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Capillary zone electrophoresis characterization of low molecular weight heparin binding to interleukin 2

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Abstract

A method based on capillary zone electrophoresis (CZE) was used to study the interaction between low molecular weight heparin (LMWH) and interleukin 2 (IL-2). The results showed that the increase of the concentration of LMWH led to the decrease of the peak height and the increase of the peak width of IL-2, but the peak areas were kept constant. The binding constant of IL-2 with LMWH was calculated as $1.2 \times 10^6\ M^{-1}$ by Scatchard analysis, which is in good agreement with the results found in the references using enzyme-linked immunosorbent assay (ELISA). The results demonstrated that the interaction between IL-2 and LMWH is of fast on-and-off kinetic binding reaction. CZE might be used to study not only slow on-and-off rates interactions, but also fast on-and-off rates ones. The binding constant can be calculated easily, and the method can be applied to study a wide range of heparin–protein interactions.
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1. Introduction

An increasing number of proteins are found to bind not only to high affinity cell surface receptors, but also to glycosaminoglycans, particularly the heparin/heparin sulfate (HS) family [1]. These polysaccharides are linear, with highly sulfated chains and extremely heterogeneous in structure [2]. Heparin, a highly sulfated variant, composes of repeating disaccharide units consisting of hexuronic acids linked to glucosamine units by $\alpha(1 \rightarrow 4)$ bonds [3]. It is well known that heparin interacts with many biologically important proteins such as proteases inhibitors, extracellular signaling molecules, lipid- or membrane-binding proteins and adhesion proteins. Some of the interactions are mediated by specific intra-chain sequence. Such specificities are assumed to be indicative of significant biological relevance and these are indeed the cases for AT III, basic and acidic fibroblast growth factors, which are activated by binding to appropriate sequences in HS [4]. The binding of heparin to protein may have highly diverse functional roles such as the control of homeostasis, the regulation of protease, cell behavior and metabolism, etc. [5].

Many methods have been used to study the interactions between heparins and proteins such as isothermal titration calorimetry [6–11], surface plasmon resonance spectrometry (SPR) [5,6,8,12,13], affinity chromatography (AC) [6,8–10,14,15], nuclear magnetic resonance spectrometry [5,6,16], X-ray [5,10,17,18], mass spectrometry [19], circular dichroism [11,14], Fourier transform infrared spectrometry [7], polyacrylamide gel electrophoresis [5], fluorescence [20], equilibrium dialysis [9,21] and capillary electrophoresis (CE) [19,22,23]. Among these methods, SPR and AC are widely used, but they require immobilization of either of the involved substances to supporting material and this poses a problem of steric hindrance [24,25]. Except for the advan-

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tages of high speed, high resolution, low sample consumption, reproducibility, flexibility and capable of using a wide range of buffers, CE can also be used to study the interactions of individual components in a mixture, and to determine binding parameters and stoichiometry in one step [22]. To this end, CE is a preferred method to study heparin–protein interactions. Affinity capillary electrophoresis (ACE) and capillary zone electrophoresis (CZE) are two of five CE methods (ACE, CZE, Frontal analysis (FA), Hummel–Dreyer and Vacancy peak) available to study interactions. Though Heegaard and Gunnarsson et al. have used ACE to study the heparin–peptide [19,22,23] and heparin–protein interactions [26], little work has been done on heparin–protein interactions using CZE [27]. Previously, the slow on-and-off kinetic bindings of granulocyte colony stimulating factor (G-CSF) to heparin were studied using CZE [28]. However, the fast kinetic systems in heparin–protein interactions were never studied by CZE.

To exploit the applicability of the assay in fast on-and-off interactions and investigate the binding kinetics of the interaction between recombinant human interleukin 2 (IL-2) and low molecular weight heparin (LMWH), we investigated the sensitivity and specificity of the assay employing IL-2 as a heparin binding protein. The interaction between IL-2 and heparin has been investigated by an enzyme-linked immunosorbent assay (ELISA) [1,29]. IL-2 binds to heparin in a dose-dependent manner. The interaction is dependent on the heparin chain length and the chains as small as 5 kDa retain the ability to bind to IL-2. However, to the best of our knowledge, the interaction has never been studied by CZE, the binding kinetics has not been investigated, and the CZE assay has never been used to study fast on-and-off kinetic systems in protein–heparin interactions. In this study, the effects of the addition of LMWH to the injection volume of a sample and the negative control of the interaction between epidermal growth factor (EGF) and heparin were investigated by CZE. The binding kinetics of the interaction between LMWH and IL-2 was represented and the binding constant was determined. The applicability of the CZE assay in both slow and fast on-and-off interactions was also discussed.

2. Experimental

2.1. Materials

Recombinant human interleukin 2 (Purity: 97% by SDS-PAGE and HPLC analyses) with a molecular weight of 15.4 kDa and with the concentration of 1 mg/mL in 100 mM acetic acid was purchased from USBiological (Swampscott, Massachusetts, USA). EGF (solid, MW: 6216) was given by Prof. Ren-Bao Gan (Institute of Biochemistry and Cell Biology, Shanghai Academy of Life Science, Chinese Academy of Sciences, Shanghai, China). Heparin (powder, MW: 15 kDa) and LMWH (powder, MW: 5 kDa) were kindly provided by Qilu Pharmaceutical Factory (Jinan, Shandong, China). Mannitol was purchased from Beijing Jingke Company (Beijing, China). Other chemicals were all analytical grade. Redistilled water was used throughout this work. Microcon YM-3 and 10 were purchased from Millipore (Billerica, MA, USA).

2.2. Sample preparation

A 10 μL of recombinant human IL-2 (1 mg/mL in 100 mM acetic acid) was diluted with water to 200 μL and ultracentrifuged by Millipore microcon YM-3 to obtain 20 μL stock solution. The stock solution was diluted with 10 mM acetic acid to various concentrations in the subsequent experiments. Benzoic acid and EGF were all dissolved and diluted with water. LMWH was dissolved and diluted using 10 mM acetic acid and water in the (IL-2)–LMWH and (benzoic acid)–LMWH interactions, respectively. Heparin was dissolved and diluted with water.

2.3. Capillary zone electrophoresis

A Beckman apparatus consists of a P/ACE MDQ system (Beckman, Fullerton, CA, USA) with a photodiode array detector was used to all experiments. A fused-silica capillary (31.2/21 cm × 50 μm i.d.) was obtained from Yongnian Optical Fibre Corp (Hebei, China). Data were collected and processed with the Beckman System software. In the present study, the temperatures of the cartridge and sample room were 25 and 20 °C, respectively. Before each measurement, the capillary was rinsed with 50 mM phosphate buffer, pH 9.0 for 3.0 min at 137.895 kPa. Samples containing mixtures of protein and LMWH were injected at the anodic end using a pressure injection mode with 3.447 kPa for 4 s and detected at the cathodic end at the wavelength of 201 nm. The running voltage was 8 kV. After each run, the capillary was flushed consecutively with 1 mol/L HCl for 2.0 min, water for 3.0 min, 1 mol/L NaOH for 2.0 min, and finally with water again for 3.0 min at 137.895 kPa. Duplicate for each sample was performed.

2.4. Quantitative model of the binding study

Binding studies often involve a proof of bindings, the number of binding sites and an estimation of the quantitative parameters [30]. Binding constant and stoichiometry are important parameters to be determined. Scatchard analysis is a common way to linearize the binding data, and the model can be expressed in the following equation:

$$\frac{r}{C_f} = -Kr + nK \tag{1}$$

where r is the ratio of the concentration of the bound ligand (or receptor) to the total receptor (or ligand) and C_f is the unbound ligand (or receptor) concentration. K is the apparent binding constant and n is the number of binding sites [31]. In this study, r is the concentration ratio of the bound protein to the total LMWH and C_f is the unbound protein concentration.

3. Results

3.1. The effect of the addition of LMWH to the injection volume of samples

The addition of LMWH to a sample solution might change the viscosity of the sample, which might influence the injection volume. Therefore, the influence of LMWH to the injection volume of benzoic acid was investigated. Fig. 1 shows the electropherograms of 0.02% benzoic acid and the mixture of 0.02% benzoic acid and 50 g/L LMWH obtained at the same experimental conditions. The peak heights of the benzoic acid are equal to each other in Fig. 1, which indicated that the addition of LMWH did not change the injection volume and sample viscosity at least below the LMWH concentration of 50 g/L. The results also demonstrated that benzoic acid had no affinity to LMWH.

3.2. The interaction between IL-2 and LMWH

IL-2 within the concentration range of 0.649–16.234 μM was injected to the capillary column to obtain the calibration plot. The peak heights of samples were found to be proportional to IL-2 concentrations. The relationship between peak height and concentration of IL-2 was $y = 902.17x + 351.41$ ($n = 6$), and the coefficient was 0.999. The corresponding concentrations of IL-2 in subsequent binding study were calculated from the calibration equation.

The samples contain a fixed concentration of IL-2, with increasing concentrations of LMWH was injected to the capillary column (Fig. 2). The electropherograms showed that with the increase of LMWH concentration, the peak widths of IL-2 increased, the peak heights decreased consecutively and regularly, but the peak areas and migration times remained nearly constant. This means that the interaction between IL-2 and LMWH occurs and the dissociation rates of the complex are so fast that the peaks of IL-2 become broadening. The results indicate that the system is of fast on-and-off kinetics.

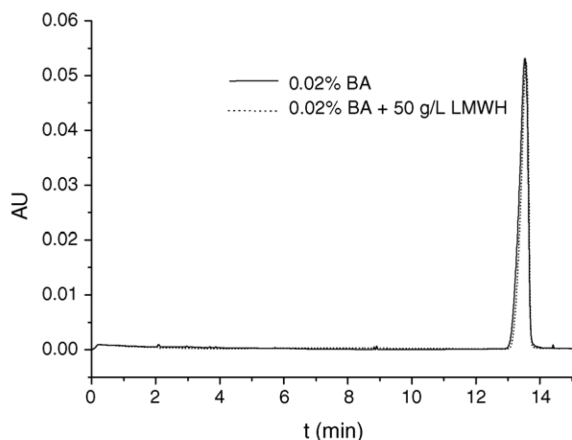

Fig. 1. Electropherograms of benzoic acid alone and the mixture of benzoic acid and LMWH as indicated in the figure. Detection wavelength: 224 nm, running buffer: 50 mmol/L phosphate, pH 7.0. Other conditions are described in Section 2.

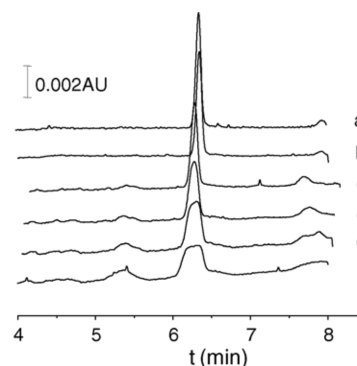

Fig. 2. Electropherograms of 0.1 g/L IL-2 mixed with various concentrations of LMWH: (a) 0 g/L, (b) 2.0 g/L, (c) 10.0 g/L, (d) 16.7 g/L, (e) 25.0 g/L, (f) 33.3 g/L. Conditions are described in Section 2.

To quantify the interaction between IL-2 and LMWH, the peak heights of IL-2 in each sample were determined and free concentrations were calculated from the calibration curve to obtain the values of r. Fig. 3 shows the Scatchard plot based on Eq. (1). From the slope of the Scatchard plot, K was calculated to be $1.2 \times 10^6\ \mathrm{M}^{-1}$ which was in excellent agreement with the K_D value of 0.5 μM (that means K is $2.0 \times 10^6\ \mathrm{M}^{-1}$) that Saloua et al. obtained by using ELISA [29]. This demonstrated that the fast on-and-off rates interactions might be studied using CZE.

3.3. Binding of EGF to heparin

It is well known that EGF has no affinity to heparin [32]. In this study, EGF and the mixture of the same concentration of EGF and an excess concentration of heparin were injected to capillary column (Fig. 4). In both electropherograms, the peaks of EGF nearly have no changes both in peak heights and areas. The results verified that EGF had no affinity to heparin.

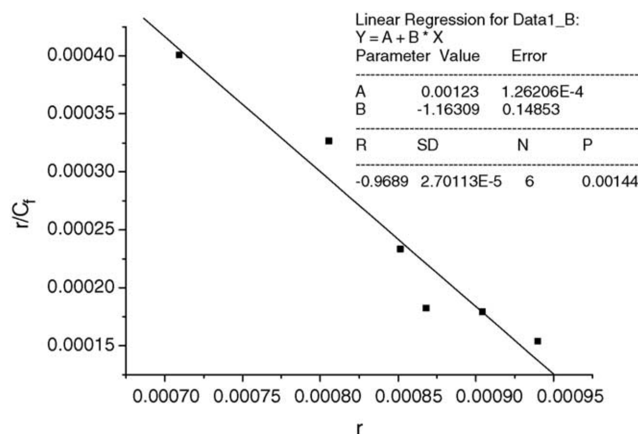

Fig. 3. Scatchard plot for the interaction between IL-2 and LMWH.

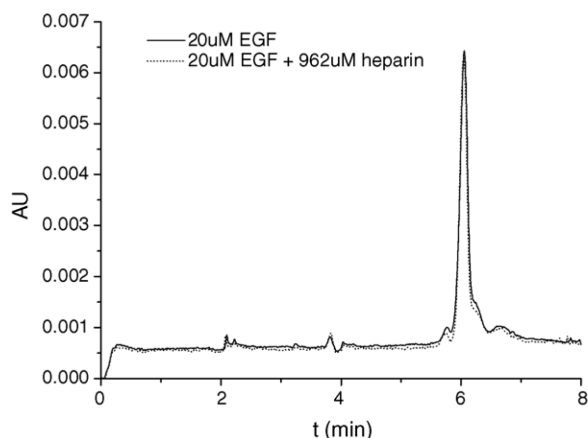

Fig. 4. Electropherograms of EGF alone and the mixture of EGF and heparin. Detection wavelength: 210 nm, running buffer: 50 mmol/L phosphate, pH 7.0. Other conditions are described in Section 2.

4. Discussion

In this study, it was found that the addition of LMWH to benzoic acid did not affect the injection volume of the benzoic acid, which means that the changes of the benzoic acid viscosity could be neglected after the addition of LMWH. In addition, the interaction between EGF and heparin confirms that if two species have no affinity to each other, the sample peak could not be affected by the addition of the other substance. Based on the above investigations, we studied the interaction between IL-2 and LMWH by CZE. It was observed that the peak height of IL-2 decreased and the peak width increased successively and regularly with the increase of the LMWH ratios in the sample, but the peak area nearly had no changes. These changes were not occasional due to the regularity. The changes in peak shape of IL-2 incubated with LMWH in Fig. 2 indicate occurrence of their interaction and the dissociation rate of the complex they formed is comparatively fast. IL-2 transfers between LMWH and (IL-2)–LMWH complex very quickly and thus no stable complex exists. Under the separation conditions, IL-2 and LMWH all have negative charges and they migrate to the cathode by the force of electroosmotic flow (EOF) contrary to their electrophoretic mobilities. The electrophoretic mobility of LMWH is larger than IL-2's because of its larger charge/mass ratio, and thus, LMWH moves slowly than IL-2, and the complex moves between them. IL-2 is released from the (IL-2)–LMWH mixed zone continuously because of the fast, reversible binding equilibrium. Finally, the whole of IL-2 leaves the LMWH zone and migrates as a zone of free form. The interaction between IL-2 and LMWH lengthens the IL-2 zone and causes the IL-2 peak broadening, but does not change its peak area. The more LMWH the sample contains, the longer the interaction lasts, the broader the IL-2 peak becomes and the lower the peak height changes. The peak heights of IL-2 were used to estimate the binding constant of the interaction between IL-2 and LMWH, and the value ($1.2 \times 10^6\,M^{-1}$) obtained here is in good agreement with that obtained by ELISA ($2.0 \times 10^6\,M^{-1}$) [29]. This demonstrated that the fast on-and-off kinetic interaction systems could also be studied by CZE contrary to traditional opinion that CZE could only be used in studying slow on-and-off kinetic interactions and ACE is exclusive in studying fast on-and-off ones based on CE methods.

CE can be performed in either ACE or CZE format, depending on the separation time and the stability of the complex [33,34]. For a system with fast on-and-off kinetics, which means that the half-life of a molecular complex is much shorter than the time of the electrophoresis, the estimation of binding constants by ACE where a ligand (or receptor) at different concentrations added to the electrophoresis buffer is feasible [35–37]. For a system with slow on-and-off kinetics, which means that the half-life of the complex is longer compared with the time of the electrophoresis, the receptor (or ligand) will be reflected in broadened or split peaks that make ACE not suitable for this type of interactions [35,36]. A general approach to assess binding constants when binding kinetics is slow is to analyze pre-incubated samples at different receptor–ligand ratios, which is the referred CZE method. Therefore, ACE is often used in fast on-and-off kinetics, while CZE more competitive in studying slow on-and-off kinetics. In cases with fast on-and-off kinetics, the lifetime of the complex is shorter than the time required for the free receptor (or ligand) molecules to leave an injected sample plug. Thus, no changes in peak areas of the receptor (or ligand) molecules are to be expected when CZE is used in this type of interaction [36]. However, the interaction between receptor and ligand lengthens the receptor (or ligand) zone and causes the peak broadening. The more ligand (or receptor) the sample contains, the wider the receptor (or ligand) peak becomes, and the lower the receptor (or ligand) peak height changes [38,39]. The changes are successively and regularly, which mean that it is not occasional or dues to other effects, but the interaction occurrence. Based on the CZE/FA method, the peak height changes of receptor (or ligand) resulting from interacting with ligand (or receptor) can be used to estimate the binding constant [40,41]. Previous reports have successfully estimated the binding constants of two fast binding kinetic systems, TrpRS–tRNA [39], DNA–netropsin [42]. From this study, we demonstrated that fast on-and-off kinetic interactions could also be studied by CZE, but not only by ACE.

Though ACE and CZE each has its own advantages and disadvantages, the obvious advantages of CZE over ACE are that the sample consumption of CZE is much lower than ACE and no neutral marker is needed in CZE. In this study, 3–4 μL samples is enough for each run, but if using ACE, 3–4 mL samples must be needed. In addition, the operation and calculation of binding constants using CZE is easier than ACE. The general approach of ACE is that a ligand (or receptor) at different concentrations is added to electrophoresis buffer, while the receptor (or ligand) at a constant concentration is injected. Scatchard analysis of the migration shifts of the receptor (or ligand) as a function of the concentration of the

ligand (or receptor) in the electrophoresis buffer allows the determination of the binding constant of the interaction [43]. In this method, the actual concentration of the injected counterpart is not needed to calibrate the binding constants (but the exact concentration of the other one is needed). However, a neutral marker is prerequisite to calibrate EOF and the binding stoichiometry cannot be obtained using this method. In addition, the ligand (or receptor) is prepared in the running buffer at different concentration and hence, the capillary must be balanced for a relatively longer time before each run and the ligand (or receptor) must be prepared each at the level of milliliter. The basic principle of CZE is that the capillary is filled with neat buffer, while the pre-incubated samples, containing different ratios of the interacting species, are injected. The peak height or area of one of the interacting species is detected to calculate binding parameters. In this method, the exact concentration of the receptor (or ligand) is needed, while no neutral marker is needed, the binding stoichiometry can be obtained easily, and the sample prepared in microlitre is enough.

5. Conclusion

This study shows that it is possible to characterize the fast on-and-off kinetic interaction between IL-2 and LMWH using CZE. The CZE approach is a highly efficient, fast, quantitative and sensitive method for the study of biomolecular interactions. It also has the advantage that the interaction can be studied at near physiological conditions. Compared with the methods such as ELISA, SPR, AC, etc., CZE approach avoids the interference when heparin or protein was immobilized on a plate. In comparison with ACE, CZE has the advantages such as lower sample consumption, no need of internal marker, easy calculating of binding stoichiometry and simple operation procedure. In addition, the CZE approach can be used in both slow and fast on-and-off kinetic interactions. The implication of this work will be that the interactions between biomoleculars, particularly the substances with minute amount of sample, can be studied in an alternative way, other than ACE. CZE appears to be of wide applicability in the study of heparin–protein interactions.

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