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Determination of binding parameters between lysozyme and its aptamer by frontal analysis continuous microchip electrophoresis (FACMCE)

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ABSTRACT

An original and simple methodology based on microchip electrophoresis (MCE) in a continuous frontal analysis mode (named frontal analysis continuous microchip electrophoresis, FACMCE) was developed for the simultaneous determination of the binding parameters, *i.e.* ligand-site dissociation constant (k_d) and number of binding sites on the substrate (n). This simultaneous determination was exemplified with the interaction between an aptamer and its target. The selected target is a strongly basic protein, lysozyme, as its quantification is of great interest due to its antimicrobial and allergenic properties. A glass microdevice equipped with a fluorescence detection system was coated with hydroxypropylcellulose, reducing the electroosmotic flow and adsorption onto the channel walls. This microdevice allowed the continuous electrokinetic injection of a mixture of fluorescently labelled aptamer and non-labelled lysozyme. By determining the concentration of the free fluorescently labelled aptamer thanks to its corresponding plateau height, mathematical linearization methods allowed to determine a k_d value of 48.4 ± 8.0 nM, consistent with reported results (31 nM), while the average number of binding sites n on lysozyme, never determined before, was 0.16 ± 0.03. These results seem to indicate that the buffer nature and the SELEX process should influence the number and affinity of the binding sites. In parallel it has been shown that the binding between lysozyme and its aptamer presents two sites of different binding affinities.

1. Introduction

Aptamers are single-stranded DNA or RNA oligonucleotide sequences which show specific binding affinity towards various targets, ranging from small molecules to proteins, and even whole cells [1–3]. These synthetic molecules are isolated from random-sequence pools by *in vitro* selection thanks to a molecular evolution technique named SELEX (systematic evolution of ligands by exponential enrichment) [4–6]. Aptamers are considered as a nucleic acid version of antibodies, with additional advantages such as low cost, small size, ease of synthesis and labelling, high affinity and increased thermal stability, as well as tolerance to wide range of pH and salt concentration. Aptamers can thus be widely employed as sensitive diagnosis agents, biomedical research tools and even therapeutics [7–9], and for the development of acoustic or optical biosensors [10–12] and for other separation methods for which aptamers are often used as affinity probes [9,13–17].

To improve the development and use of aptamers in a wide range of domains, it is necessary to develop methods allowing a simple, fast and accurate evaluation of the binding parameters (*i.e.* dissociation constant and stoichiometry) with the target. Methods available for studying biomolecular interactions include spectroscopy, gel filtration chromatography, microdialysis, isothermal calorimetry, surface plasmon resonance and separation techniques such as gel electrophoresis flow induced dispersion analysis (FIDA) [17,21] or capillary electrophoresis (CE) [18–20]. Among the various modes of electrokinetic separations, frontal analysis continuous capillary electrophoresis (FACCE) is an alternative method that involves the continuous electrokinetic injection of the incubated mixture during separation, leading to fronts that are processed as for FACE [31]. FACCE presents the advantage of being simpler in the instrumental process and providing larger fronts for precise height determination.

Since about 15 years, microchip electrophoresis (MCE) has become a powerful alternative to conventional separation methods. Indeed MCE presents further advantages in terms of speed, low sample and reagent consumption, high throughput and inte-

Abbreviations: CE, capillary electrophoresis; FACMCE, frontal analysis continuous microchip electrophoresis; MCE, microchip capillary electrophoresis; PB, phosphate buffer.

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gration capabilities. In this context, different papers have evidenced the interest of MCE for binding characterization. Among them, Gong et al. have used different methods (direct injection or frontal analysis) to study protein–DNA binding [32–34]. In parallel Le Saux et al. exemplified the implementation of continuous frontal analysis in a quartz microchip with UV detection, through measuring inclusion constants of model compounds into β-cyclodextrin by competitive assays [36]. MCE in a continuous frontal analysis mode was also carried out to characterize the binding between a glycopeptide antibiotic teicoplanin (Teic) immobilized on magnetic microspheres, and fluorescently labelled D-Ala–D-Ala terminus peptides [37]. Even if these papers have evidenced the ability of MCE to determine binding constants, most of them used a microdevice with at least a simple-cross or more complicated layout and thus required an optimization of the voltage sequence to drive the sample and electrolyte within the chip. Moreover none of these works allowed to determine the binding stoichiometry of the studied systems; on the contrary, this latter was supposed a priori or from other studies and used to calculate the dissociation constant.

In this work, we report the development of an original and simple methodology based on continuous frontal analysis MCE (that we named frontal analysis continuous microchip electrophoresis, FACMCE) for the determination of the binding parameters, *i.e.* ligand-site dissociation constant (k_d) and number of binding sites on the substrate (n). The proof of the methodology was demonstrated with lysozyme and its aptamer. Lyzozyme is an acid hydrolase which destructs bacterial cells walls, that can be used as biomarker to diagnose or monitor the treatment efficiency of blood diseases such as leukemia [38], or the rejection of organs transplants [39]. Despite its clinical relevance, this system presents some experimental difficulties and has never been completely characterized. Being a strongly basic protein (pI = 11.35), lysozyme is positively charged under physiological pH and could strongly be adsorbed onto the channel wall due to hydrophobic and electrostatic interactions. The aptamer–lysozyme complex should thus also be adsorbed on the channel walls, causing disruption of the interaction. In addition, under physiological pH, the electrophoretic mobility of the lysozyme-binding aptamer had nearly the same absolute value as the electroosmotic mobility, leading to very long migration times. In order to prevent protein and complex adsorption and to perform rapid separations, a neutral coating was performed on the glass microchip as previously described [43]. The continuous frontal analysis is carried out in a single channel device avoiding any analyte injection step. To deeply investigate the performances of the methodology, the experiments were performed according to two configurations: with (1) varying ligand (*i.e.* aptamer) concentrations and fixed substrate (*i.e.* lysozyme) concentration, and (2) varying substrate concentrations and fixed ligand concentration, the fluorescently labelled ligand being the unique detectable compound. In the first configuration, the determination of the interaction parameters is based on the free ligand concentration, which is directly accessible. Conversely, in the second configuration, the determination of the interaction parameters is based on the free substrate concentration, that is indirectly accessible through the free ligand concentration. The interaction parameters between lysozyme and its aptamer (k_d and n) were determined according to mathematical linearization methods. The results obtained with both configurations were compared to further investigate the aptamer–lysozyme interaction.

2. Theoretical [44]

Let us consider an interaction between a substrate (S) and a ligand (L) that can be described by a 1: n complex (C). In this case, the equilibrium can be written (Eq. (1)):

$$S + nL \Leftrightarrow C \tag{1}$$

The binding constant ($K_f = 1/K_d$) is then defined as follows (Eq. (2)):

$$K_f = \frac{[C]}{[S][L]^n} \tag{2}$$

where [C], [S] and [L] are the equilibrium concentrations of the complex, free substrate and free ligand, respectively.

In the continuous frontal analysis mode, an incubated substrate/ligand mixture is continuously introduced into the capillary. In most cases, the substrate and the ligand do not play symmetric roles: the partner showing more potential interaction sites (in most cases, the larger one) is usually considered as the substrate, whereas the other partner, supposed to show a unique binding mode, is considered as the ligand. In this case, n corresponds to the number of binding sites on the substrate. This situation is particularly true for systems of biological interest, such as the interaction between proteins and small molecules. Let us assume that the accessible parameter is the concentration of free ligand, from the height of the plateau following the migration front of the ligand.

If the concentration of the ligand is varied whereas the concentration of the substrate is constant, the binding constant can be obtained from the mass balance equations for the substrate and ligand, yielding the equilibrium complex and free substrate concentrations (Eqs. (3) and (4)):

$$[C] = [S]_0 - [S] = \frac{[L]_0 - [L]}{n} \tag{3}$$

$$[S] = [S]_0 - [C] = [S]_0 - \frac{[L]_0 - [L]}{n} \tag{4}$$

where $[L]_0$ and $[S]_0$ are the initial concentrations of the ligand and substrate in the incubated mixture, respectively.

A deeper study employing the multi-site binding model for the determination of the binding parameters expresses the independent binding of one ligand L to one site s of a given binding strength of a substrate S according to a monomolecular reactional scheme (Eq. (5)):

$$s + L \Leftrightarrow s \cdot L \tag{5}$$

The binding parameters are in this model the number of binding sites n belonging to each category and the ligand-site interaction constant k_f (=$1/k_d$), defined as follows (Eq. (6)):

$$k_f = \frac{[s \cdot L]}{[s][L]} \tag{6}$$

The mass balance equation can be expressed as (Eq. (7)):

$$[s]_0 = [s] + [s \cdot L] = n[S]_0 \tag{7}$$

where $[s]_0$ and [s] are the initial and free concentrations of the sites.

Data processing can be achieved either by non-linear or linear regressions [45]. In this study, data were treated by performing linearization methods. The binding constant (k_f) as well as the number of binding sites (n) were simultaneously determined from the equilibrium free ligand concentration [L] according to three mathematical methods for data linearization proposed for the FACCE mode. The mean number of ligands bound to the substrate, r, is defined as (Eq. (8)):

$$r = \frac{[L]_0 - [L]}{[S]_0} = n\frac{[s \cdot L]}{[s] + [s \cdot L]} = n\frac{k_f[L]}{1 + k_f[L]} \tag{8}$$

and can be directly determined from the free ligand concentration.

Table 1a reports the rearranged equations used in these methods. Even if all equations are equivalent in their algebraic form, the impact of the precision of [L] value depends on whether it appears on numerator or denominator.