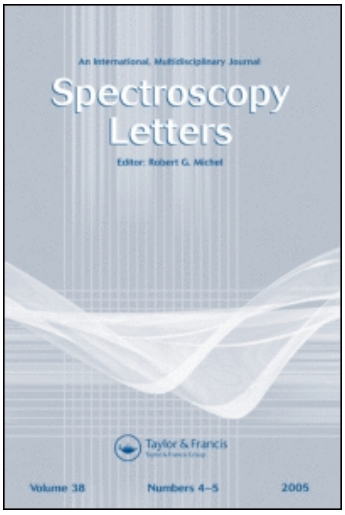

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A Mathematical Model for Interaction of Spectroscopic Probe with Polysaccharides

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A MATHEMATICAL MODEL FOR INTERACTION OF SPECTROSCOPIC PROBE WITH POLYSACCHARIDES

—The binding interaction between azure A and heparin

Key words: Heparin, Azure A, Interaction.

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ABSTRACT

Heparin has a high anionic charge density and is known to interact with a large number of cationic dye through electrostatic interaction. To gain insight into the mechanism of interaction of the dye and heparin, we studied the cationic dye–azure A by a spectrophotometric method. The research was based on the fact that the absorption spectra of the free and bound dye are different. It is considered that the combination of heparin with azure A is

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due to the noncovalent binding forces. The Scatchard model is discussed from a new standpoint. The solution equilibrium of the reaction system was studied. A new linear regression equation has been developed. From this the binding ability of azure A on heparin can be estimated from the equilibrium constant K, and the maximum binding number N. The linear regression equation can determine these two parameters. The factors which influence the sensitivity of a dye binding heparin assay are discussed, and the Sandell index is used to express the sensitivity of heparin detection. It is found that the concentration of the azure A, sodium chloride solutions have significant effect on the interaction of heparin and azure A.

INTRODUCTION

Dye binding heparin assays are commonly used in biochemical and clinical laboratories, primarily due to their high sensitivity and convenience [1-4], but they are not well understood at the molecular level. A further theoretical study on this kind of reaction should be beneficial not only with regard to the quantitation of heparin but also to a deep understanding of the interaction between heparin and small ions or molecules.

Spectroscopic probe has been widely used to inquire into the interaction of proteins or nucleic acids with small substances or molecules[5-8]. However, no systematic investigation on the interaction between polysaccharide and spectroscopic probe has been found in the literature. The present work is undertaken in an attempt to clarify the general principles involved in polysaccharides–small substances interaction. Heparin[9,10] is a heterogeneous mixture of oligosaccharides of varying length and sulfation levels. Azure A[1,2] has been used as a staining reagent in the determination of heparin. It will be shown that the azure A–heparin binding reaction can be successfully treated by using a new method proposed in this paper.

EXPERIMENTAL

A Kontron Uvikon 860 spectrophotometer (Kontron Company, Swiss) was 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 company, China) was used for the pH measurements.

Heparin, sodium salt, $\geq$ 160 IU/mg, was obtained from Shanghai, China and used without further purification. All calculations reported by heparin are in terms of a molecular weight of 12,000 Da[11]. The aqueous heparin solution (5.21×10^{-5} mol/L) was prepared by dissolving 0.05 g heparin reagent in 80 mL deionized water. This stock solution of heparin was pipetted 2 mL into 100 mL volumetric flask, and then diluted to the mark with water. This stock solution was stable for several weeks when kept in the dark at 4°C.

The azure A (Structure of azure A is shown in figure 1) was purchased from Shanghai, China. The azure A stock solution (3.43×10^{-3} mol/L) was prepared by dissolving 0.5 g dye in 500 mL deionized water. The operating solution of azure A was prepared by diluting 5 mL stock solution with water into 30 mL.

All other reagents were of analytical or guaranteed reagent grade.

Azure A operating solution was transferred into a series of 12x100mm test tubes, then heparin solution, aliquots of NaCl solution were added to each test tube in different amounts. The mixtures were diluted to a certain volume with water and mixed either by inversion or vortexing. After 15 min and before 1 hr, spectra or absorbances of these solutions were measured with reference to water. All runs were thermostated at room temperature, and were performed in triplicate.

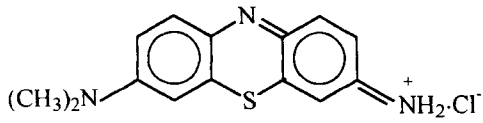

Fig. 1. Structure of azure A dye.

RESULTS AND DISCUSSION

Dye–heparin spectra

Figure 2 shows the absorption spectra of azure A dye and azure A–heparin complexes from 400 to 800 nm. They are obtained by keeping the pH, azure A concentration constant, and changing the heparin concentration. With the increase in heparin concentration, the absorption peak at 620 nm decreases, while a new absorption peak at 510 nm appears. This peak, attributable to the heparin–dye complex, is apparently different from the absorption peak of the dye because the wavelengths corresponding to both absorbance maximum are different. A well–defined isobestic point is observed at 550 nm. Figure 2 indicates that there are interactions between azure A and heparin[7,8].

In view of the molecular structure of azure A, and heparin, it is not possible to reach a conclusion that azure A combines perferentially with a particular group on heparin to form a complex. A reasonable explanation of these molecular events is that azure A interacts with heparin by non-specific, electrostatic forces. Owing to the presence of the sulfate, carboxyl groups, the whole heparin molecules are negatively charged under the conditions of figure 2. However, the azure A species, D^+, have positively charges. Therefore, heparin, azure A species should be bound together by

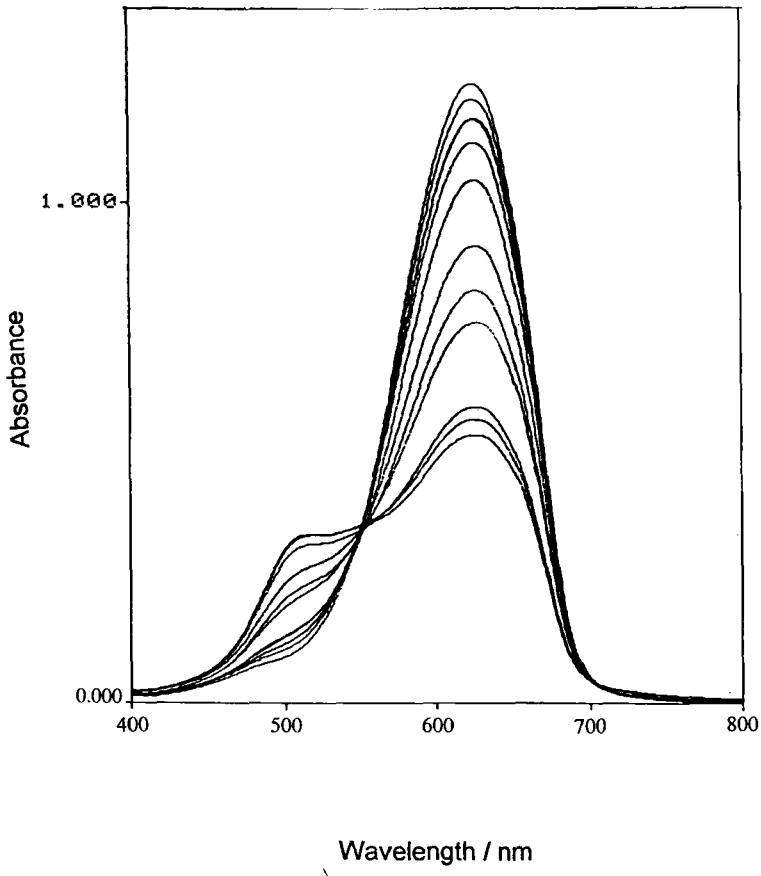

Fig. 2. Absorption spectra of azure A–heparin mixtures. Azure A operating solution constants at 4.76×10^{-5} mol/L. pH = 8.05. In order of increasing peak absorbances at 510 nm, heparin usage are: 0.0, 1.74, 3.47, 5.21, 6.94, 13.89, 15.62, 19.09, 24.30, 26.03, 27.78 $\times 10^{-8}$ mol/L in total assay volume.

electrostatic forces (In the text ion charges will be omitted for simplicity):

D + Heparin ---------- D' (1)

where D represents the azure A species. D' refers to heparin bound D. With an increase in heparin concentration will shift equilibrium (1) from the left to the right, causing spectral changes as can be seen in figure 2.

A new method for the treatment of the dye binding reaction

Define D_F as the concentration of free azure A, D_B as the concentration of bound azure A, and D_T as the total analytical concentration of azure A.

$$D_T = D_F + D_B \quad (2)$$

The absorbance at 510 nm is attributed to the fact that both the dye and the dye–heparin complex. This situation can be analyzed according to Beer's law, as follows:

$$A = \varepsilon_F D_F + \varepsilon_B D_B = \varepsilon_F D_T + (\varepsilon_B - \varepsilon_F) D_B \quad (3)$$

where ε_F, is the molar absorptivity of free dye, ε_B is that of the bound dye.

Rearranging Eq. (3) yields

$$D_B = (A - \varepsilon_F D_T) / (\varepsilon_B - \varepsilon_F) \quad (4)$$

where A is the absorbance of dye–heparin mixtures, and $\varepsilon_F D_T$ the absorbance of a zero-heparin solution. Both A and $\varepsilon_F D_T$ can be measured.

Let

$$\Delta A = A - \varepsilon_F D_T \quad (5)$$

$$\Delta\varepsilon = \varepsilon_B - \varepsilon_F \quad (6)$$

then Eq. (4) may be simplified as

$$D_B = \Delta A / \Delta\varepsilon \quad (7)$$

If ε_B and ε_F are determined previously from the absorbance of solutions with an excess of heparin and without heparin, then $\Delta\varepsilon$ is a known quantity.

Define the average binding number of dye molecules per heparin molecule as

$$n = D_B / C_H \quad (8)$$

where C_H represents the analytical concentration of heparin.

Determination of the equilibrium constant and the maximum binding number

According to Eq. (1), the equilibrium constant of the total binding reaction should be

$$K = D_B / [Hep] D_F \quad (9)$$

where [Hep] represents the concentration of unoccupied binding sites on heparin

$$[Hep] = N C_H - D_B = (N - n) C_H \quad (10)$$

where C_H is the analytical concentration of heparin, N the total number of binding sites per heparin molecule, n the average binding number of dye molecules per heparin molecule.

$$K = n C_H / D_F (N - n) C_H \quad (11)$$

then

$$n = K N D_F / (1 + K D_F) \quad (12)$$

Substituting Eq. (8) and Eq. (12) into Eq. (7) gives

$$\Delta A/\Delta\varepsilon = C_H K N (D_T - D_B) / [1+K (D_T - D_B)] \quad (13)$$

Rearranging this equation yields

$$\Delta A = \Delta\varepsilon(1 + KD_T)/K - \Delta\varepsilon N(D_T\Delta\varepsilon/\Delta A - 1) C_H \quad (14)$$

where $\Delta\varepsilon N$ is a constant, and $\Delta\varepsilon(1 + KD_T)/K$ has a fixed value at given azure A concentration D_T. In fact, $D_T\Delta\varepsilon$ is the maximum value of ΔA, which can be measured directly at high heparin / dye concentration ratios.

$$\Delta A_{max} = \Delta\varepsilon D_T \quad (15)$$

There is a linear relationship between ΔA and $(\Delta\varepsilon D_T/\Delta A-1)C_H$ according to Eq. (14). From the slope $\Delta\varepsilon N$ and intercept $\Delta\varepsilon(1 + KD_T)/K$ of the regression line, K and N can be calculated.

Calculated Eqs. (2 ~ 14) yields:

Table 1 gives a group of data. Using these data, a $\Delta A \sim (\Delta\varepsilon D_T/\Delta A - 1)C_H$ regression equation is obtained:

$\Delta A = 0.316 - 9.19\times10^5 (\Delta\varepsilon D_T/\Delta A - 1) C_H \qquad R = -0.999$

From the slopes and intercepts of this equations, $K = 5.16\times10^5$, $N = 143.81$.

The data of figure 3 are obtained from Table 1.

Test of scatchard model

In the Scatchard model, the binding equation is given by[12]

$$n / D_F = K N - K n \qquad (16)$$

where K is the intrinsic binding constant.

Using the data listed in Table 1, a plot of n/D_F vs. n is drawn, and it is found that the plot conforms to Eq. (16).

$n / D_F = 6.36\times10^7 - 4.30\times10^5 n \qquad R = -0.9588$

Therefore the Scatchard model is suitable for this study.

Influence of experimental conditions on dye binding reaction

The Sandell index (S, $\mu g\ cm^{-2}$)[13], which represents the number of micrograms of the determinant per millilitre of a solution having an absorbance of 0.001 for the path length 1 cm, is a suitable parameter for expressing the sensitivities of heparin–dye assay. The relationship between the Sandell index and the above three parameters is

$$S = F / N (\varepsilon' - \varepsilon) = F / N \Delta\varepsilon \qquad (17)$$

where F is the average molecular weight of the heparin.

TABLE 1. Data used for linear regressions. pH = 8.05, $D_T = 4.76 \times 10^{-5}$ mol/L, $\Delta\varepsilon = 6.39 \times 10^3$ L/mol, $\Delta A_{max} = 0.304$, at 510 nm.

C_H (mol/L)	ΔA	n/D_F	n	$(\Delta\varepsilon D_T/\Delta A - 1)C_H$
3.47×10^{-8}	0.030	3.15×10^6	135.16	3.17×10^{-7}
6.94×10^{-8}	0.060	3.54×10^6	135.30	2.82×10^{-7}
10.42×10^{-8}	0.091	4.10×10^6	136.66	2.44×10^{-7}
13.89×10^{-8}	0.121	4.76×10^6	136.36	2.10×10^{-7}
17.36×10^{-8}	0.151	5.68×10^6	136.12	1.76×10^{-7}
20.83×10^{-8}	0.180	6.96×10^6	135.24	1.44×10^{-7}
24.30×10^{-8}	0.209	9.04×10^6	134.61	1.11×10^{-7}
27.78×10^{-8}	0.225	10.23×10^6	126.75	0.98×10^{-7}
31.25×10^{-8}	0.240	11.97×10^6	120.19	0.83×10^{-7}
34.72×10^{-8}	0.252	13.92×10^6	113.59	0.72×10^{-7}
38.19×10^{-8}	0.261	15.85×10^6	106.97	0.63×10^{-7}
41.66×10^{-8}	0.267	17.23×10^6	100.29	0.58×10^{-7}
45.14×10^{-8}	0.273	19.39×10^6	94.64	0.52×10^{-7}
48.61×10^{-8}	0.281	24.92×10^6	90.45	0.40×10^{-7}
52.08×10^{-8}	0.287	32.06×10^6	86.23	0.31×10^{-7}

It is shown in Table 2 that an increase in salt concentration causes a significant decrease in K and $\Delta\varepsilon$ values, and increase S values, thus decreasing the sensitivity of the azure A–heparin assay. This effect may be explained as a competition between anion and heparin for the same binding sites on dye species. Even when no salt is added to the solutions, the concentration of anions coming from the addition of buffer is still much higher than that of the dye species. Azure A species have positively charges. Therefore, heparin, azure A species and NaCl should be bound together by electrostatic forces. The N is evaluated with the salt's concentration increase, and most likely due to NaCl bound to dye.

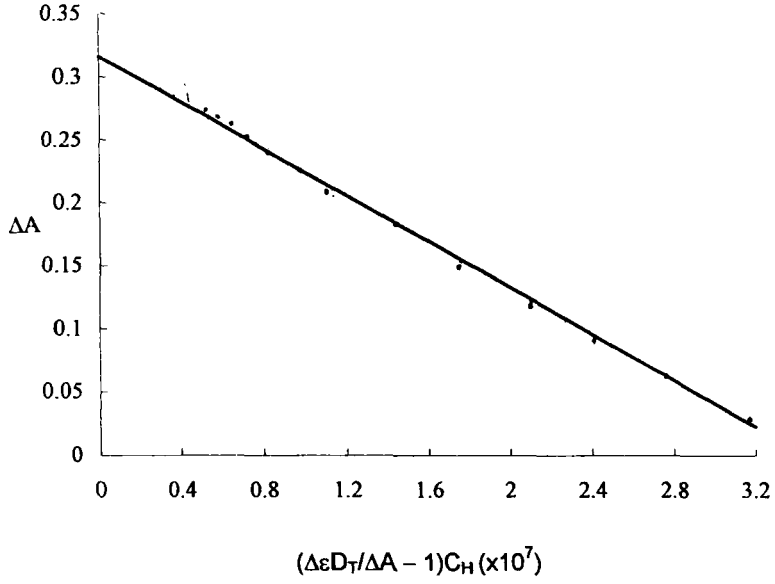

Fig. 3. Regression lines of $\Delta A \sim (\Delta\varepsilon D_T/\Delta A - 1)C_H$

TABLE 2. Effect of NaCl concentration on binding of azure A on heparin. pH = 8.05, $D_T = 4.76 \times 10^{-5}$ mol/L, at 510 nm

NaCl(mol/L)	Δε	N	K	S	R
0	6.39×10^3	143.81	5.16×10^5	0.0130	-0.999
14.26×10^{-3}	5.36×10^3	156.65	3.24×10^5	0.0143	-0.997
28.52×10^{-3}	4.28×10^3	174.99	2.22×10^5	0.0160	-0.997
42.78×10^{-3}	2.97×10^3	234.98	1.02×10^5	0.0171	-0.997

TABLE 3. Effect of azure A concentration on binding of azure A on heparin pH = 8.05, at 510 nm

D_T	$\Delta\varepsilon$	N	K	S	R
28.56×10^{-6}	6.34×10^{3}	143.24	4.04×10^{5}	0.0132	-0.985
38.08×10^{-6}	6.38×10^{3}	143.05	4.50×10^{5}	0.0131	-0.998
47.60×10^{-6}	6.39×10^{3}	143.81	5.16×10^{5}	0.0130	-0.999
57.12×10^{-6}	6.36×10^{3}	149.34	7.31×10^{5}	0.0126	-0.999

The influence of azure A concentration on the binding reaction is shown in Table 3. From this test we know that an increase in azure A concentration causes an increase in K, while $\Delta\varepsilon$ and N remain practically constant. *S* is slightly decreased with increase dye concentration. However, the dye concentration cannot be too high because of the restriction of spectral measurement.

Heparin has three O-sulfate groups, two N-sulfate groups, and two carboxyl groups per tetrasaccharide unit. The O-sulfate and N-sulfate groups are completely dissociated, even below pH 3.0. In this region, therefore, azure A binds only to the O-sulfate and N-sulfate groups. But the carboxyl group is weakly acidic, and the pKa of D-glucuronic acid in heparin is 3.6. On increasing the pH above 3.0, the carboxyl groups gradually dissociate and combine with dye, and reaching a constant level above pH 5.0, where dissociation of the carboxyl groups is complete. Accordingly, the carboxyl group dissociates at high pH values, so heparin is subjected to bind to dye over a wider pH range[14]. So pH has not significant effect on the interaction of heparin and azure A (among pH 4.5 ~ 10). It has been experimented by assay (Data not shown).

CONCLUSIONS

It seems that this method is useful and convenient for the investigation of small substance–heparin interaction, because the parameters defined in this paper can be determined easily.

To use the method, the experiment could be carried out under any conditions. That is to say, the dye / heparin concentration ratios could not effect this $\Delta A \sim (\Delta\varepsilon D_T/\Delta A - 1)C_H$ linear regression equation.

Experimental conditions such as ion strength, and concentration of dye have different effects on the maximum binding number, the equilibrium constant, the molar absorptivities of free and bound dye, thereby influencing the sensitivity of a heparin assay.

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