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BINDING INTERACTION BETWEEN CHONDROITIN SULFATE AND METHYLENE BLUE BY SPECTROPHOTOMETRY

S. G. Zhou, Q. C. Jiao,* L. Chen, and Q. Liu

Pharmaceutic Biotechnology Key Laboratory,
College of Life Science, Nanjing University, 210093
Nanjing, People's Republic of China

ABSTRACT

Chondroitin sulfate (CS) has a variety of biological activities, most of them due to chondroitin sulfate's sulfonic acid groups and carboxyl groups as well. To gain insight into the mechanism of interaction between the spectroscopic probe and chondroitin sulfate, we have used Methylene Blue (MB), one cationic dye, by a spectrophotometric method. This paper developed a new experimental method for determining the maximum binding number Ne, which expresses the binding ability of this dye with CS. Meanwhile, By using an interaction theory model we have established, the maximum binding number Nc can be calculated from the linear regression equation. The research results show that the Ne value is in agreement with that of Nc.

*Corresponding author. E-mail: jiaoqc@jlonline.com

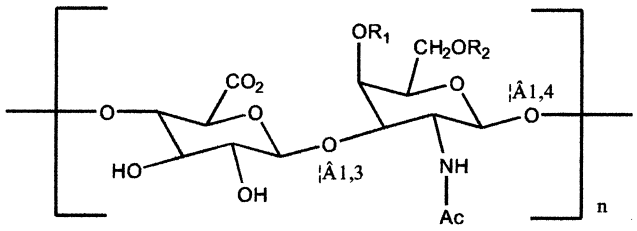
Key Words: Chondroitin sulfate; Methylene Blue; Maximum binding number; Spectroscopic probe

INTRODUCTION

Chondroitin sulfate (CS), one of the main components of connective tissues of mammals, belonging to glycosaminoglycan (GAG) family, is widely distributed among gristle, sinew, ligament, blood vessel wall and so on. There are different types of CS such as CS-A, CS-B, CS-C due to their differences in glycuronic acid of which they are made up and in the location of sulfonic acid group on glycosamine. Generally, CS refers to the mixture of different CS.

CS has a variety of biological activities, including anti-coagulant, anti-tumor, anti-arthritis and anti-HIV activities etc[1–3]. CS is applied in treating atherosclerosis, angina, miocardial infarction, hyperlipemia and hypercholesterolemia because of its activity of bringing down blood lipids[4]. CS has prosperous prospects as an immune modulation agent in immunotherapy[3,4]. In addition, CS is also widely used as auxiliary materials in medicament and cosmetics[5,6]. Sulfonic acid group and/or carboxyl group play an important role in affecting biological activities of CS[4]. The structure of CS is shown in Fig. 1[7].

However, no systematic investigation on binding interaction between CS and spectroscopic probes has been found in the literature. Jiao et al.[8–11] suggested that such negatively charged polysaccharides may interact with cationic dyes like Azur A, Alcian Blue and Methylene Blue. It is inferred that cationic dyes interact with CS by electrostatic binding force[10]. A change



CS-A R_1=SO_3H, R_2=H **CS-C** R_1=H, R_2=SO_3H

Figure 1. Structure of chondroitin sulfate.

in spectrum of dyes is expected and the mechanism is thought to be a conformation change caused by the interaction between the positive charge of dye and the negative charges of CS. It can be used in studying the characterization of interaction between CS and spectroscopic probes by a spectrophotometric method.

In order to understand deeply the characterization of binding interaction between polysaccharides or GAGs and spectroscopic probes, and to establish a model to advance studies on these substances, we have been carrying out a serial of investigations. The cationic spectroscopic probe used in this paper is Methylene Blue(MB). Fig. 2 shows its chemical structure:

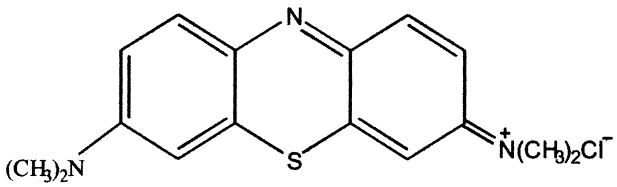


Figure 2. Structure of MB.

In this study, we describe the binding characterization between CS and MB by a spectrophotometric method, which is more rapid and simple than usual methods based on physicochemical properties of CS[12–13].

EXPERIMENTAL

Apparatus

A Hitachi U-3000 spectrophotometer (Tokyo, Japan) is used for recording absorption spectra. A Kontron Uvikon 860 is used for measuring the absorbance at a given wavelength, using 1cm path length.

Reagents

CS, sodium salt, was obtained from Shandong Freda Fine Chemicals Co. Ltd. Further purification was processed. CS contains $50 \sim 70$ repeating disaccharide units, and its molecular weight ranges from 10,000 to 30,000. So all calculations reported by CS were in terms of a molecular weight of 20,000. The CS stock solution (2.5×10^{-5} mol/L) was prepared by

dissolving 50.0 mg CS reagent in 100 mL deionised water. And the operating solution of CS (2.5×10^{-7} mol/L) was prepared by pipetting 1 mL stock solution into a 100 mL volumetric flask, and then diluting to the mark with water. This stock solution is stable for several weeks when kept in the dark at 4 °C.

The MB was purchased from Shanghai, China. The MB stock solution (1.34×10^{-3} mol/L) was prepared by dissolving 50.0 mg dye in 100 mL deionised water, and the operating solution (2.23×10^{-4} mol/L) of MB was prepared by diluting 5 mL stock solution with water into 30 mL. Due to light sensitivity of the dyes, the dye stock solution must be stored in the dark.

All other reagents used were of analytical or guaranteed reagent grade.

Methods

MB operating solution was transferred into a series of 12×100 mm test tubes, then CS operating solution was added to each test tube in different amounts. The mixtures were diluted to a certain volume of 6 mL with water and mixed by vortexing. After 5 min and before 2 hr, spectra or absorbances of these solutions were measured with reference to water. All runs were thermostated at 20 °C are performed in triplicate.

RESULTS AND DISCUSSION

MB-CS Spectra

Figure 3 shows the absorption spectra of MB and MB-CS complexes from 400 to 800 nm. MB operating solution constants at 1.86×10^{-5} mol/L, pH 8.0. CS concentrations are 0.0, 4.16, 8.33, 12.50, 16.67 and 20.83×10^{-8} mol/L from top to bottom at 664 nm. They were obtained by keeping the pH and dye concentration constant and changing the CS concentration. With an increase in CS concentration in the mixture of CS and MB, the absorption peak at 664 nm decrease, while a new absorption peak at 570 nm appears. This new peak, attributable to the MB-CS complexes, is apparently different from the absorption peak of MB because the wavelengths corresponding to both absorbance maxima are different. The decrease in absorbance at 664 nm and the increase in absorbance at 570 nm are in proportion to CS concentration. The results also show that assays at 664 nm are much more sensitive than those at 570 nm.

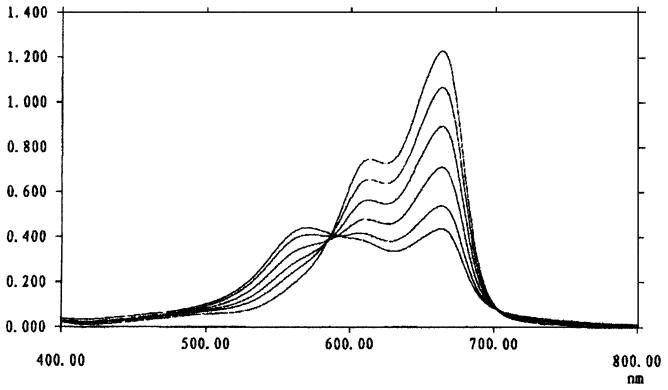


Figure 3. Absorption spectra of MB-CS mixtures.

Effect of Dye/CS Molar Ratio on Absorption Spectra

A certain amount of CS is transferred into a series of tubes, then MB is added in increasing amount. The mixtures are diluted to a total volume of 6 mL with water and mixed by vortexing. Absorption spectra of these solutions are measured with reference to water. Results are shown in Fig. 4.

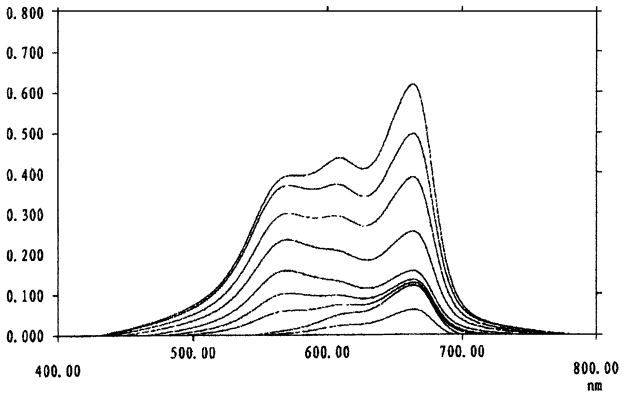


Figure 4. Absorption spectra of MB-CS complexes in the different MB concentrations.

MB usage are: 1.49, 2.97, 3.72, 5.58, 7.43, 11.15, 14.87, 18.58, 20.44×10^{-6} mol L^{-1} from bottom to top. To understand the experimental phenomenon in Fig. 4, we draw other graphs as shown in Fig. 5a and Fig. 5b. If C_D represents the dye concentration, and C_{CS} refers to CS concentration. Define the dye/CS molar ratio as:

$$\mathbf{m} = C_D/C_{CS} \tag{1}$$

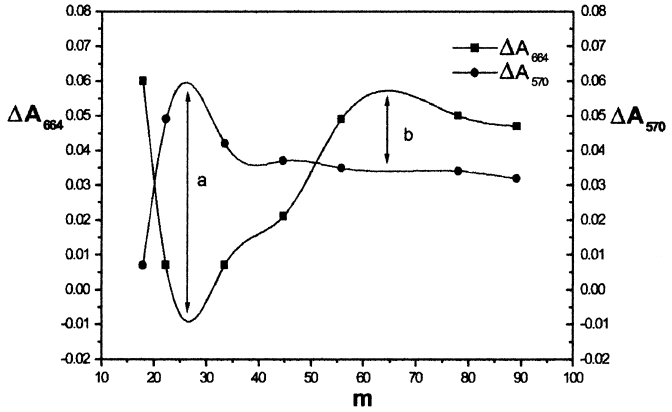


Figure 5a. The relation between m and ΔA_{570}, m and ΔA_{664}.

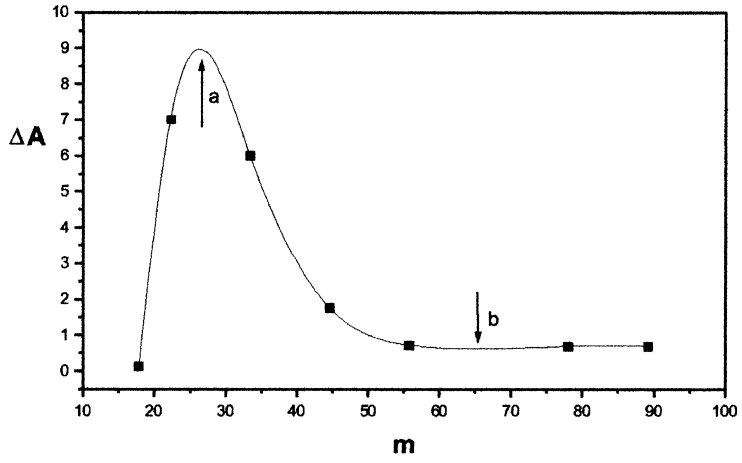


Figure 5b. The relation between m and A.

A_{570} is the absorbance of the MB-CS complex at 570 nm, and ΔA_{664} is the absorbance at 664 nm. ΔA_{570} is determined by the subtraction of two neighboring absorbances at 570 nm and ΔA_{664} is obtained the same way. Let

$$= \Delta A_{570}/\Delta A_{664} \tag{2}$$

The relation between **m** and ΔA_{570}, **m** and ΔA_{664}, **m** and β are presented in Fig. 5. The experimental phenomena in Fig. 4 can be explained in such a way. When **m** is low, the positively charged dye molecules are attracted by the negative charges of CS, but are sparsely spread on the long chain of CS molecule according to the principle of minimum energy. Hydrophobicity interaction between dye molecules is too weak to aggregate. Due to dye molecules are so far away from each other, therefore, the new absorption peaks at 570 nm is not generated.

The increases of absorbance at 570 and 664 nm are due to the increase of MB. With dye concentration increases, dye molecules bound on CS molecules become more and are close enough to aggregate to induce hypochromism and hypsochromism. So the absorption peaks at 570 nm appear. We conclude that whether hypochromism and hypsochromism will occur depends on not only the electrostatic interaction between dye molecules and CS but also the concentration of dye binding on CS.

Determination of Maximum Binding Number Ne

From Figs. 5a and 5b, we know that when **m** is less than 27(**a** point), β increases sharply with the rapid increase of ΔA_{570} and decrease of ΔA_{664}. This indicates that nearly all the dye molecules binds with CS. With m increasing, binding sites on CS are fewer, and only part of dye molecules added bind with CS, so the value of β decreases. When **m** is up to about 65(**b** point), β reaches the lowest point. This means no more interaction between CS and MB takes place because no binding sites on CS are left, so ΔA_{570} and ΔA_{664} remains constant. We conclude that the maximum binding number Ne of MB with CS is about 65.

Calculation of Maximum Binding Number Nc

One theoretical model for calculating the maximum binding number Nc of spectroscopic probes and polysaccharides was established by Jiao etc[9–11]. It is:

$$\Delta A = \Delta\varepsilon(1 + KD_T)K - \Delta\varepsilon N_c(D_T\Delta\varepsilon/\Delta A - 1)C_P \tag{3}$$

Table 1. Data from MB-CS Assay Used for Linear Regressions

C_{CS} (mol/L)	ΔA	$(D_T\Delta\varepsilon/\Delta A\text{-}1)C_{CS}$
6.25×10^{-7}	0.240	1.917
8.33×10^{-7}	0.312	1.773
10.42×10^{-7}	0.411	1.432
12.50×10^{-7}	0.498	1.200
14.58×10^{-7}	0.577	1.008
16.67×10^{-7}	0.683	0.715
18.75×10^{-7}	0.758	0.539
20.83×10^{-7}	0.803	0.449
22.92×10^{-7}	0.831	0.400

pH 8.0, $D_T = 1.86\times10^{-5}$ mol L^{-1}, $\Delta\varepsilon = 5.24\times10^{4}$ at 664 nm, $\Delta A_{max} = 0.976$.

$\Delta A = A - \varepsilon_F D_T$; $\Delta\varepsilon = \varepsilon_B - \varepsilon_F$; D_T is dye concentration, ε_F and ε_B refer to the molar absorptivity of free and bound dyes respectively. C_P is the concentration of polysaccharide, and K is a constant.

Absorbances of MB-CS complexes at 664 nm are used to calculate the maximum binding numbers of MB with CS because of its high sensitivity. Data are listed in Table 1.

From these data, using the linear regression equation, we obtain the regression equation that is:

$$\Delta A = 0.966 - 0.380\times10^{7}(D_T\Delta\varepsilon/\Delta A - 1)C_{CS}, \quad R = -0.998$$

The maximum binding number $Nc = 73$ is obtained.

The value of Nc we got by calculating accords with that of Ne obtained from Fig. 5.

CONCLUSIONS

This method is useful and convenient for investigation of binding interaction between CS and spectroscopic probes. The maximum binding number of MB with CS can be determined easily. The results got by experiment and by calculating are in well agreement. The linear regression equation is helpful in studying characterization of binding interaction between spectroscopic probes and GAGs.

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