **1974**, *21*, 979.
20. Atherton, B.A.; Cunningham, E.L.; Splittgerber, A.G. Anal. Biochem. **1996**, *233*, 160.

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