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Analysis of the Interaction Mechanisms of Polysaccharide Homologs Binding with Serum Albumin Using Capillary Electrophorsis

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Summary: Different polysaccharide homologs (as the ligand) binding to serum albumin (bovine serum albumin (BSA)) as the receptor were investigated by capillary electrophoresis (CE). The Hummel–Dreyer method (HD), frontal analysis method(FA), and vacant peak method(VP) were used to measure the interaction parameters of the polysaccharide-BSA system. A workable CE method was selected by comparing different analytical methods and a feasible binding model was established by evaluating the different theoretical equations. The binding mechanism of polysaccharide-BSA was also explored under physiological conditions. The results showed that both HD and FA method can be used to analyze the polysaccharide-BSA system, with the most suitable CE method being the HD, and the double logarithmic equation gives the best fit to the data. The binding parameters indicate that the interaction between polysaccharides and BSA had moderate affinity, which had only one type of binding site. This work provides a reference for future studies of polysaccharide-protein interactions.

Keywords: Capillary electrophoresis, Polysaccharide, Bovine serum albumin, Hummel-Dreyer method, Frontal analysis method

Introduction

Polysaccharides are long chains of monosaccharides linked by glycosidic bonds. Three important polysaccharides, starch, cellulose, and chitin, are widely found in plants, animals, and microorganisms. In humans, polysaccharides play important functions in immune regulation, cell recognition, and anti-viral and anti-cancer activities. Thus, polysaccharides represent potential scaffolds to develop biological active drugs [1-3]. The biological effectiveness of polysaccharides is dependent on their transportation to targets in the body via the blood. Forming complexes with transport proteins is essential for polysaccharides to exert their biological activity. Polysaccharide-protein interactions meditate a diverse range of biological processes, including cell communication and trafficking, tumor genesis and progression, immune responses, fertilization, and infection [4-6]. Therefore, quantifying polysaccharide-protein interactions is essential for characterizing such processes, as well as facilitating polysaccharide drug development. However, polysaccharide binding with biologically active molecules (including proteins) does not involve a characteristic part of the polysaccharide acting as a signal, thus making it difficult to analyze the interactions by a general instrument-based method. Accordingly, there is an urgent need to establish effective analytical methods. There is structure-activity relationship existed between the polysaccharides of structure and biological activity, the relationship is related to its molecular weight, solubility, viscosity, etc. Although biological activity of a variety of polysaccharides are different. For instance, pachyman polysaccharides have antitumor activity, laminarin has hypoglycemic activity、reducing blood lipid activity, oat polysaccharides have antibacterial activity. But most polysaccharides of outstanding biology activity have common features: they have main chain structure of dextran. Therefore dextran is typical representative of most polysaccharides of outstanding biology activity. Dextran (DEX) is a polysaccharide that can replace a portion of whole blood in blood transfusion processes, and can be used as an expansion agent of the plasma volume (a plasma substitute). The DEX has the strongest physical activity, and it can activate macrophages and neutrophils [7-9]. Therefore, DEX can increase interleukins, cytokinins, and specific antibody levels [10], and fully stimulate the immune system [11-14]. DEX is a typical polysaccharide and investigations of the interactions between different dextran homologs and proteins are of significance in our efforts to understand comprehensively polysaccharide transport and metabolism processes.

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Serum albumin is the most abundant plasma protein with important physiological functions [15-17] that involve storage and transport of endogenous metabolites and exogenous drug molecules. Compared with other proteins, serum albumin is a relatively small protein, has greater solubility and better stability, and has higher affinity for its specific ligands, which can be separated and purified more easily. In addition, the tertiary structure of serum albumin has been determined, which is helpful in drug-binding studies, and has often been used as a model protein in drug-protein interaction studies. BSA comprises three domains and contains 582 amino acid residues. Because of the high sequence homology between BSA and human serum albumin, as well as their low cost and availability, they are commonly used in research examining the binding of drugs to serum albumin [18-19]. Previous work has shown that CE and microchip CE are rapid and low-cost tools for studying interactions between carbohydrates/drugs and proteins [20-23]. Similar to other techniques, there is a prerequisite that the complex of the binding pair under investigation must alter some properties (e.g., size, shape, or charge in CE; reflection index in surface plasmon resonance (SPR) when CE is used to study molecular interactions. This alteration can produce detectable changes that relate to the equilibrium concentration of free (or bound) species [24-27]. It is likely that the polysaccharide molecule does not have a detectable spectral characteristic signal (e.g., fluorescence). The classical method for detecting polysaccharides is the molecular derivatization method, so that polysaccharides can be detected by ultraviolet detector. According to the object of this paper, dextran has a large number of hydroxyl groups and no double bonds, so it has no maximum ultraviolet absorption. However, the aldehyde group on the oxidized dextran makes it has the ultraviolet absorption. It is mainly the formation of hydrate between aldehyde groups and H_2O molecules after oxidized process. For the interaction between polysaccharides and biomarmolecules, there is no detection value. So in this paper, the polysaccharides are oxidized to obtain ultraviolet absorption, then, the interaction between polysaccharide oxidative derivatives and biomarmolecules is studied. The process of oxidative is to provide an indicator for the detection of dextran by CE. Thus, the CE method is a suitable approach to measure the mechanism of polysaccharide binding to proteins by UV detector. Three CE modes are often used to study the binding between a carbohydrate and a protein [28-32], namely zone CE, affinity CE, and frontal analysis. To extract a valid binding constant, a particular linear or nonlinear regression plot has to be determined for each carbohydrate-protein binding pair [33-34]. This is tedious and time consuming, especially for carbohydrate drug screening that aims to quantitate the binding between different carbohydrates to a protein. Thus, a fast and accurate method that can scan the interactions between different carbohydrates and the same protein needs to be developed. In this study, the HD, FA, and VP method were used to characterize the binding reaction between three homologs of DEX (with different molecular weights) and BSA. A method for detecting polysaccharide-protein interactions was established and the binding parameter of DEX with BSA was calculated based on theoretical model equations. The binding mechanism between polysaccharide homologs and serum albumin has been reported less previously [35-36]. The active constituent from polysaccharides needs to be first combined with plasma albumin to form compounds. The compounds and free drug which is in plasma reaching the equilibrium state. The binding of compounds is reversible, when the active constituent from polysaccharides reaches the target position, it can be released from the plasma albumin and can acting on the objects. The different polysaccharides have different protein-binding rates, the different binding rates directly affect the acting time and intensity of polysaccharides in organisms. Therefore, this innovative work study on the interaction of polysaccharides homologues binding with serum albumin has important clinical value and has important significance to evaluate resistance and efficacy of polysaccharides. It can solve a problem that how to give full play to maximum resistance under the premise of avoiding toxicity through on research on the interaction of polysaccharides homologues binding with serum albumin. Moreover, It also play a certain guiding role in the aspect of grug design and pharmacokinetics associated with polysaccharides.

Experimental

Reagents and Materials

BSA (≥98%) was purchased from Shanghai Huamei Bio-Engineering (Henan, China), DEX (molecular weights of 10,000 Da, 70,000 Da, and 150,000 Da; ≥98%) was purchased from J & K Technology Co., Ltd.(Beijing, China) and ACS (99.5–105.0%) was purchased from Alfa Aesar Chemical Co(Ward Hill, MA, USA). Other reagents were analytical grade and sub-boiled distilled deionized water was used. The solution was prepared at a concentration of 0.1 mol L^{-1} pH=9.6 buffer solution of borate.

Instruments

P/ACE™ MDQ capillary electrophoresis and an uncoated fused silica capillary column (60.2 cm × 50 μm I.D, effective length 50 cm, USA Beckman-Coulter (CA,USA))were used to determine the polysaccharide-protein interaction. A KQ-250DB CNC ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd(Kun Shan, Jiang Su, China)) was used to prepare solutions and clean glassware. A little solution was performed on Eppendorf pipettes (Kenqiang Instruments Ltd (Shang Hai, China)). A ZD-2 precision pH meter (Shanghai Leici Instrument (Shang Hai, China)) was used to control the pH of the working solution. The sample masses were accurately weighed using a BS224S electronic balance (Sartorius (Aubneng, France)).

Methods

The polysaccharide derivatives

The purpose of oxidized is to indicate the role of dextran in detection. After derivatization processing, the DEX which does not have a detectable spectral characteristic signal can change into oxidized dextran (ODEX) which have UV signal [37].

Preparation of ODEX: taking proper amount of DEX precision, DEX was dissolved using phosphate buffer solution (pH 4.5). An equimolar amount of potassium periodate (relative to glucose units on dextran). The reaction mixture was stirred for 1.5 h at room temperature and at 1500r/min, then there are added suitable amount of propanetriol (to terminate the further oxidation of dextran and the breakage of glycosidic bonds). Stirring continuouily at a rate of 1500 r/min and room temperature. The reaction mixture was collected within a dialysis bag. The mixture was dialyzed using water as a medium at 4 °C, 2 d. The ODEX can be obtained by freeze drying the dialysis solution.

The Preparation of Running Buffer in the CE Method and Sample Solution

A borate buffer solution (0.1 mol L^{-1}) was used to prepare a 1.0×10^{-5} mol L^{-1} BSA solution, ODEX solutions with different concentrations, and mixed solutions of ODEX-BSA (1.0×10^{-5} mol L^{-1}) at different concentrations. The sample solutions were filtered through a 0.45-μm mixed cellulose ester filter and degassed using an ultrasonic bath (Kunshan Ultrasonic Instrument Co., Ltd (Kun Shan, Jiang Su, China)) for 5 min prior to use.

The Establishment of Analysis Methods of the ODEX-BSA Interaction System

Before CE analysis, the capillary column was flushed consecutively with 0.1 M NaOH, deionized water, and the borate buffer for 3 min prior to injection, followed by a 5-min rinse with the corresponding running buffer. The same rinse procedure was performed for each CE analysis.

Between each program run, the capillary column was flushed with 0.1 M NaOH, deionized water, and running buffer for 3 min. Each experiment was measured three times and the averages were reported.

The simplified HD method: This method has two injections of drugs (sample and blank) at particular drug concentrations. The combined conditions of the polysaccharide and BSA are analyzed using the experimental data obtained from the two injections. The borate buffer solution with different concentrations of ODEX was used as the background electrolyte. A borate buffer was used as a blank test sample and the borate buffer only containing BSA was used as a sample to carry out the simplified HD capillary electrophoresis analysis (positive injection). The detection wavelength ensured that both protein and ODEX have ultraviolet (UV) absorption (214 nm). Electrophoresis conditions were: separation voltage: 25 kV; pressure injection: 0.3 psi, 3 s; column temperature: 25 °C.

FA method: Using the borate buffer as the blank, and the running buffer solution and the borate buffer with different concentrations of ODEX-BSA as the test samples, experiments were carried out using the capillary electrophoresis FA method (positive injection). The detection wavelength ensured that both protein and ODEX have UV absorption (214 nm). Electrophoresis conditions were: separation voltage: 25 kV; pressure injection: 0.3 psi, 3 s; column temperature: 25 °C.

VP method: Using the borate buffer with different concentrations of ODEX-BSA as the blank and the running buffer solution and the borate buffer as the test sample, experiments were carried out using the VP capillary electrophoresis analysis method (positive injection). The detection wavelength ensured that both protein and ODEX have UV absorption (214 nm). Electrophoresis conditions were: separation voltage:

25 kV; pressure injection: 0.3 psi, 3 s; column temperature: 25 °C.

Results and Discussion

The Preparation of ODEX

The mianly principle of Preparation of ODEX is that hydroxyl (OH) groups of dextran can be oxidized to form active aldehyde groups. The determination of ODEX by Ultraviolet spectrophotometry suggested that ODEX has typical UV absorption characteristics (Fig. 1).

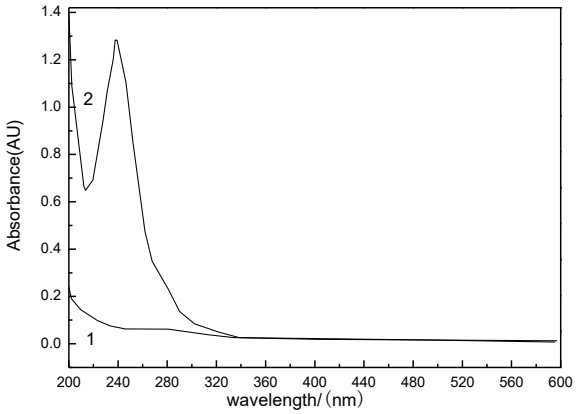


Fig. 1: UV spectrum. 1: UV spectrum of DEX; 2: UV spectrum of ODEX.

The CE Analytical Method of Interactions between Polysaccharide and BSA

The interaction between BSA and polysaccharides was studied by capillary electrophoresis. The binding of the polysaccharide to BSA is a non-covalent interaction and the interaction is a reversible equilibrium process, *i.e.*, P + nD ⇌ [D_nP], where P represents a protein, D represents the polysaccharide, and D_nP represents protein-polysaccharide complexes. In CE, the binding ⇌ dissociation equilibrium of proteins and polysaccharides as well as the component separation is measured. Because of the differences between the mass-to-charge ratio of free polysaccharide, free protein, and that of the protein-polysaccharide complex, and the different electrophoretic migration rate, the HD, FA, and VP method can be applied to separate the three species in the reversible equilibrium reaction. Further analysis can be done through detection of the signal [38-43].

The Electropherogram of BSA in Interaction with ODEX

The experiments used the simplified HD method, FA method, and VP method to analyze the interaction of ODEX with BSA using three different ODEX molecular weights. The results showed that the difference in peak height determined only by the VP method was small. This may be owing to the different concentrations of ODEX-BSA borate buffer used as the running buffer. The large concentration requires a long time to separate completely. Because of the limited length of the capillary column and the short separation time in this experiment, it was not possible to separate completely the samples and this gave a small peak height difference. Therefore, the experiments used the simplified HD method and the FA method to analyze the results. Because the interactional spectra of BSA binding with ODEX at three different molecular weights were similar, the results of the interaction of BSA and ODEX with a molecular weight of 70,000 was used as a representative analysis, and is shown in Figs. 2 and 3.

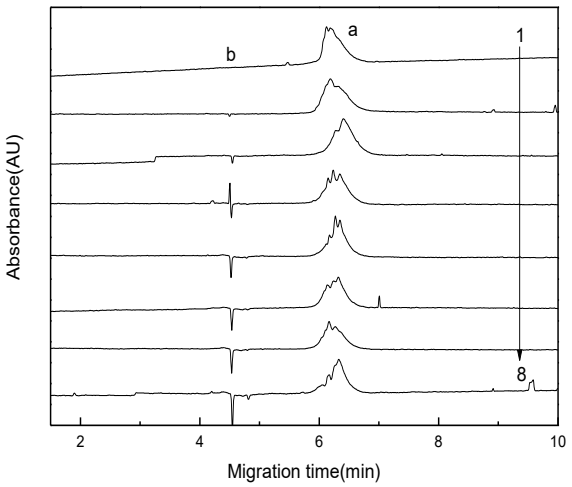


Fig. 2: Electropherograms of BSA in interaction with ODEX using simplified Hummel-Dreyer (MW=70000 D). *a:* bound polysaccharide*; b:* Free protein and complex; $c_{(ODEX)}/10^{-6}$mol·L^{-1},1 to 8: 0.0, 5.0, 15.0, 30.0, 50.0, 60.0, 90.0, 100.0. Conditions: BGE: the borate buffer solution with different concentrations of ODEX; blank test sample: the borate buffer; sample: the borate buffer only contained BSA; positive injection; separation voltage: 25 kV; pressure injection: 0.3 psi, 3 s; column temperature: 25 ºC; uncoated fused silica capillary60.2 cm (effective length 50cm)×50 μm ID; detection 214 nm.

The polysaccharide-protein system used the UV absorption change in the solution system to detect the formation of the polysaccharide-protein complex, and the established CE method should be suitable for use in the analysis of other polysaccharide-protein complexes.

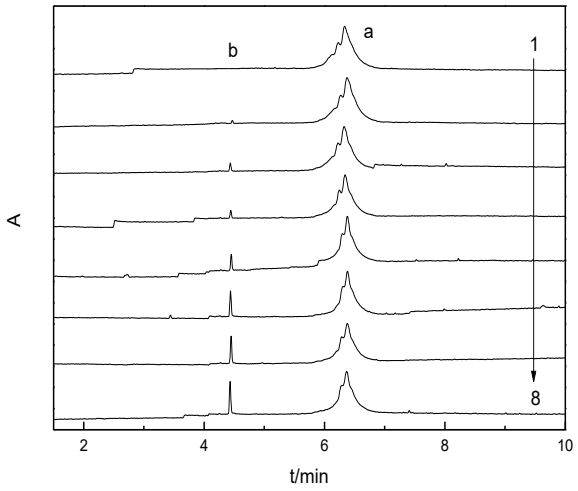


Fig. 3: Electropherograms of BSA in interaction with ODEX using frontal analysis (MW=70000 D). *a:* free polysaccharide*; b:* Free protein and complex; $c_{(DEX)}/10^{-6}$mol·L^{-1},1 to 8: 0.0, 5.0, 15.0, 30.0, 50.0, 60.0, 90.0, 100.0. Conditions: empty and the running buffer solution: the borate buffer ; test sample: the borate buffer with different concentrations of ODEX-BSA; positive injection; separation voltage: 25 kV; pressure injection: 0.3 psi, 3 s; column temperature: 25 ºC; uncoated fused silica capillary60.2 cm (effective length 50cm)×50 μm ID; detection 214 nm.

In the analysis of the results presented in Figs. 2 and 3, two peaks appeared in the respevtive figures called peak *a* and peak *b*. Peak *a* in the figure is a UV absorption peak of the ODEX-BSA complex and BSA. With increasing polysaccharide concentration, peak *a* reduces in intensity. This illustrates that ODEX interacts with BSA to form a complex, and reduces the absorption peak arising from BSA. Peak *b* in the figure is the UV absorption peak of the polysaccharide-ODEX complex and with increasing polysaccharide concentration, peak *b* was observed to increase gradually. In the figure, the ODEX peak was in front of the BSA peak. This is because in the polysaccharide and BSA mixed sample, a fraction of the ODEX in the sample was reversibly binding with BSA to form a ODEX-BSA complex. Under this experimental condition, the solute molecules of the ODEX, BSA, and ODEX-BSA complex all carried a negative charge and their electrophoresis direction was opposite to the electro-osmosis direction. As the electro-osmosis rate was greater than the solute electrophoretic rate and the charge-to-mass ratio of ODEX was less than that of BSA and the BSA-ODEX complex, the migration rate of ODEX was greater than that of ODEX-BSA and BSA, and ODEX reached a peak before BSA. Because the charge/mass ratio of BSA and the ODEX-BSA complex were similar, the ODEX-BSA complex and BSA were not completely separate and a positive peak was observed (peak *a*). In the simplified HD method, the negative peak (peak *b*) was the fraction of ODEX in solution that was lower than that in the background buffer and the difference value was the dextran binding the protein. In the FA method, the ODEX peak was positive (peak *b*) when injecting the blank buffer.

ODEX and BSA Binding Constants (K) and the Number of Binding Sites

This report analyzed the binding parameter of the ODEX-BSA complex and respectively used the Lineweaver–Burk double reciprocal equation, double logarithmic equation, and Scatchard equation to process the above system.

Lineweaver–Burk double reciprocal equation [25]:

$$\frac{1}{A_0 - A} = \frac{1}{A_0} + \frac{1}{KA_0[D_t]} \qquad (1)$$

where A_0 is the absorbance of the BSA solution in the absence of polysaccharides, A is the absorbance of the BSA solution with polysaccharides, and $[D_t]$ is the concentration of polysaccharides. Plotting $1/(A_0–A)$ according to $1/[D_t]$ gives the binding constant K according to the slope.

The double logarithmic equation [44]:

$$\lg[(A_0 - A)/A] = \lg K + n\lg[D_t], \qquad (2)$$

where A_0 and A are the absorbance of the BSA solution in the absence and presence of polysaccharides, respectively, and $[D_t]$ is the concentration of polysaccharides. Plotting

lg[(A_0–A)/A] according to lg[D_t] gives the binding constant K and the binding sites n according to the slope.

In the Scatchard method [27], the equation is:

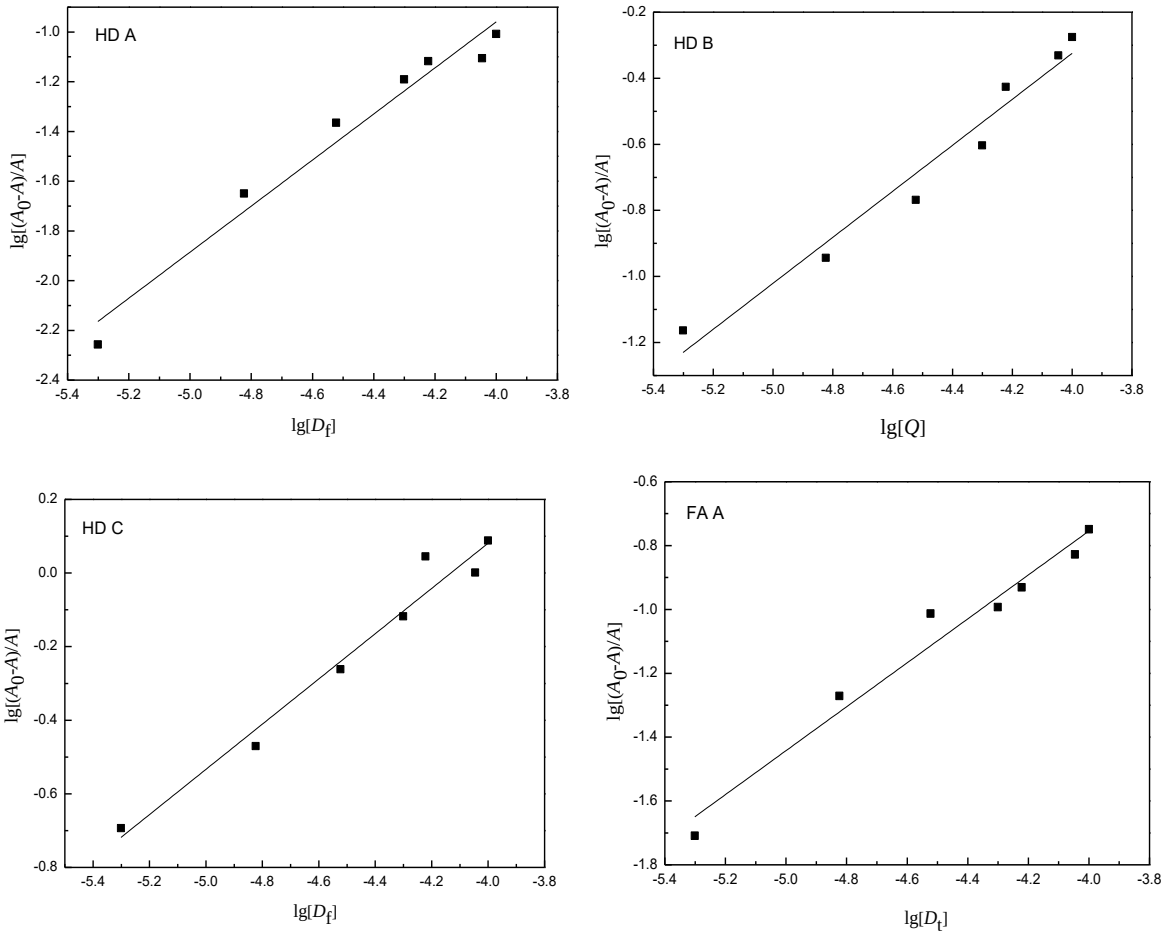
$$A_0 / A = K\,[D_t]A_0 / (A_0 - A) - nK\,[P_t], \quad (3)$$

where [D_t] and [P_t] are the total concentrations of the polysaccharides and BSA in the system, and A_0 and A are the absorbance of the BSA solution in the absence and presence of the polysaccharide, respectively. Plotting A_0/A according to [D_t] $A_0/(A_0–A)$ gives the binding constant K and the binding sites n according to the slope.

The three different equations were used to fit the experimental data. After comparing the fittings using the equations, the results showed that the linear fit of equations (1) and (3) were not ideal, but the fitting results of equation (2) were better. The graphed double logarithmic diagram (Fig. 4) of lg[(A_0–A)/A] according to lg[D_t], obtained K and n and the results are presented in Table-1.

Table-1: The binding parameters of ODEX to BSA.

Method	MW(Dalton, D)	K (L·mol^{-1})	n	Fitting equation	R
HD	10000	5.5693×10^2	0.93	y=2.7458+0.9262 x	0.9610
	70000	2.8854×10^2	0.70	y=2.4602+0.6962 x	0.9571
	150000	4.0495×10^3	1.02	y=3.1281+1.0184 x	0.9616
FA	10000	0.9959×10^2	0.69	y=1.9982+0.6881 x	0.9631
	70000	6.5253×10^2	0.80	y=2.8146+0.7956 x	0.9555
	150000	3.2181×10^2	0.66	y=2.5076+0.6570 x	0.9785



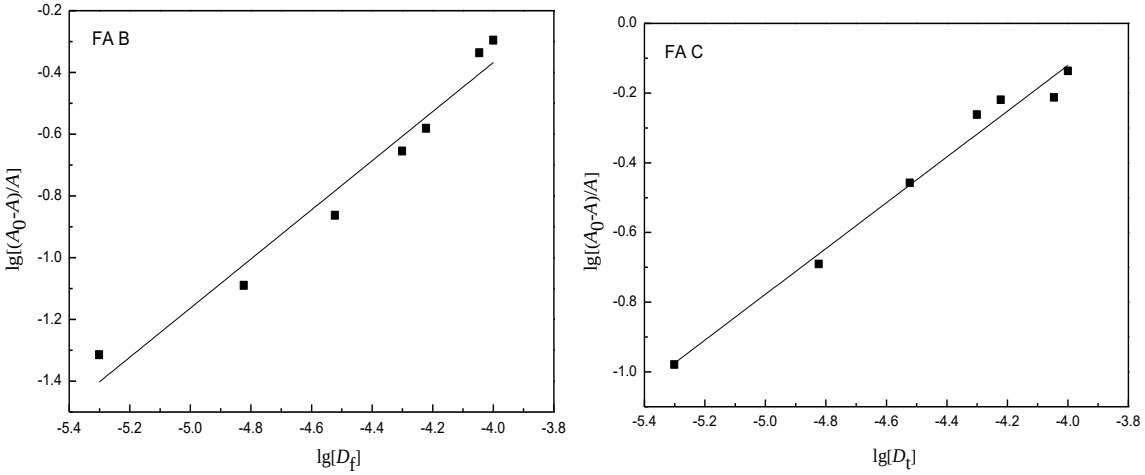


Fig. 4: Linear fitting results of BSA in interaction with ODEX using simplified Hummel-Dreyer (A: MW=10000 D; B: MW=70000 D; C: MW=150000 D) and Linear fitting results of BSA in interaction with ODEX using frontal analysis (A: MW=10000 D; B: MW=70000 D; C: MW=150000 D).

It can be seen from Fig. 4, and Table-1 that ODEX and BSA presented binding sites and there is a moderate binding affinity between ODEX and BSA. The binding constant is significantly weaker than that of the binding action between small drug molecules and BSA. The main reason for this weaker interaction is probably because ODEX has a large molecular weight that cannot enter the cavity of BSA. Thus, ODEX most likely binds weakly to the surface of BSA. The binding constants between ODEX at different molecular weights and BSA are also different. The molecular weight has an optimum value. In this range, the larger ODEX molecular weight gave a stronger binding constant. This may be owing to the greater molecular weight, the longer ODEX molecular chain, and the larger combined surface area of BSA, as well as the stronger combined effect. If the combined area of ODEX and BSA reached saturation, the binding constant will remain the same over a certain range. A comparison of the results obtained using the different methods with the same molecular weight ODEX showed clear differences. Different homologs have the most suitable method respectively. After comparing the results, the simplified HD method was found to give the best data analysis, and the FA method can be applicable only when the *K* value is in the range of 10^3–10^8 L mol^{-1}. For a weak binding reaction with a $K < 10^3$ L mol^{-1}, the system is difficult to measure accurately.[26] Therefore, the simplified HD method is best suited for the ODEX-BSA system, and this method showed that the ODEX-BSA system is a fast binding reaction.

Conclusions

The simplified HD, FA, and VP methods were used to study the interaction of BSA and ODEX with three different molecular weights, and a double logarithmic equation was used to determine the *K* values and the number of binding sites (*n*). The measured binding parameters illustrated that the bonding strength of ODEX and BSA is moderate with a single type of binding site. After comparing the results of the fitting routine using the three methods, it was found that the FA method and the simplified HD method are suitable to analyze the ODEX-BSA system, but the simplified HD method is the best method to use. By analyzing the applicable degree of the model, it was concluded that the double logarithmic equation is the most effective equation to fit data derived from the ODEX-BSA system. In this report, a detection method was established to measure the interaction mechanism of the ODEX-BSA system. This method not only could provide a reference for research of other similar interaction mechanisms involving polysaccharides-protein complexes, but also can help us understanding the medicine efficacy of polysaccharides, It will provide some useful inspiration and guidance for molecular design、selection of polysaccharide medicaments and new drug development.

Acknowledgments

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