A rational route to the development of a competitive capillary electrophoresis immunoassay: assessment of the variables affecting the performances of a competitive capillary electrophoresis immunoassay for human serum albumin

A RATIONAL ROUTE TO THE DEVELOPMENT OF A COMPETITIVE CAPILLARY ELECTROPHORESIS IMMUNOASSAY: ASSESSMENT OF THE VARIABLES AFFECTING THE PERFORMANCES OF A COMPETITIVE CAPILLARY ELECTROPHORESIS IMMUNOASSAYS FOR HUMAN SERUM ALBUMIN

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1. Introduction

Affinity capillary electrophoresis (ACE) is considered a powerful modern analytical tool for extracting qualitative and quantitative information about inter- and intra-molecular binding properties of different interacting systems. These systems are generally constituted by ligand-receptor couples like antigen-antibody, enzyme-substrate, receptor-hormone, drug-protein. The most relevant features of ACE are high separation efficiency, short analysis time, low sample consumption and ease of execution. Moreover, the affinity interactions can be studied in aqueous buffered solutions whose composition easily simulates the naturally occurring conditions where the binding interactions take place [1-2]. The experimental approaches used in ACE techniques depend on the thermodynamic and kinetic features of the interactions while the monitored variables are the change in mobility or relative migration time for weak to moderate molecular interactions and the peak area or height for strong molecular interactions [3-4]. In the latter case, it has also opened the route to the development of capillary electrophoresis immunoassay with fluorescence detection mostly in the competitive format. This immunoassay is based upon non- equilibrium separations of pre-equilibrated mixtures of ligand and receptor and allows the quantification of the bound and free fractions of the labelled reagent - the antigen in the more usual competitive assay - by the corresponding peak areas obtained in electrophoretic runs performed without the other binding partner (the antibody) in the separation

buffer. Despite the equilibrium not being sustained during the separation, the affinity constant is not significantly altered when the binding interaction is very strong [5-8]. The high affinity constant of the antibody-antigen system together with the laser-induced fluorescence detection allow one to reach very high sensitivity also in terms of mass, due to the very low amounts of sample detected. However, these high analytical performances can be obtained if the experimental conditions are adequate: the labelling procedure, the affinity features of the labelled reagent, the antiserum affinity and the separation conditions adopted should be tested and optimized to achieve the best results.

The present paper aims to describe the main steps followed to set up a competitive immunoassay from the antiserum titration curve, the determination of the affinity constant in the non-equilibrium separation conditions of pre-equilibrated mixtures to the definition of the pre-incubation conditions prior to separation required to get the best performances. The test system used is constituted by the human serum albumin, the corresponding labelled protein and a polyclonal antiserum.

2. Materials and methods

2.1. Chemicals and apparatus

All chemicals used were of analytical grade. The dimethyl sulfoxide (DMSO), sodium dihydrogen phosphate bihydrate, disodium hydrogen phosphate monohydrate, sodium chloride, sodium hydroxide, potassium hydroxide, hydrochloric acid, tris(hydroxymethyl)aminomethane (TRIS), ethylenediaminetetraacetic acid (EDTA), sodium carbonate, hydroxylamine, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), Tween 20, sodium azide, fluorescein isothiocyanate (FITC) were from Sigma (Milan, Italy). The human serum albumin (HSA, lyophilized powder purified by crystallization) and the rabbit anti-human albumin antiserum (pAb, provided as IgG fraction purified by ion exchange chromatography) were also supplied by Sigma and used without further purification steps. Protein concentration was determined by using the Bradford reagent from Sigma. The 6-mL polyethersulfone Vivaspin concentrator (cut-off 10000 MW), used to concentrate the labelled protein, was obtained from Vivascience AG (Hannover, Germany). The 0.5-mL polypropylene microtubes with low protein adsorption from Kartell (Milan, Italy) were used both for dilutions and as reaction vials. All buffered and washing solutions were prepared with ultrapure water purified by reverse osmosis in a Purelab Prima System from Elga (Bucks, UK) and filtered before use through 0.45-μm cellulose acetate filters from Alltech (Milan, Italy). The spectrophotometric measurements were performed on a double beam spectrophotometer Cary 1E from Agilent Technologies (Milan, Italy). The centrifuge used was a BR4i model from Jouan (Jouan Italia, Milano, Italy). The P/ACE MDQ system (Beckman, Fullerton, CA, USA) was

equipped with a diode-array UV detector and a 5 mW air-cooled argon ion laser detector (excitation at 488 nm, emission at 520 nm) was controlled by 32 Karat Software.

2.2. Capillary electrophoresis separations

All the electrophoretic separations were performed in fused silica capillaries (50 μm i.d., 60 cm total length, 50 cm to the window) purchased from Beckman Coulter and housed in a liquid-thermostated cartridge at 25°C. Each new capillary was pre-treated before use by flushing with 1M potassium hydroxide (15 min), 1M hydrochloric acid (15 min) and washed with water (15 min) at 50 psi. Before each run the silica capillary was rinsed with 1M sodium hydroxide for 15 min at 50 psi, followed by a 10 min rinse with deionized water and run buffer at 50 psi. The samples were injected by pressure (0.5 psi x 3 s with an injected volume of about 3 nL, 0.3% of the plug length to the window) at the anodic end of the capillary. The electrophoresis run buffer was 0.1 M CAPS, pH 9.0 and the applied voltage was 20 kV.

At the end of each run desorption of protein from the silica was performed by sweeping the capillary's inner surface with SDS micelles as described in literature [9-10]. Then, the capillary was washed with water for 5 min at 50 psi.

2.3. Preparation of FITC-labelled HSA

The labelling reactions were performed according to a previously described procedure [11]. Protein/FITC molar ratios of about 1:2, 1:5 and 1:10 were used for the labelling procedure. 10, 25 and 50 mg of HSA were each dissolved in 0.4 mL of 0.1 M sodium carbonate buffer, pH 9.4; then 50 μl of 0.6 mg mL^{-1} FITC in freshly distilled DMSO were added under stirring to obtain the three different above mentioned reaction molar ratios. The mixtures were left in the dark for 2 hours at room temperature, then the reaction was stopped by the addition of 50 μL of freshly prepared 1M hydroxylamine pH 9.0. The solutions were concentrated and purified by spin-filtering them three times at 10000 *g* at 4°C. After each centrifugation step the initial volume was restored by the addition of water. The final concentrated samples were reconstituted to 0.5 mL by the addition of 0.02 M sodium phosphate buffer, 0.1 M sodium chloride and 0.001M EDTA, pH 7.4. Thereafter, the mean substitution degrees were determined by the Bradford method for the protein and by spectrophotometric determination at 494 nm for the FITC (ε = 68000 L mol^{-1} cm^{-1}) and was calculated as the ratio of the FITC to protein molar concentration. After the characterization 0.005% Tween 20 (w/v) and 0.005% sodium azide (w/v) were added to the stock protein solutions that were frozen in 30 μL aliquots until use.

2.4. Preliminary binding studies

10 μL of 100 nM HSA-FITC were mixed in polypropylene microtubes with 10 μL of 1:6400 to 1:50 diluted anti-HSA antiserum. The reaction mixtures were vortexed for 2 min and then incubated for 10 min at room temperature before injection. All the dilutions were made in a 20 mM sodium phosphate buffer, 0.13 M NaCl, 1 mM EDTA, pH 7.4. The electrophoretic peaks of both free and bound protein fractions were integrated by means of 32 Karat Software. The bound to free protein ratio (A_B/A_F) was plotted as a function of the antiserum dilution to obtain the titration curve.

2.5. Determination of the affinity constant

10 μL of 50 to 1000 nM HSA-FITC (reaction molar ratio of 1:5) was mixed in polypropylene microtubes with 10 μL of 1:400 diluted anti-HSA antiserum. The reaction mixtures were vortexed for 2 min and then incubated for 10 min at room temperature before injection. All the dilutions were made in a 20 mM sodium phosphate buffer, 0.13 M NaCl, 1 mM EDTA, pH 7.4. The electrophoretic peaks of both free and bound protein fractions were integrated by means of 32 Karat Software. The affinity constant, K, and the antibody site concentration, Ab_0, were calculated by plotting the peak area of the bound fraction as a function of the peak area of the free fraction in order to obtain a non-linear trend of the experimental data that was interpolated according to the mathematical model reported in equation 1 and previously developed and described in a previous paper [11].

$$A_B = A_F\,(K\,Ab_0\,a_B/a_F) / (1+K\,A_F/a_F) = (m\,n\,A_F) / (1+m\,A_F) \qquad \text{eq.1}$$

In this equation A_B and A_F represent the areas of bound and free labelled HSA; a_F and a_B are the response factors experimentally determined in the absence of antiserum ($a_F=A_F/C$) and in the presence of antiserum in conditions of protein saturation ($a_B=A_B/C_{sat}$). m and n are respectively expressed as K/a_F and $a_B \cdot Ab_0$ allowing the determination of K and Ab_0. This model is based on the assumption that the amount of free and bound fractions of the labelled protein are not perturbed during the electrophoretic separation.

2.6. Immunoassay procedure

10 μL of 1:400 diluted anti-HSA antiserum was mixed with 10 μL of 0.1 to 125 mM unlabelled HSA and 10 μL of 100 nM FITC-HSA. Different addition orders of the reagents, as well as different incubation times were tested (as later discussed). The mixtures with the three components were then incubated for a defined time (see details in the discussion) at room temperature and then injected. All dilutions were made in a 20 mM sodium phosphate buffer, 0.13 M NaCl, 1 mM EDTA, pH 7.4. The electrophoretic peaks of both free and

bound labelled protein fractions were integrated by means of 32 Karat Software. The calibration curves were obtained by plotting the variation of the analytical signal of the labelled protein as a function of the HSA addition and by interpolating the experimental points with a 3-parameter logistic function. A normalization between 0 and 1 was performed to re-scale the curves in the same range.

3. Results and discussion

The preliminary experimental work was focused on the preparation, the purification and the spectrophotometric characterization of the conjugates between HSA and FITC. The labelling of HSA with FITC was performed using three different reaction molar ratios according to the experimental procedure already described [11]. The reaction molar ratios and the corresponding average substitution degrees are reported in Table 1. The experimental results show that a 5-fold excess of FITC regarding the protein is the minimum required to obtain a substitution degree greater than 1. The use of labelled proteins with high substitution degrees leads to an increase of the corresponding analytical signal with a positive effect on the sensitivity. However, the labelling procedure can occur by generating a population of protein molecules with different surface charges that can produce different electrophoretic behaviour and different protein reactivity towards its own receptors. The result is an increase in the heterogeneity of behaviour that could compromise the binding measurements. For these reasons, the chosen criterion was to operate in experimental conditions to obtain average substitution degrees of around 1 which is a condition where the formation of 1:1 labelled product is mainly favoured. Thus, the HSA-FITC conjugate prepared with a reaction molar ratio of 1:5 was used to continue the experimental work.

Once the labelled protein was prepared more appropriate separation conditions were investigated. The choice of the running buffer composition was made to keep the run current low and to get high reproducibility and efficiency of the electrophoretic peaks. To preserve the binding properties, the pH should be in the range between 7 and 9 where is good the stability of the antibody molecules and relevant the electrosmotic flow. In this range we tested buffers composed by inorganic ions like phosphate and borate as well as organic zwitterionic ions like TRIS, HEPES and CAPS. This preliminary screening of different buffer formulations allowed us to choose 0.1 M CAPS buffer at pH 9.3 as the most suitable. The same buffer has been already described in the separation of proteins and immunoaffinity capillary electrophoresis. [12] The choice of the run voltage should be performed in order to speed up the separation because short migration times reduce the probability of protein adsorption phenomena and immunocomplex dissociation. This, it was also chosen a run potential of 20 kV that ensures good separation of the free and the bound fractions of the labelled

protein, where the latter is the immunocomplex. Figure 1 shows the protein signal obtained in these experimental conditions.

Preliminary binding studies were performed in order to measure the binding properties of the anti-HSA antiserum to the labelled HSA according to the experimental conditions described in Section 2.4. The experimental conditions allowed us to get electrophoretic patterns suitable for the simultaneous quantification of the free labelled protein and the immunocomplex formed at defined concentrations of both reagents. Figure 2 shows the electropherograms achieved. Pattern A was observed at high antiserum dilution (i.e. 1:200) and shows - aside from the free protein peak - a broad peak at lower migration time that can be attributed to the 1:1 immunocomplex formation. Pattern B was attained with lower antiserum dilution (i.e. 1:100) and shows the appearance of narrow peaks at lower migration times probably associated to multiple immunocomplex forms. Pattern C was obtained with an antiserum dilution of 1:50 and shows only two narrow signals of which the one at about 3 minutes could be related to the aggregation of more antibody molecules to the protein. These different profiles show how much the antigen-antibody complexation may vary in function of the antiserum concentration used. Nevertheless, in spite of the different immunocomplex electrophoretic behaviour, all the signals attributed to the protein bound fractions were considered together.

As in the case of immunochemical techniques in the microplate formats, this data was used for the building of a titration curve by plotting the bound to free protein ratio *versus* the antiserum dilution as reported in the graph of Figure 3. This curve provides an estimate of the antiserum dilution that produces an analytical signal equal to 50% of maximum in the tested experimental conditions. This is conventionally called the antibody titer. The antiserum dilution that satisfies this condition is 1:400. The antibody dilution selected through the titration curve allows to get high analytical signals (i.e. the peak areas) without the risk of working with an excess of antibody binding sites. In fact, the latter condition does not promote the competition of the unlabelled protein with the labelled protein for the antibody binding sites that is fulfilled only when the binding site concentration is lower than that of the protein (labelled plus unlabelled).

The next step of the experimental work involved the determination of the affinity constant K between the antiserum and the labelled protein and the antibody binding site concentration Ab_0. It was performed according to a mathematical model reported in section 2.5. The trend of the experimental points as well as the non-linear fit were shown in Figure 4. The estimated values of the parameters a_F and a_B, as well as the affinity binding constant and the antibody site concentration, are instead shown in Table 2.

The obtained value of the binding constant does not appear to be so high considering that this is a polyclonal antiserum not subjected to any affinity purification procedure. In fact, the affinity constant of polyclonal

antiserum for its specific antigen can easily exceed 10^8 M^{-1}. This affinity constant value can be due to the chosen experimental conditions; the separation conditions adopted to measure the strength of the protein-antiserum interaction use a strong electric field and a basic pH buffer that could produce a decrease in the equilibrium constant. However, the value is high enough to be considered acceptable for the assessment of a sensitive immunoassay in capillary electrophoresis. The importance of having high equilibrium constant is due to the inverse relationship between K value and the detectable analyte. In fact, starting from the expression $K=[AbAg]/[Ab][Ag]$ it is possible to affirm that when $[AbAg]\approx[Ab]$ - a condition where approximately all the antibody sites are bound to Ag (the analyte) - then $K\approx1/[Ag]$. Therefore, the sensitivity is affected by K value and it is higher with higher constant.

The values of a_F and a_B which represent the response factors for the free and the bound protein are significantly different from each other and in particular the value measured for the bound protein is higher than that measured with the free protein. This may indicate that the binding to the antibody amplifies the fluorescence signal of the labelled protein as expected by the increased rigidity of the complex.

After the binding properties of the antiserum-labelled HSA pair were assessed, the experimental work was focused on the definition of the calibration curves in order to find the best results in terms of detection limit and dynamic range. The immunoassay format was typically competitive, thus based on the competition between a fixed concentration of labelled protein and a variable concentration of HSA (the analyte) for a fixed concentration of antibody binding sites. The change in the area of the labelled protein was expressed as a function of the increase of the protein concentration by obtaining a trend of the experimental points characterized by a direct proportionality of Y-values with respect to X-values.

The separation conditions were kept constant whereas the addition order of the different reagents in the pre-incubation step was changed. In particular, three different cases were considered:

(1) antiserum and HSA-FITC were mixed and incubated for 10 minutes, then HSA was added,

(2) HSA-FITC and HSA were mixed and incubated for 10 minutes, then antiserum was added;

(3) antiserum and HSA were mixed and incubated for 10 minutes, then HSA-FITC was added.

In cases (**2**) and (**3**), a further pre-incubation step of 10 minutes was performed before injection to let the competition take place, whereas in case (**1**) this time had to be extended to 30 minutes. Each tested condition allowed us to get a competition curve, shown in Figure 5, from which it was possible to measure the corresponding Limit of Detection (LoD), calculated as the protein concentration corresponding to three standard deviations of the blank analytical signal. Well known is the fact that the competition can be assessed by operating in the presence of an antibody site concentration lower than the concentrations of the

labelled and unlabelled protein (taken together). This condition is a basic feature of the competitive format in order to achieve high detection sensitivity but it is not the only one; even the addition order of the reagents which play a role in the competition has to be considered as fundamental.

Case (**1**) – that corresponds to curve A - can clearly disadvantage the competition between the labelled and the unlabelled protein by mostly promoting the formation of the labelled immunocomplex. That is the reason why the corresponding LoD is not only the highest value obtained (26 nM) but it was obtained by greatly extending (30 minutes) the last pre-incubation step (with the unlabelled protein) in order to make the competition somehow possible. On the contrary, the case (**2**) – that corresponds to curve B - allowed us to get a LoD of 22 nM; the simultaneous pre-incubation of labelled and unlabelled protein with the antiserum maintains the balance of the competition and this has a direct effect on the detection limit that is clearly lower than the previous one. Case (**3**) – that corresponds to curve C - gave a sharp gain in sensitivity (5.6 nM) due to the preliminary interaction of the unlabelled protein with the antiserum which promotes a more balanced competition. In conclusion, the experimental data allow us to affirm that the order of addition of the three subjects of the competition plays a determining role in the assay sensitivity.

The dynamic range for the three competition curves appears to be inversely proportional to the LoD; the more sensitive the assay is the more reduced the dynamic range appears to be. This reduction is due to the greater proximity of the experimental points to the limit of detection of the calibration curve.

4. Conclusion

The experimental work described shows that the development of a competitive immunoassay in capillary electrophoresis can be achieved by following a path similar to that generally applied in conventional microplate immunoassays even if it does have some peculiarities. In the capillary format, a high level of protein labelling with fluorescent probes must not always be considered a positive feature because it may generally lead to multi-labelling patterns. Furthermore, the strength of the electric field together with non-equilibrium conditions of separation may reduce the apparent equilibrium constant of the system with a direct effect on the performance of the assay, so the chosen experimental conditions may indeed make the difference. Finally, the use of short incubation steps to speed up analysis obliges us to consider the order of addition of the reagents as a leading parameter for maximizing the competitive phenomena in favour of the analyte, thus improving detection sensitivity.

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Table 1: HSA-FITC reaction molar ratios for and corresponding average substitution degrees

reaction molar ratio	substitution degree
1:2	0.7
1:5	1.0
1:10	1.4

Table 2: adimensional parameters, affinity constant and antibody site concentration calculated according to the mathematical model reported in the text.

m	$(2.96 \pm 0.542) \times 10^{-6}$
n	$(1.23 \pm 0.477) \times 10^{6}$
a_F	$(4.53 \pm 0.19) \times 10^{12}$
a_B	$(1.39 \pm 0.60) \times 10^{13}$
K	$(1.34 \pm 0.312) \times 10^{7}\ M^{-1}$
Ab_0	$(8.82 \pm 3.95) \times 10^{-8}$ M

Figures

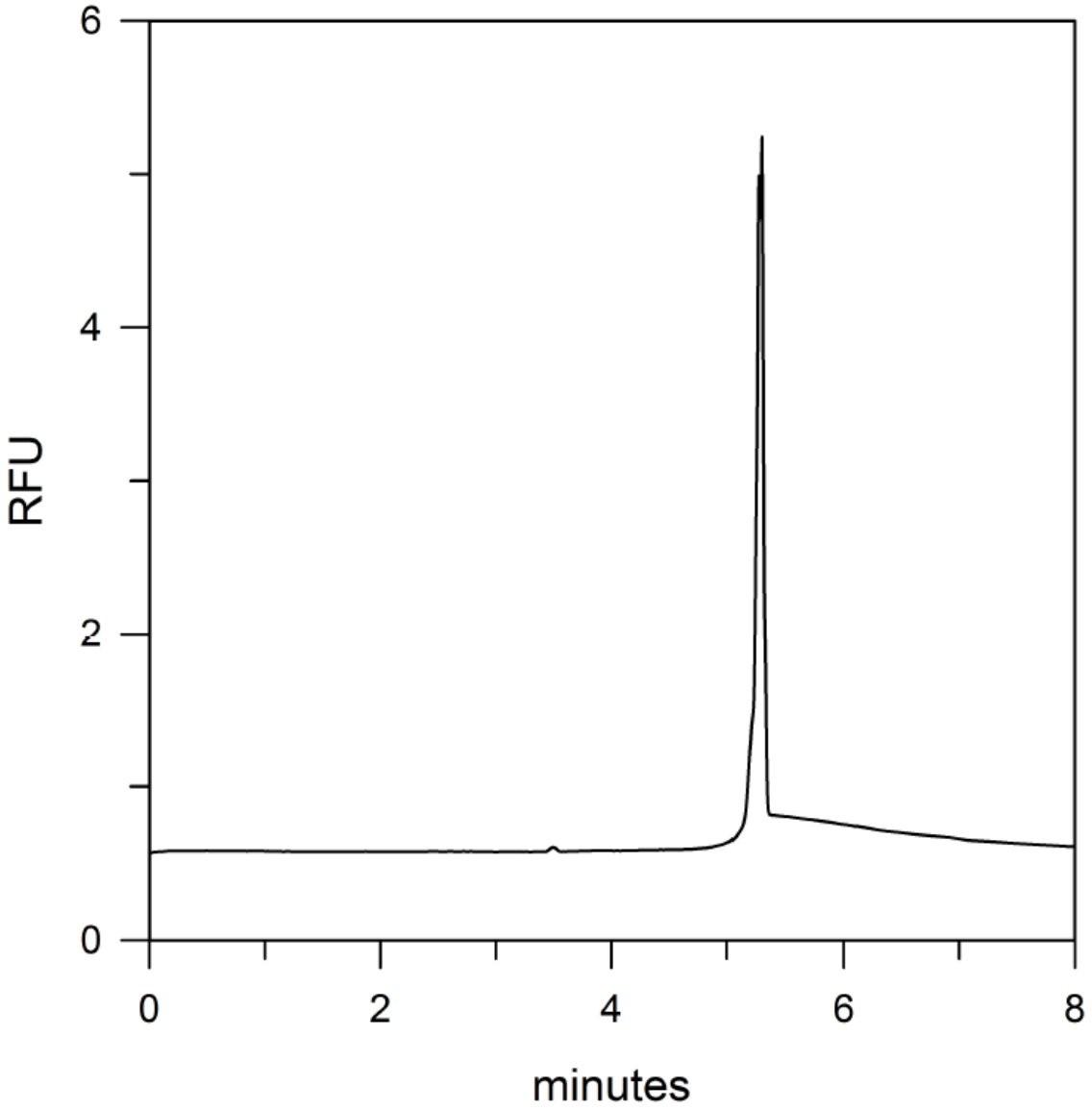

Figure 1: electropherogram of the labelled HSA (reaction molar ratio of 1:5) at 200 nM. Separation conditions: run buffer 0.1 M CAPS pH 9.3; run voltage 20 kV; injection 0.5 psi x 3 s; excitation wavelength 488 nm, emission wavelength 512 nm.

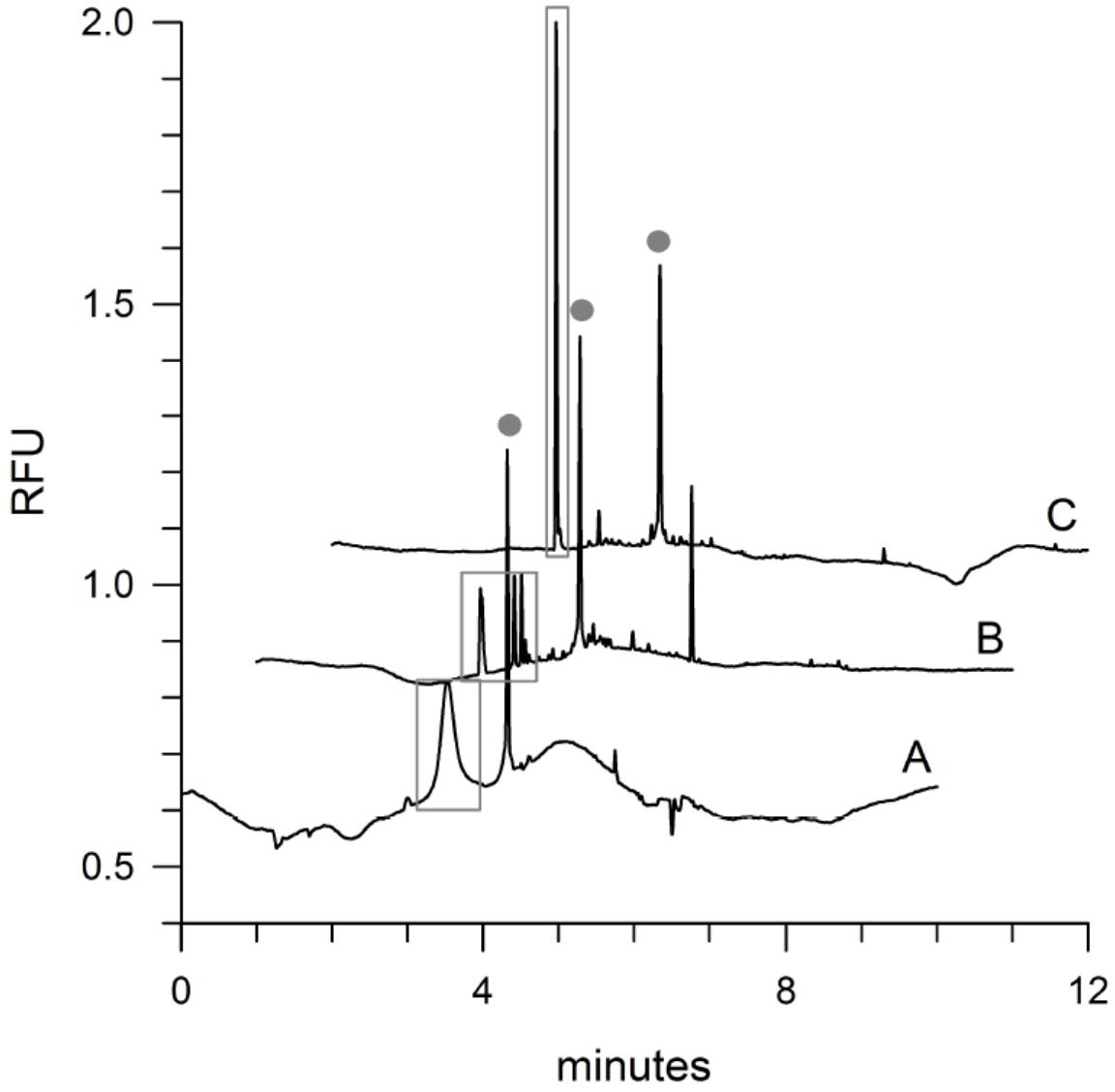

Figure 2: electrophoretic patterns obtained at different antiserum dilutions: A with 1:200 pAb dilution; B with 1:100 pAb dilution; C with 1:50 pAb dilution.The labelled HSA concentration was 100 nM. Separation conditions as in Fig.1. The peaks included in the grey boxes for each pattern are considered different types of immunocomplexes; the grey circles mark the free protein fraction for each pattern.

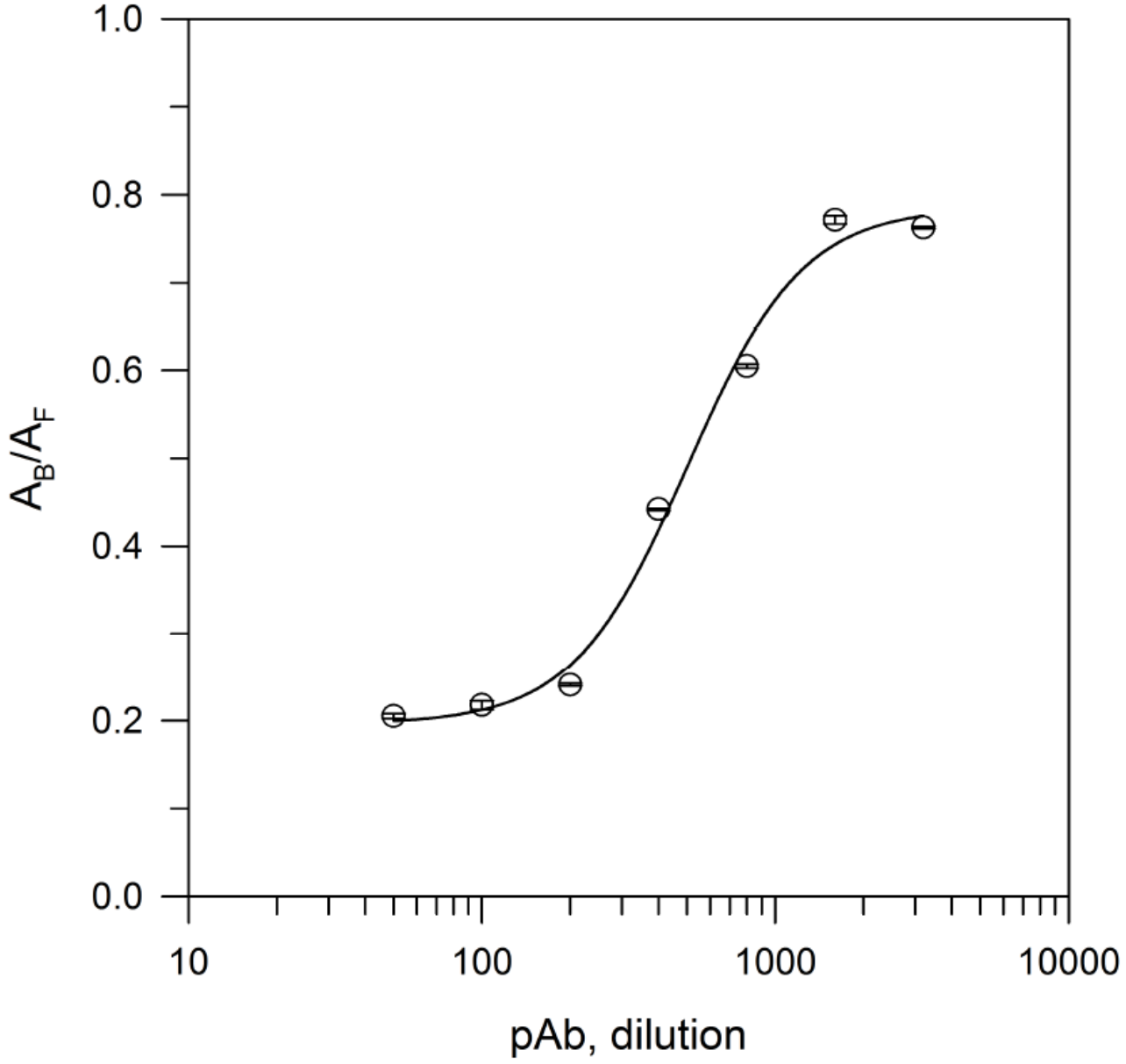

Figure 3: titration curve obtained with the anti-HSA antiserum; A is the area of the free protein at different antiserum dilutions; A_0 is the protein area in the absence of antiserum; the antiserum dilutions used are 1:6400, 1:3200, 1:1600, 1:800, 1:400, 1:200, 1:100, 1:50. Each experimental point is the average of three replicated measures. Separation conditions as in Fig.1.

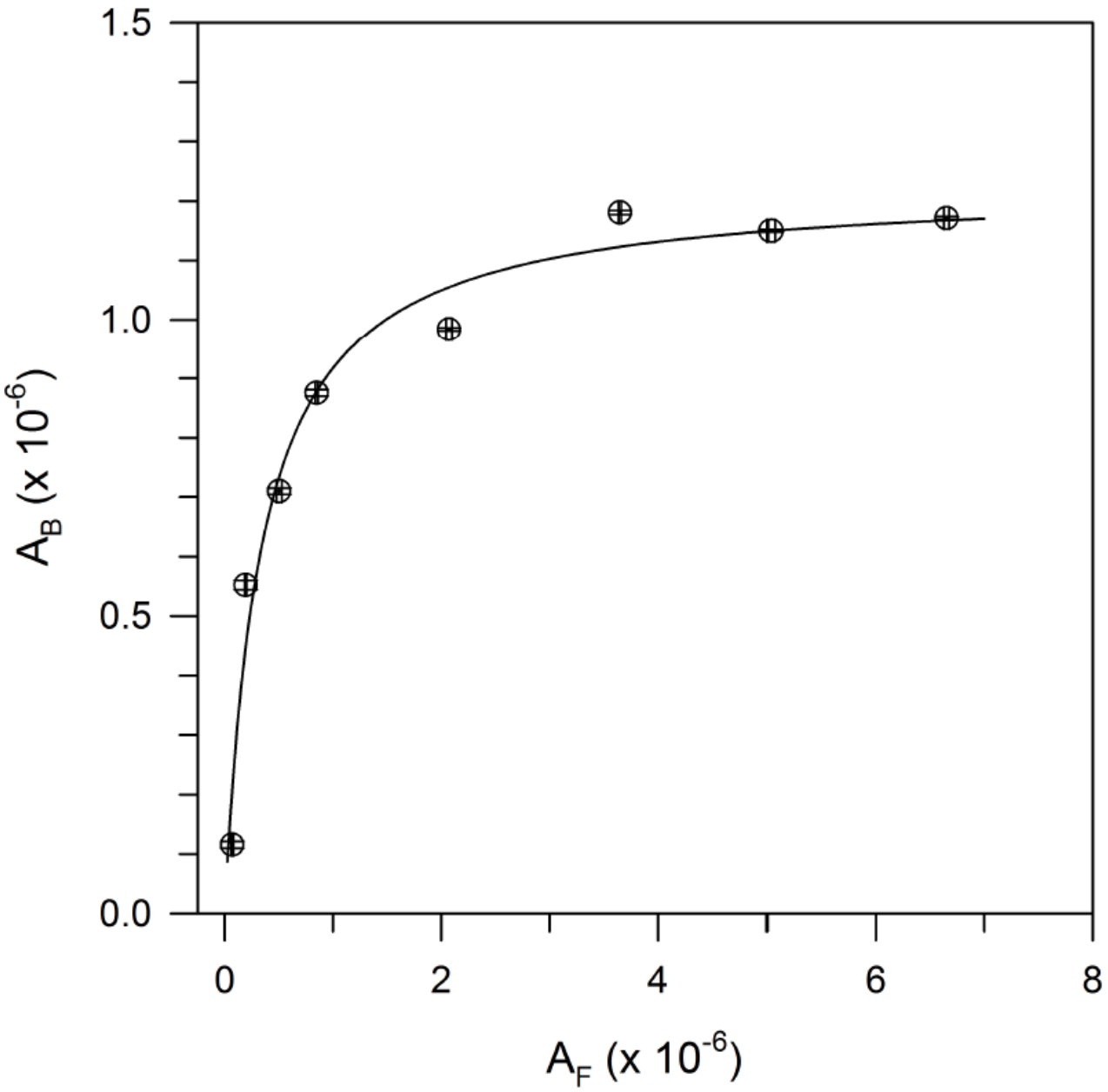

Figure 4: binding isotherm according to the eq 1. A_B is the peak area of the immunocomplex, A_F is the peak area of the labelled protein. Each experimental point is the average of three replicated measures. Separation conditions as in Fig.1.

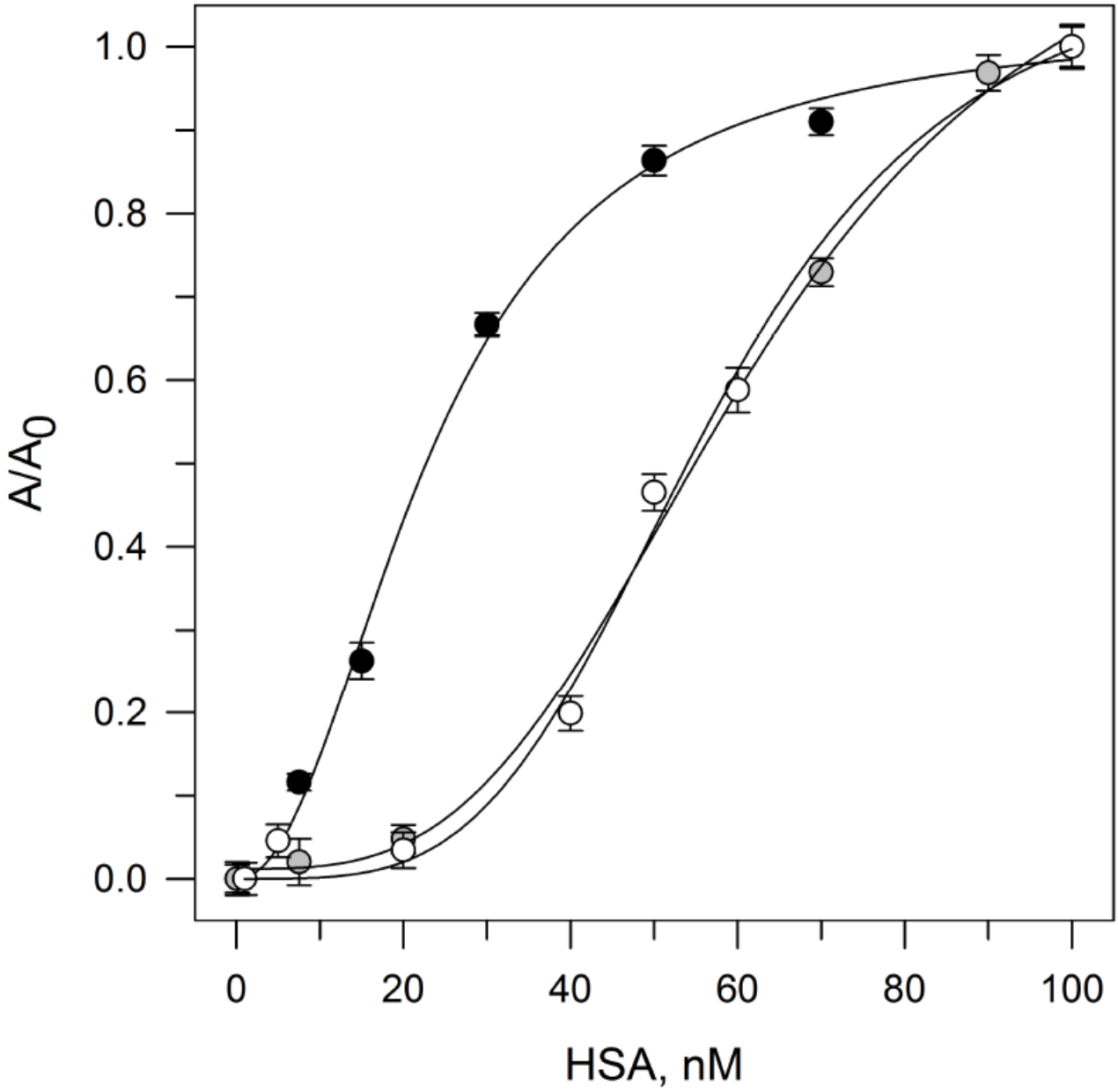

Figure 5: competition curves obtained in different experimental conditions. White circles: preincubation of antiserum with HSA-FITC followed by the addition of HSA. Each experimental point is the average of three replicated measures. Grey circles: preincubation of HSA-FITC with the HSA followed by the addition of antiserum. Black circles: preincubation of antiserum with HSA followed by the addition of HSA-FITC.