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RESEARCH PAPER

Modified capillary electrophoresis based measurement of the binding between DNA aptamers and an unknown concentration target

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Abstract The recognition of targets such as biomacromolecules, viruses and cells by their aptamers is crucial in aptamer-based biosensor platforms and research into protein function. However, it is difficult to evaluate the binding constant of aptamers and their targets that are hard to purify and quantify, especially when the targets are undefined. Therefore, we aimed to develop a modified capillary electrophoresis based method to determine the dissociation constant of aptamers whose targets are hard to quantify. A protein target, human thrombin, and one of its aptamers were used to validate our modified method. We demonstrated that the result calculated by our method, only depending on the aptamer's concentrations, was consistent with the classical method, which depended on the concentrations of both the aptamers and the targets. Furthermore, a series of DNA aptamers binding with avian influenza virus H9N2 were confirmed by a four-round selection of capillary electrophoresis–systematic evolution of ligands by exponential enrichment, and we identified the binding constant of these aptamers by directly using the whole virus as the target with the modified method. In conclusion, our modified method was validated to study the interaction between the aptamer and its target, and it may also advance the evaluation of other receptor–ligand interactions.

Keywords Capillary electrophoresis · Aptamer · Complicated target · Dissociation constant

Introduction

Aptamers are single-stranded oligonucleotides isolated in an in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX) [1, 2]. With the ability of folding into different three-dimensional structures, aptamers can bind to a wide range of targets with high affinity and specificity [3, 4], similar to antibody–antigen interaction. Besides, compared with antibodies, aptamers are easier to produce and modify, and they are more inexpensive, stabler, and more suitable for stringent conditions such as extreme temperatures and pH, which make them useful in the fields of pathogen diagnostics and chemical analysis [5].

In the detection method with aptamers, the binding affinity between the aptamer and its ligand is a critical factor for the sensitivity. The binding ability can be measured by techniques such as gel electrophoresis [6], capillary electrophoresis (CE) separation [7] and surface plasma resonance based detection [8, 9], among others [10]. In addition, in single-molecule measurements, atomic force microscopy [11], fluorescence resonance energy transfer [12] and laser tweezers[13] have also been used to determine the dissociation constant of aptamer–ligand interactions. All these methods have been successfully applied to measure the binding affinity between aptamers and their targets when the background information on the targets was clear and accurately quantified, and it was possible to prepare the

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solution with only one concentration of target in single-molecule measurements.

However, the applications of the aforementioned methods are limited because they are not appropriate for the aptamers selected for some complex targets, such as biomacromolecules and biological particles, and it is very difficult to determine the characteristics of these complicated targets that cannot be purified or are even totally unknown to us. We developed a modified method, derived from the classical CE-based method [14, 15], by which the binding constant of a complex of an aptamer and its target can be calculated even if the concentration of the target cannot be determined. Human thrombin and one of its previously reported aptamers (thrombin aptamer, TBA) [16] were used to verify the modified method and determine the application conditions. Furthermore, the method was applied to measure the binding ability of the newly acquired DNA aptamers against the whole viral particle of avian influenza virus H9N2, and the results were recalculated with the classical method. The results showed that there is good correlation between the modified CE method and the classical method in the measurement of the binding capability of aptamers and their targets.

Experimental

Materials

Human thrombin, glycerin and bovine serum albumin (BSA) was purchased from Sigma. Skim milk was purchased from BD. Tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (1 M, pH 8.3) and 4× Tris–glycine (TG) buffer (100 mM Tris, 768 mM glycine, pH 8.5) were purchased from Solairbo and diluted to the desired concentration. All oligonucleotides used were synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China), and some aptamers were labelled with 6-carboxyfluorescein (FAM) at the 5′ end for detection by laser-induced fluorescence. Avian influenza virus strain A/Chicken/Hebei/3/98(H9N2) was obtained from Harbin Veterinary Research Institute, China.

Equipment preparation

All CE experiments were performed using P/ACE MDQ CE equipment (Beckman Coulter, USA) with bare fused-silica capillaries of 75-μm inner diameter and 360-μm outer diameter with a total length 60.2 cm (50 cm to the detection window). A UV detector was used in the CE-SELEX procedure and a laser-induced-fluorescence detector (λ_{ex}= 488 nm, λ_{em}=520 nm) was used in K_d determination. Before everyday use, the capillary was rinsed with methanol, 1 M HCl and H_2O, each for 5 min, with 0.1 M NaOH for 20 min and finally with H_2O for 3 min. Between runs the capillary was rinsed with H_2O and running buffer, each for 2 min. All data were processed with 32 Karat (version 4.0, Beckman Coulter).

Method derivation

The new method in the present research is derived from a classical CE method [17] which is used to determine the binding constant based on the concentrations of the targets and their corresponding peak area according to following equation (a detailed description is given in Fig. 1):

$$K_d = \frac{[T](1 + 1/R) - [Apt]}{1 + R}, \quad (1)$$

where R is $(A_1+A_2)/A_3$, [T] is the concentration of the target and [Apt] is the concentration of the aptamer.

The detailed derivation steps of the method are as follows. Two equal volumes of an aptamer with different concentrations were incubated with the same amount of target, and the two mixtures were injected into the capillary one by one. Although the binding constant cannot be determined directly without the concentration of the target being known according to Eq. 1, we will get two equations as $K_d(1+R_1)+[Apt]_1=[T](1+1/R_1)$ and $K_d(1+R_2)+[Apt]_2=[T](1+1/R_2)$. If the first equation is divided by the second one, [T] will be eliminated, and the equation can be changed into Eq. 2:

$$\frac{K_d(1 + R_1) + [Apt]_1}{K_d(1 + R_2) + [Apt]_2} = \frac{R_2(1 + 1/R_1)}{R_1(1 + 1/R_2)}. \quad (2)$$

Finally, after mathematical transformation, Eq. 2 can be simplified and changed into Eq. 3:

$$K_d = \frac{1}{R_1 - R_2}\left(\frac{[Apt]_2}{1 + 1/R_2} - \frac{[Apt]_1}{1 + 1/R_1}\right), \quad (3)$$

which was used in the modified method for affinity determination.

Method verification using thrombin and TBA

Human thrombin in concentrations of 50, 200 and 500 nM was applied and mixed with a series of concentrations of fluorescently labelled TBA (5′-FAM-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′) solutions with concentrations ranging from 10 to 500 nM. After incubation in sample buffer (1× TG) for 30 min at room temperature, the mixtures were introduced by hydrodynamic injection at 0.5 psi for 5 s, and separated at +30 kV and 20 °C in running

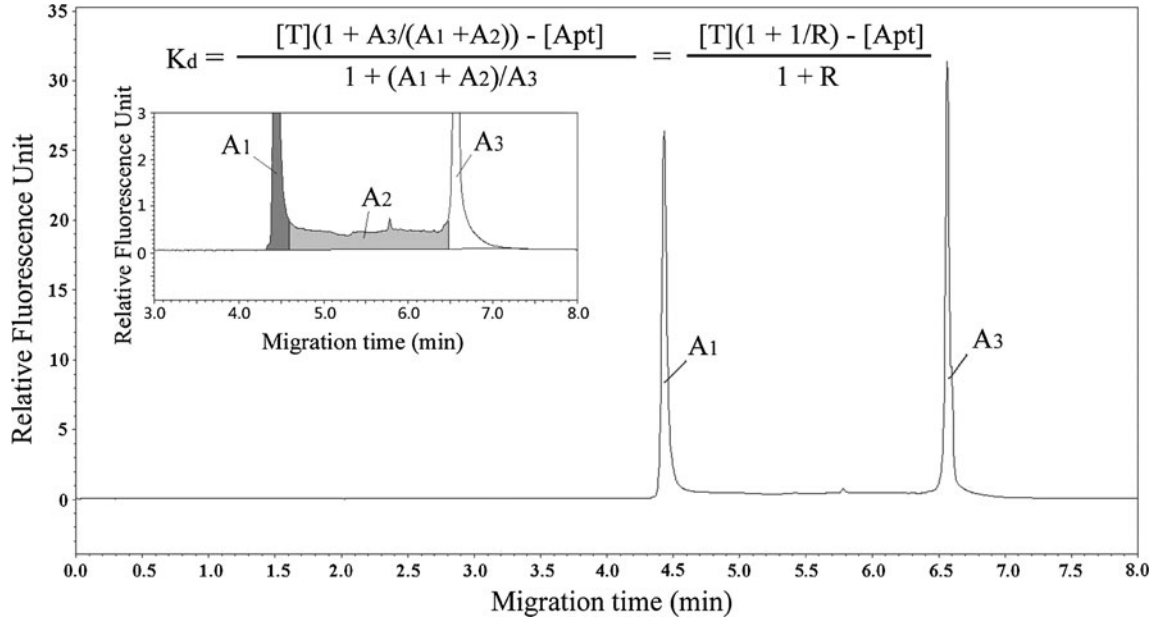

Fig. 1 K_d determination in the classical method. The mixture contained 80 nM thrombin aptamer (TBA) and 100 nM thrombin. The capillary electrophoresis (CE) conditions were as follows: running buffer 2× tris(hydroxymethyl)aminomethane (Tris)–glycine at pH 8.5, 0.5-psi injection for 5 s, separation voltage +30 kV. The *inset* shows the detailed view for A_1, A_2 and A_3 which represents the areas of peaks of the complexes (A_1), the aptamer dissociated from the complex during electrophoretic separation (A_2) and the free aptamer (A_3), respectively. In the equation, [T] and [Apt] are total concentrations of the target and aptamer, respectively

buffer (2× TG). The binding constants were calculated firstly according to the classical method, and were then compared with the results calculated by the modified method. Furthermore, the modified method was verified using the contaminated thrombin samples with some interfering reagents such as 1 % (v/v) glycerin, 10 μg/mL skim milk and 10 μg/mL BSA.

K_d determination for aptamers against avian influenza virus H9N2

DNA aptamers were selected against haemagglutinin (HA) protein of avian influenza virus H9N2 by a CE–SELEX process, which is described in detail in the electronic supplementary material. After selection, purified HA was used as the target to assess the binding affinity by both the classical and the modified method. Since HA is an important surface protein of avian influenza virus, the virus solution could be used directly to determine the values of K_d according to the modified method, once the final aptamers had been confirmed to be capable of binding to the whole virus. The HA–aptamer equilibrium mixture was prepared in 50 mM Tris–HCl (pH 8.3), and the running buffer was changed to 100 mM Tris–HCl (pH 8.3). A plug of the equilibrium mixture was injected into the capillary at 0.5 psi for 5 s and separated under +30 kV at 20 °C.

Result and discussion

K_d determination by the classical method

A series of mixtures containing different concentrations of thrombin and TBA were tested, and all the results were calculated with Eq. 1. As the results in Table 1 show, in the group of 50 nM thrombin, consistent K_d values (31.25± 0.97 to 34.72±3.10 nM) were achieved with TBA concentration ranging from 10 to 80 nM. Similar results can also be seen in the 200 nM thrombin group with TBA concentration ranging between 50 and 200 nM, and in the 500 nM thrombin group with TBA concentration ranging between 50 and 400 nM. However, obvious errors (Table 1) can be seen in the final values of K_d when higher concentrations of TBA were used in all three groups.

A linear transformation of Eq. 1, transformed to $U=-K_dR+[T]$, where $U=[Apt]/(1+1/R)$, could be used to explain these errors. Figure 2 shows the results of the linear analysis, and a linear relationship between U and R is seen when low concentrations of TBA were used (solid dots in Fig. 2), such as TBA concentration ranging from 10 to 80 nM in the group of 50 nM thrombin, TBA concentration ranging from 50 to 200 nM in the group of 200 nM thrombin and TBA concentration ranging from 100 to 400 nM in the group of 500 nM thrombin, which were the same as the results in Table 1. When relatively high TBA concentrations

Table 1 K_d determination by the classical method

TBA (nM)	50 nM thrombin		200 nM thrombin		500 nM thrombin	
	R	K_d (nM)	*R*	K_d (nM)	*R*	K_d (nM)
10	1.29±0.03	34.35±0.74	NT	NT	NT	NT
25	1.17±0.03	31.25±0.97	NT	Non	NT	NT
50	0.81±0.05	34.72±3.10	4.75±0.04	33.44±0.28	8.23±0.12	55.39±0.80
80	0.59±0.02	34.29±2.31	4.04±0.10	33.70±0.85	NT	NT
100	0.30±0.03	90.77±14.01	3.53±0.05	34.62±0.61	7.63±0.17	53.98±1.23
120	0.15±0.05	329.03±131.51	NT	NT	NT	NT
150	0.14±0.05	476.26±347.66	NT	NT	NT	NT
200	0.13±0.05	407.54±242.07	1.68±0.11	45.16±4.44	6.89±0.06	47.21±0.45
250	NT	NT	0.46±0.14	460.22±213.60	NT	NT
300	NT	NT	0.53±0.11	272.24±139.37	4.41±0.12	58.10±1.83
400	NT	NT	0.41±0.06	227.12±53.32	2.99±0.34	70.22±11.71
500	NT	NT	NT	NT	1.51±0.13	136.07±20.16

NT no test, *TBA* thrombin aptamer

were used, low values of *R* were achieved, resulting in the low values of *U* in Fig. 2 (hollow dots) and the low values of K_d in Table 2. The low *R* values were mainly due to the incorrect baseline analysis because of the low proportion of the binding sequences when higher concentrations of aptamers were used, and especially when lower concentrations of targets were used at the same time. Thus, high concentration of aptamers and low concentration of targets were supposed to be the application condition of the classical method.

K_d determination by the modified method

According to Eq. 3, when two samples with the same concentration of thrombin were tested, the *R* values and the corresponding aptamer concentrations could be used to calculate the binding constants. As the data in Table 2 show, compared with the classical method, only the results in the 200 nM thrombin group showed a significant difference, which might be mainly due to the accuracy of the target concentration. A similar situation was also seen in the calculation of the aptamer binding affinity for avian influenza virus H9N2. However, it could still be concluded that the K_d values calculated by the modified method were similar to those calculated by the classical method. In addition, errors were also found in the results calculated by the modified method involving the data obtained from the tests using high aptamer concentrations. For example, K_d (−21.50± 2.27 nM) was much less than zero, calculated with 50 nM thrombin, and 80 nM and 100 nM TBA. According to the comparison between the two methods, we can draw a conclusion that the modified method has the same application conditions as the classical method and gives results consistent with the classical method.

K_d determination of the contaminated thrombin samples

As the data in Table 3 show, when 1 % (v/v) glycerin, 10 μg/mL skim milk and 10 μg/mL BSA, respectively, were added to the target solutions, the K_d values were 24.63± 1.21, 31.78±1.84 and 48.15±7.57 nM, similar to the values of 28.98±0.94, 42.75±4.58 and 64.89±5.10 nM (as shown

Table 2 Comparison between the classical method and the modified method

Method	K_d (nM) determined in different thrombin concentrations		
	50 nM thrombin	200 nM thrombin	500 nM thrombin
Classical method[a]	33.65±0.96	36.73±1.77	56.98±2.85
Modified method[b]	42.75±4.58[c]	28.98±0.94[d]	64.89±5.10[c]

[a] Statistical calculation according to the data in Table 1 after rejecting the wrong data

[b] Statistical calculation according to the data in Table S

[c] No difference compared with the classical method, $P>0.05$

[d] Significant difference compared with the classical method, $P<0.01$

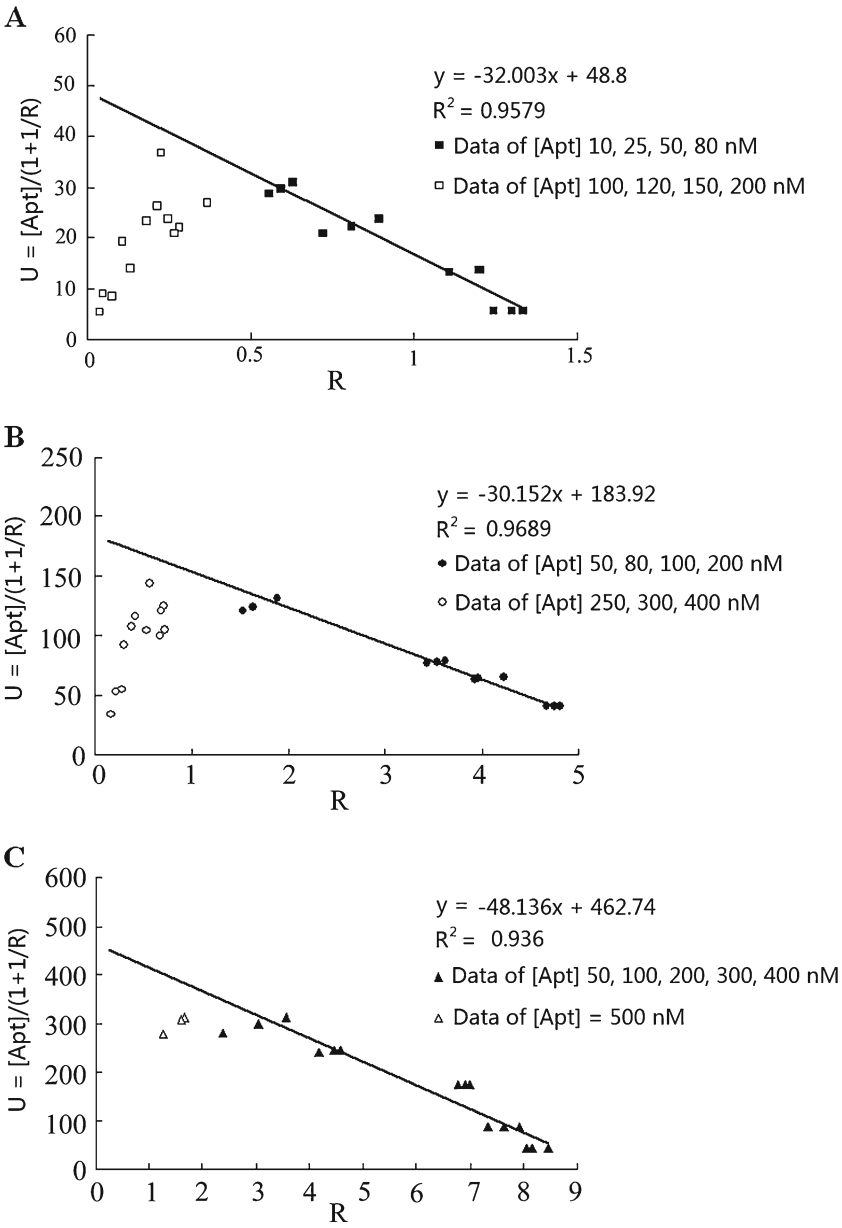

Fig. 2 The linear analysis of the relationship between *U* and *R*. The results for the 50 nM thrombin group (**a**), the 200 nM thrombin group (**b**) and the 500 nM thrombin group (**c**) are displayed. The *line* in each graph is drawn according to the data corresponding to the *solid symbols*, and the correlation coefficient and the fitted formula are shown in the *upper-right corner* in **a**–**c**

in Table 2) when no interfering reagents are added. Therefore, the interfering reagents added to the target samples do not affect the determination of the binding affinity significantly, and it is estimated that other reagents would also not influence the interaction between TBA and thrombin directly because of the high specificity of TBA. However, if an excessive amount of interfering reagents is involved, they would significantly affect the chemical conditions of the mixture, such as the ionic strength and pH, which are important factors affecting the aptamer–target interaction. That might be the reason for the lower K_d values found when BSA was added to the mixture. Indeed, a high concentration of target might have a similar influence on the aptamer–target interaction (a higher K_d resulted in the 500 nM thrombin group) as shown in Table 2. Thus, preliminary purification as well as appropriate enrichment is recommended to improve the accuracy of CE detection.

Table 3 K_d determination of the contaminated thrombin samples

Interfering reagents	TBA (nM)	*R*	K_d (nM)
1 % (v/v) glycerin	100	0.36±0.01	48.15±7.57
	50	0.56±0.04	
10 μg/mL skim milk	100	0.85±0.02	31.78±1.84
	50	1.39±0.03	
10 μg/mL BSA	100	0.78±0.02	24.63±1.21
	50	1.38±0.03	

BSA bovine serum albumin

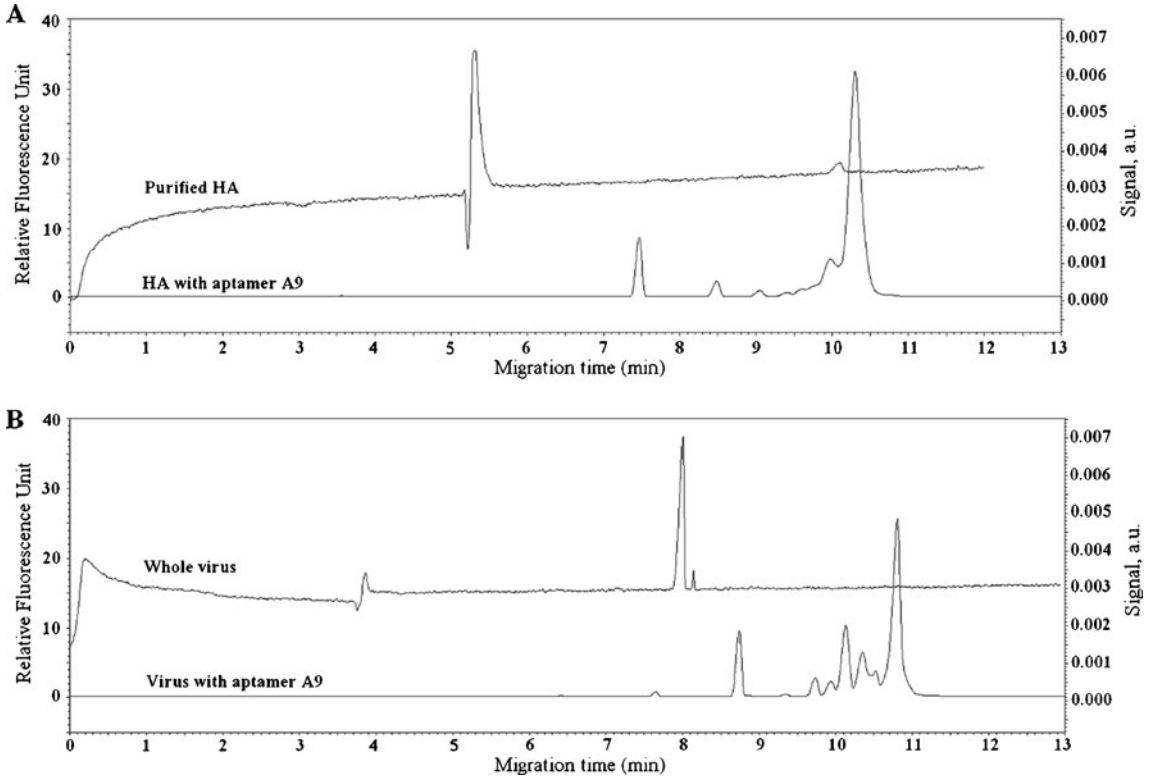

Fig. 3 CE detection of A9 binding to purified haemagglutinin (*HA*) and to whole virus. **a** A9 binding to purified HA. The migration time of the complex was at 7.5 min. **b** A9 directly binding to whole virus. The migration time of the complex was at 8.7 min. The CE conditions were as follows: running buffer 100 mM Tris–HCl at pH 8.3, 0.5-psi injection for 5 s, separation voltage +30 kV

Assessing the binding capacity of aptamers against avian influenza virus H9N2

Purified HA protein of avian influenza virus H9N2 was applied as the target for aptamer selection through a four-round CE–SELEX procedure. Two aptamers, namely, A9 and B4, were confirmed to be capable of binding directly to avian influenza virus H9N2 (Fig. 3). Since the binding target in the virus solution could not be quantified, only the modified method could be used to determine the binding affinity of A9 and B4 directly against the whole particle. As the data in Table 4 show, the K_d values of A9 were in the same range regardless of whether purified HA (K_d=46.23±5.46 nM) or the whole virus (K_d=42.54±9.75 nM) was used as the target in the modified method. However, there was a significant difference between the two methods (31.48±0.96 nM in the classical method, 46.23±5.46 nM in the modified method, P<0.01) when purified HA was used as

Table 4 The binding capacity of aptamers against avian influenza virus H9N2

Group of different targets		Results for A9		Results for B4	
		20 nM aptamer	40 nM aptamer	20 nM aptamer	40 nM aptamer
Purified protein, 25 nM HA	*R*	0.55±0.02	0.43±0.01	1.20±0.08	0.60±0.03
	K_d in classical method (nM)	31.48±0.96[a]		14.40±1.46[a]	
	K_d in modified method (nM)	46.23±5.46		7.38±1.09	
Whole virus	*R*	0.49±0.03	0.36±0.02	0.58±0.02	0.30±0.01
	K_d in modified method (nM)	42.54±9.75[b]		6.37±0.70[b]	

A9 5′-**GCT GCA ATA CTC ATG GAC AG**C CTC CTG GGG TCA GGC TCA GAC ATT GAT AAA GCG ACA TCG **GTC TGG AGT ACG ACC CTG AA**-3′, *B4* 5′-**GCT GCA ATA CTC ATG GAC AG**G GGC CGC GCC TGG TCG GTT GGG TGG GTG GCG CCC GGG ACG **GTC TGG AGT ACG ACC CTG AA**-3′, HA haemagglutinin

[a] Significant difference compared with the modified method using the purified protein as the target, P<0.01

[b] No difference compared with the modified method using the purified protein as the target, P>0.05

the target. Similar results were observed in the determination of the B4 binding affinity. This kind of difference might result from the inaccurate quantitative concentration of the aptamer which was directly applied in the classical method. Nevertheless, the results from these two methods were of the same order of magnitude, and it could be concluded that the modified method was applicable not only for the purified protein but also for the whole virus. The successful application of the whole virus to determine the binding constant suggests that some bioparticles whose multiple binding sites are independent each other could also be used as targets for K_d determination of the aptamers, as long as the irrelevant ingredients have little influence on the binding of the aptamers to the binding sites of the targets.

In addition, 25 nM purified HA and 12 μg/mL viruses were applied because some delayed migrations occurred in CE detection (data not shown) as high concentrations of the virus (or purified HA) solution may lead to non-specific adsorption. Further optimization of the CE condition for HA and the whole virus might be helpful to improve the concentrations of HA and virus.

Conclusions

In summary, a modified CE-based method has been developed especially to measure the binding affinity of DNA aptamers and their corresponding targets such as biomacromolecules or bioparticles that cannot be quantified accurately. Appropriate enrichment and preliminary purification may be favourable to a more accurate result, so they are strongly recommended. As a CE-based method, optimization of the CE conditions is necessary to ensure a typical peak of the target can be detected, and the target should be used in the proper quantity to ensure excellent repeatability. We anticipate that the method described here will be useful not only for the measurement of the binding affinity of aptamers but also for measurement of other molecules.

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