ZongWen Wang
YingWei Fan
JinFa Chen
Ying Guo
WeiHua Wu
Ye He
LiangJun Xu
FengFu Fu

Key Lab of Analysis and Detection for Food Safety of Ministry of Education, Fujian Provincial Key Lab of Analysis and Detection for Food Safety, Department of Chemistry Fuzhou University, Fuzhou, Fujian, P.R. China

Received March 12, 2013
Revised April 16, 2013
Accepted April 17, 2013

Research Article

A microfluidic chip-based fluorescent biosensor for the sensitive and specific detection of label-free single-base mismatch via magnetic beads-based "sandwich" hybridization strategy

A novel microfluidic chip-based fluorescent DNA biosensor, which utilized the electrophoretic driving mode and magnetic beads-based "sandwich" hybridization strategy, was developed for the sensitive and ultra-specific detection of single-base mismatch DNA in this study. In comparison with previous biosensors, the proposed DNA biosensor has much more robust resistibility to the complex matrix of real saliva and serum samples, shorter analysis time, and much higher discrimination ability for the detection of single-base mismatch. These features, as well as its easiness of fabrication, operation convenience, stability, better reusability, and low cost, make it a promising alternative to the SNPs genotyping/detection in clinical diagnosis. By using the biosensor, we have successfully determined oral cancer-related DNA in saliva and serum samples without sample labeling and any preseparation or dilution with a detection limit of 5.6×10^{-11} M, a RSD ($n = 5$) < 5% and a discrimination factor of 3.58–4.54 for one-base mismatch.

Keywords:
Biosensor / Magnetic beads / Microfluidic chip / Oral cancer / Single nucleotide polymorphism
DOI 10.1002/elps.201300131

1 Introduction

Many studies have reported that the individual differences in drug metabolism or susceptibility to diseases such as cancer and some genetic diseases are caused by SNPs and short insertion/deletion variations (InDels) [1, 2]. These studies have led to an increasing demand to detect specific genes in clinical samples for diagnostic purposes. For above reason, many hybridization-based DNA biosensors have been developed for the sensitive and specific detection of DNA [3–5]. Especially, the recently developed hybridization-based DNA biosensors that take advantage of modern optoelectronics, show great promise for rapid, sensitive, and cost-effective DNA detection in clinical diagnostics [6, 7]. However, practical application of these biosensors is greatly hampered by one or more of the following disadvantages: poor resistibility to the complex matrix of real biological samples, poorer specificity and stability, sophisticated operation, time-consuming fabrication, long analysis time, and so on. Spurred by the aforementioned limitations of conventional electrochemical and optical biosensors, the microfluidic chip-based biosensor was proposed for the high-throughput analysis of biomolecule and has received increasing attention [8, 9]. For example, some microfluidic chip-based biosensors have been developed for the high-throughput analysis of antigens by combining the specific binding of antibody–antigen and nanoparticle amplification [10, 11]. Compared with conventional electrochemical and optical biosensors, microfluidic chip-based biosensors promise advantages such as easiness of fabrication, higher automation ability, better reusability, and high-throughput analysis etc. Especially, the utilization of magnetic beads (MBs) has made the fabrication of microfluidic chip-based biosensor easier. To date, several microfluidic chip-based biosensors, which used MBs as carrier for the immobilization of DNA probe in microchannel, have been developed for the high-throughput and rapid analysis of DNA species [12–15]. However, most microfluidic chip-based biosensors previously reported have obvious drawbacks, such as lower sensitivity, poorer discrimination ability for the single-base mismatch and poor resistibility to complex matrices of real biological samples due to the use of pump-driving mode. Not long ago, a microfluidic chip-based biosensor, which

Correspondence: Dr. FengFu Fu, Key Lab of Analysis and Detection for Food Safety of Ministry of Education, Fujian Provincial Key Lab of Analysis and Detection for Food Safety, Department of Chemistry, Fuzhou University, Fuzhou, Fujian 350108, P. R. China
E-mail: fengfu@fzu.edu.cn
Fax: +86-591-22866135

Abbreviations: $\mathbf{C_P}$, capture probe DNA; **MB**, magnetic bead; $\mathbf{S_P}$, signal probe DNA

Colour Online: See the article online to view Scheme 1, Figs. 1, and 3–5 in colour.

used electrophoretic driving mode and "duplex" hybridization strategy, was developed for the detection of DNA [16]. The biosensor has robust resistibility to complex matrices of real biological samples, however it needs a complex and time-consuming sample-labeling procedure. All above limitations of previous microfluidic chip-based biosensors have seriously impeded the practical application of microfluidic chip-based biosensors in clinical detection.

In an effort to resolve aforementioned limitations, in this contribution, we design a novel microfluidic chip-based biosensor based on the combination of electrophoretic driving mode, MBs-based "sandwich" hybridization strategy and sensitive LIF detection. Compared with the existing microfluidic chip-based biosensors, the proposed microfluidic chip-based DNA biosensor does not need sample labeling and is much more robust to complex matrices of real samples. In addition, the proposed microfluidic chip-based DNA biosensor has better reusability, excellent discrimination ability for the detection of single-base mismatch, and relatively high sensitivity. It can be used for the detection of oral cancer-related DNA species in saliva and serum sample without sample labeling and any preseparation or dilution. The success in the present biosensor served as a significant step toward the practical application of the biosensor in clinical diagnosis.

2 Materials and methods

2.1 Reagents and apparatus

Tris, EDTA, hydrochloric acid (HCl), Tween-20, and sodium chloride (all of analytical grade) were obtained from the Shanghai Chemical Factory (Shanghai, China). Streptavidin-functionalized MBs (1 μm, 10 mg/mL) were purchased from Dynal Biotech, USA. The buffer solutions are as follows: the immobilization buffer is a mixture of 2 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5); the washing buffer is a mixture of 1 M NaCl, 0.5 mM EDTA, and 5 mM Tris-HCl (pH 7.5); the running and hybridization buffer is a mixture of 900 mM Tris-HCl, 20 mM EDTA, and 0.1% v/v Tween-20 (pH 7.75). All buffer solutions were filtered with a 0.22 μm Millipore membrane filter before use. The water used in this experiment was Milli-Q water (18.2 MΩ/cm).

All oligonucleotides used in this study were synthesized by the Shanghai Sangon Biological Engineering Technology Services Reagents Co. (China). The DNA sequences are given in Table 1. The target DNA is a DNA species related to oral cancer. The single-base mismatch DNA has one different base (underlined one) in comparison with the target DNA. The 100 μM stock solutions of each DNA were prepared by dissolving above DNA in water and stored at −40°C. More dilute DNA solutions were prepared by diluting the stock solution to the desired concentration with water except for the signal probe, which was diluted to the desired concentration with running buffer.

A LIF detector (Shandong Normal University, China), which has an excitation wavelength of 473 nm and an emission wavelength of 525 nm, was used for the determination of fluorescence signal. Data collection, processing, and analysis were performed with a chromatography workstation (model HW-2000, Qianpu Software Shanghai, China). A microchip high-voltage source (XCDY, Chinese Academy of Sciences, China) was used to supply voltage for the electrokinetic injection of samples or target DNA and the electrophoretic driving of MBs in this study.

Table 1. The details of the DNA sequences used in the experiment

Capture probe	5′biotin-TTT TTT TCA GCC GTC TTC-3′
Signal probe	5′-AAA AAC TTC TCC-FAM-3′
Target DNA	5′-GGA GAA GTT TTT GAA GAC GGC TGA-3′
Single-base mismatch DNA	5′-GGA GAA GTT TTT GAA C<u>A</u>C GGC TGA-3′
	5′-GGA GAA GTT TTT GAA A<u>A</u>C GGC TGA-3′
	5′-GGA GAA GTT TTT GAA T<u>A</u>C GGC TGA-3′
Noncomplementary DNA	5′-TCA GCC GTC TTG AAA AAC TTC TCC-3′

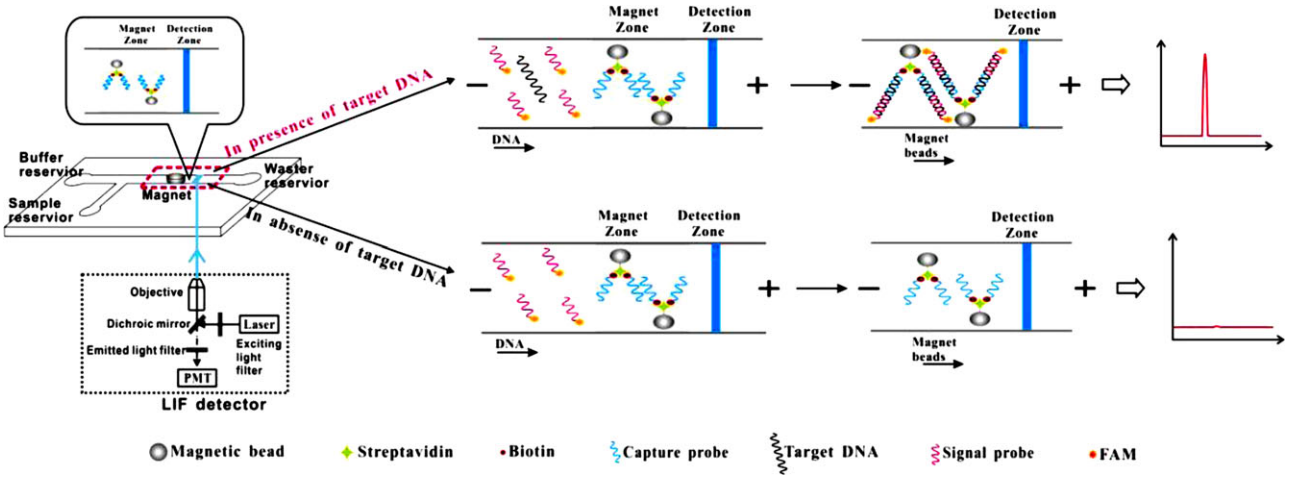
FAM, carboxyfluorescein tag.

2.2 Fabrication of the "single-T" microfluidic chip

The "single-T" microfluidic chip used in the experiment has a 50 μm-wide, 15 μm-deep, and 15 mm-long separation channel. The main process of fabricating the glass microfluidic chip was the same as that described previously [17]. First, the indium oxide glass was cleaned with acetone and Milli-Q water in an ultrasonic bath for 5 min, respectively. Then, the indium oxide glass was spin-coated with BP218 positive photoresist. After soft baking at 120°C, standard lithography was performed to define the microstructure in the glass. Then, the microchannel was formed via wet etching technology. In another cover glass, three holes with 3.0 mm diameters were drilled as buffer reservoir, sample reservoir, and waste reservoir, respectively, and then the cover glass was thermally bonded to the etched glass at 590°C to fabricate the microchip.

2.3 Fabrication of the microfluidic chip-based fluorescent DNA biosensor

The immobilization of capture probe DNA (C_P) on the surface of MBs was carried out by a procedure recommended by Dynal Biotech. About 3 μL of streptavidin-functionalized MBs solution was first mixed with 4 μL of 4 μM capture probe DNA solution and 4 μL of the immobilization buffer solution. Then, the mixed solution was incubated at 37°C for 15 min to immobilize the capture probe DNA on the surface of MBs by the specific interaction between streptavidin and biotin. The C_p-modified MBs were then separated and redispersed in 10 μL of washing buffer solution again. Second, two magnet buttons (2 mm diameter and 2 mm thick) were fixed on the top and bottom of the separation channel of microfluidic chip, respectively (see Scheme 1). The site of the magnet buttons



Scheme 1. The experimental principle of the microfluidic chip-based fluorescent DNA biosensor.

is 5 mm away from the waste reservoir. The microchannel was then filled with running buffer solution using a syringe, and 1 μL of C_p-modified MB solution was added into the waste reservoir. Subsequently, the peristaltic pump, which connected to the buffer reservoir, was operated for 60 s to bring the C_p-modified MBs to the magnet buttons. Finally, the waste reservoir was rinsed with running buffer, and the entire C_p-modified MBs were immobilized in the microchannel by the magnet buttons to form the DNA biosensor.

2.4 Genotyping of SNPs and determination of target DNA

Before measurement, the exciting light beam of the LIF detector was adjusted to focus on the detection area located between the magnet buttons and waste reservoir as shown in Scheme 1. In this study, the free solution electrophoresis was adopted to drive the target DNA to the detection area. First, the target DNA or sample was premixed with equal volume of 2 μM signal probe DNA (S_p), and then 5 μL of the mixture was added into the sample reservoir. At the same time, about 5 μL of running buffer was added into the buffer and waste reservoirs. Then, the mixture of target DNA and S_p was injected into the microchannel for detection by adding a +1000 V voltage between the waste and sample reservoir for 60 s, followed by a +800 V voltage for 480 s. As shown in Scheme 1, when the mixture of target DNA and S_p flowed through the C_p-modified MBs, the target DNA hybridized with S_p and C_p to form "sandwich" structure. Thereby, the target DNA and S_p were captured by the magnet. Then, a +800 V voltage was added between the waste and buffer reservoir for 210 s in order to remove the target DNA and S_p, which nonspecifically adsorbed on the surface of the separation channel and MBs. Finally, the magnet buttons were removed, and a +2200 V voltage was added between the waste and buffer reservoir to drive the MBs to the detection area for the measurement of fluorescence signal at an emission wavelength of 525 nm (excitation wavelength is 473 nm). After one measurement, the microfluidic chip was used for the next determination by repeating the procedure of Sections 2.3 and 2.4 until all experiments were completed.

3 Results and discussion

3.1 The experimental principle of the microfluidic chip-based fluorescent biosensor

As we mentioned above, the microfluidic chip-based fluorescent biosensor utilized a MB-based "sandwich" hybridization strategy. As shown in Scheme 1, two probes were designed according to "sandwich" strategy. The first probe is capture probe (C_p, colored blue), which was functionalized with a biotin group at one end and then was immobilized on the surface of streptavidin-functionalized MBs. The capture probe is complementary to part of the target DNA. The second probe is a signal probe (S_p, colored red), which was modified with a FAM (carboxyfluorescein) tag at one end and is complementary to another part of the target DNA. In the presence of target DNA (when the mixture of target DNA and S_p flows through the C_p-modified MBs), the target DNA can effectively hybridize with the signal and capture probes to form a stable "sandwich" structure on the surface of the MBs. Thereby, the target DNA and S_p were captured by the magnets, which will lead to an obvious fluorescence signal when the MBs are electrokinetically driven to detection area for fluorescence detection by removing the magnets. In the absence of the target DNA (when only S_p flows through the C_p-modified MBs), S_p cannot be captured by the magnets since S_p cannot hybridize with the capture probe to form a stable "sandwich" structure on the surface of the MBs. This will result in no fluorescence signal when the MBs are electrokinetically driven to the detection area for fluorescence detection.

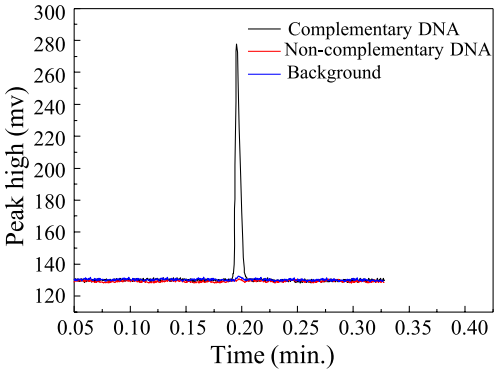


Figure 1. The fluorescence signal of the biosensor for the detection of complementary DNA, noncomplementary DNA, and background. The concentration of complementary DNA and noncomplementary DNA are both 10 nM. Other conditions: MBs introduction time is 60 s; run buffer is a mixture of 900 mM Tris-HCl, 20 mM EDTA and 0.1% v/v Tween-20 (pH 7.75); the voltage used to inject the sample is 800 V for 480 s; the concentration of signal probe is 1 μM.

3.2 Fluorescence characterization of the DNA biosensor

In order to test whether the DNA biosensor actually worked, the fluorescence signal of the biosensor was investigated before and after the target DNA solution was injected into the microchannel for analysis. As shown in Fig. 1, before the target DNA solution was injected into the microchannel for analysis, a signal close to baseline was detected by the LIF detector (see background in Fig. 1) when the C_p-modified MBs flowed through the detection area. After the mixture of target DNA and signal probe DNA was electrokinetically injected into the microchannel for determination, a high fluorescence signal was detected by the LIF detector when the MBs flowed through the detection area (see complementary DNA in Fig. 1). This is because the target DNA hybridized with S_p and C_p to form a stable "sandwich" structure on the surface of the MBs when it flowed through the C_p-modified MBs and thereby the S_p was completely captured by the magnets. In the case of noncomplementary DNA, a negligible fluorescence signal (close to background) was detected by the LIF detector when the MBs flowed through the detection area (see noncomplementary DNA in Fig. 1). This is because noncomplementary DNA cannot hybridize with S_p and C_p to form a stable "sandwich" structure on the surface of the MBs when it flowed through the C_p-modified MBs, and thereby no S_p was captured by the magnets. The result in Fig. 1 clearly reveals that the DNA biosensor does indeed work as expected. The same experiment was repeated six times (see Fig. 2) and the RSD ($n = 6$) of the peak area and the peak height were calculated to be 3% and 5% respectively, indicating that the DNA biosensor has good reproducibility. Since the peak area has a better reproducibility than the peak height, the peak area of fluorescence signal was used for the quantification of target DNA.

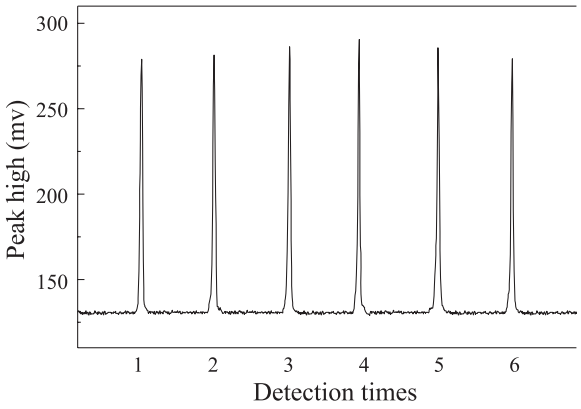


Figure 2. The reproducibility of the same six experiments. Experimental conditions are the same as that of Fig. 1.

3.3 Optimization of the microfluidic chip-based DNA biosensor

As we mentioned above, the proposed biosensor utilized a "sandwich" hybridization strategy. In general, a "sandwich" strategy is chosen in conventional electrochemical and optical biosensors where the hybridization can take place over longer time (one or even a few hours) [18, 19]. However, for microfluidic chip-based DNA biosensors, the hybridization time of C_p, S_p and target DNA has only one minute or even tens of seconds since the sample or target DNA in the microchannel was continuously transported by the electrophoresis. Therefore, the improvement of the hybridization efficiency of C_p, S_p and target DNA is a key factor to improve the sensitivity of the proposed biosensor. So far, the microfluidic chip-based DNA biosensor employed "sandwich" strategy has not been reported in the literature.

The flow-rate of the target DNA in the microchannel directly affects the hybridization efficiency of the "sandwich," and finally influences the sensitivity of the biosensor. In the experiment, the flow-rate was set by controlling the voltage between the waste and sample reservoir. In order to obtain the optimum voltage, four different voltages (600, 800, 1000, and 1200 V) were investigated in our experiment. The results shown in Fig. 3A reveal that the biosensor has the highest sensitivity when the 800 V voltage was used to inject and drive the sample or target DNA. A lower voltage will reduce the injection amount of the target DNA and lead to a lower sensitivity. On the other hand, a higher voltage will decrease the hybridization time and lead to more Joule heat, which will depress the hybridization efficiency of the "sandwich" and finally results in a lower sensitivity too.

The injection time of the sample or target DNA directly affects the injection amount of the target DNA, and finally affects the sensitivity of the biosensor. The experimental results indicate that the sensitivity of the biosensor linearly increases with the increase of injection time in the range of 1–8 min. When the time is longer than 8 min, the sensitivity of biosensor shows no change. Therefore, 8 min was selected as the best injection time.

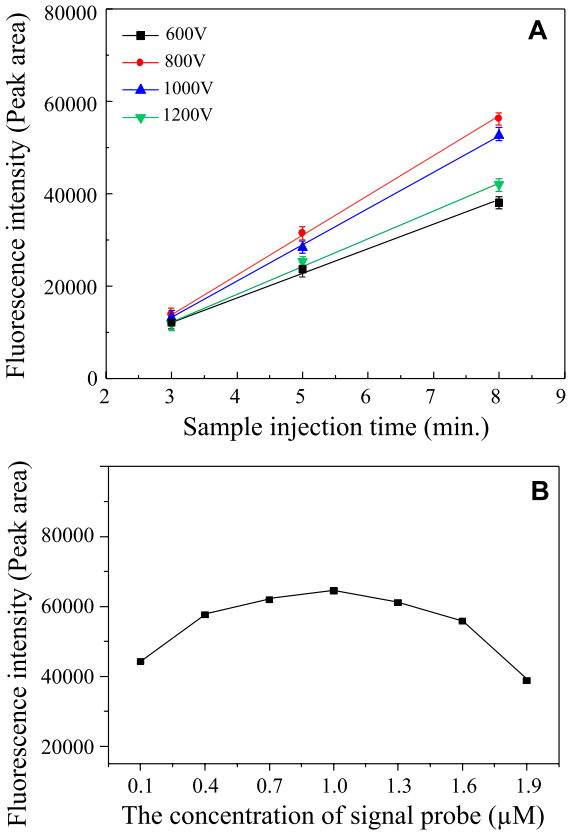


Figure 3. (A) Effect of the voltage used to inject the sample on the fluorescence intensity. Other conditions are the same as that of Fig. 1 except the voltage used to inject the sample. (B) Effect of the concentration of signal probe DNA (S_p) on the fluorescence intensity. Other conditions are the same as that of Fig. 1 except the concentration of S_p.

As we mentioned above, in our biosensor, the C_p-modified MBs were immobilized in the microchannel for the capture of target DNA. Therefore, the density of C_p on the surface of the MBs and the amount of the MBs immobilized in the microchannel directly affects the sensitivity of the biosensor. The density of C_p on the surface of the MBs was obtained by detecting the concentrations of the C_p solution before and after it reacted with the MBs, respectively. The experimental results show that the density of C_p on the surface of the MBs is about 250 pM/mg MBs. In the experiment, the C_p-modified MBs were introduced into microchannel by a peristaltic pump. Therefore, the amount of C_p-modified MBs immobilized in the microchannel was exactly determined by controlling the operating time and flow rate of the peristaltic pump. The experimental results showed that too few MBs will lead to an obvious decrease in the detection sensitivity, whereas, too many MBs will hinder the hybridization of target DNA and probe DNA due to spatial restriction, which will finally result in a lower sensitivity too. The experimental results show that the biosensor has the highest sensitivity and the best stability when the C_p-modified MBs are introduced into the microchannel by the peristaltic pump for 60 s with a rate of 18 nL/min (about 0.054 μg MBs). In our biosensor, two magnet buttons were used to immobilize the C_p-modified MBs in the microchannel. We have studied the influence of magnetic force on the immobilization of the C_p-modified MBs. We found that the magnetic force is strong enough to immobilize the C_p-modified MBs in the microchannel even if the magnet buttons are smaller than 2 mm (diameter and thick). In view of the convenience of removing and setting, two magnet buttons with a diameter of 2 mm and a thickness of 2 mm were used in the experiment. We also observed the structure of the MBs plug in the micro channel via an inverted microscope (Optec BD200, China), and we found that the MBs are mainly accumulated in the center of micro channel because two magnets have a attractive configuration, which resulted in a most strong magnetic force in the central axis of micro channel [20].

The concentration of S_p not only affects the sensitivity but also affects the linear range of the biosensor. The experimental results show that the fluorescence signal increases with the increase of the S_p concentration in the range of 0.1–1 μM, then the fluorescence signals turn to decrease with the increase of the S_p concentration when the concentration of S_p is higher than 1 μM (see Fig. 3B). This is because that the excess of S_p hinder the hybridization of target DNA, S_p, and C_p. Therefore, 1 μM of S_p was selected in the experiment.

As previously described, the electrophoretic driving mode was used to transport the sample or target DNA in the biosensor. Therefore, the chemical component and pH of buffer solution not only affects the transport of the target DNA by affecting EOF but also affects the hybridization efficiency of target DNA, S_p, and C_p, which finally influences the sensitivity and stability of biosensor. In the experiment, we found that the target DNA is easily adsorbed on the surface of the microchannel and the hybridization efficiency of target DNA, S_p and C_p was seriously inhibited when a lower concentration TE buffer solution (10 mM) was used as running and hybridization buffer. It was reported that the surfactant can effectively avoid the aggregation of MBs and prevent the adsorption of DNA on the surface of microchannel, and that a higher concentration TE buffer solution improves the hybridization efficiency of target DNA, S_p, and C_p since the positive ions of the buffer can shield the negative charge of the phosphate on the DNA backbone [16]. In the experiment, a mixture of 900 mM Tris-HCl, 20 mM EDTA, and 0.1% v/v Tween (pH 7.5) was used as the running and hybridization buffer. In this buffer, the DNA can be transported smoothly with electrophoretic driving mode and the biosensor showed a satisfactory stability and sensitivity. However, it should be noted that too high concentration of the buffer solution would lead to a greater current, which will result in poor reproducibility due to Joule heating.

3.4 The specificity and sensitivity of the DNA biosensor

Under the above optimum conditions, the discrimination ability of the biosensor was investigated by detecting a 24-base

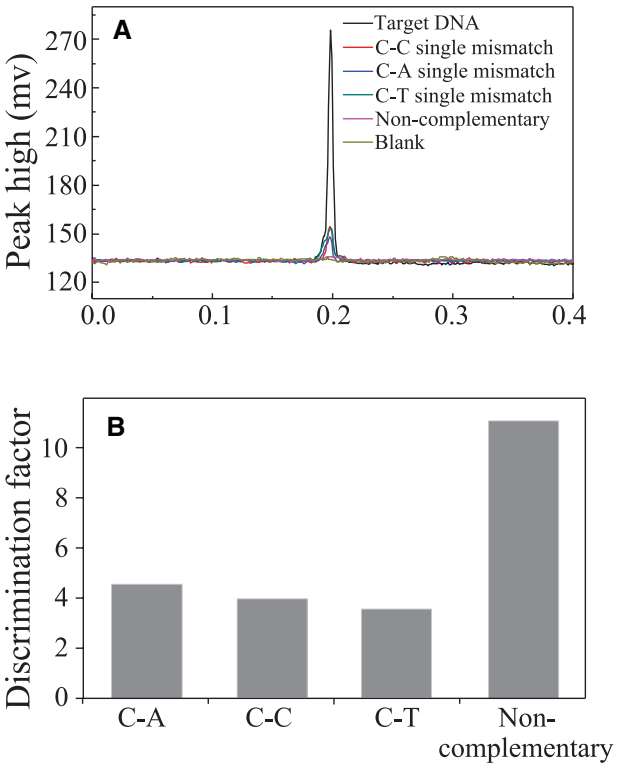


Figure 4. (A) The fluorescence signals of the detection of complementary DNA, single-base mismatch DNA, noncomplementary DNA, and background. Experimental conditions are the same as that of Fig. 1. (B) The discrimination factor of the biosensor for single-base mismatch in 24 bases oral cancer-related DNA. Data shown in Fig. 4A were used for the calculation.

oral cancer-related target DNA, three one-base mismatch DNA (with a G-to-C, G-to-A, and G-to-T substitution at position 16 from 5′ end, respectively) and a noncomplementary DNA (see Table 1). From the results shown in Fig. 4A, we clearly observe that: (i) a fluorescence signal close to baseline was detected for noncomplementary DNA, indicating that noncomplementary DNA cannot hybridize with S_p and C_p to form a stable "sandwich" structure on the surface of the MBs and thereby no S_p was captured by the magnets; (ii) the highest fluorescence signal was detected for the target DNA. This is because that target DNA completely hybridize with S_p and C_p to form a stable "sandwich" structure on the surface of the MBs when it flowed through the C_p-modified MBs and thereby S_p and target DNA was completely captured by the magnets; (iii) compared with the target DNA, a much lower fluorescence signal was detected for three one-base mismatch DNA, implying that one-base mismatch DNA cannot completely hybridize with S_p and C_p to form a stable "sandwich" structure on the surface of the MBs when it flowed through the C_p-modified MBs and thereby only a small part of S_p and target DNA was captured by the magnets. These results demonstrate that only a complementary DNA sequence can completely hybridize with S_p and C_p-modified MBs to form a stable "sandwich" structure on the surface of the MBs and thereby lead to a significant fluorescence signal, indicating that our biosensor has excellent discrimination ability for single-base mismatch and can be used for the genotyping/detection of SNPs.

To quantitatively evaluate the discrimination ability of our biosensor, we define the single-base mismatch discrimination factor as the ratio of the net fluorescence signal obtained with the complementary target DNA to that obtained with one-base mismatch DNA. A larger discrimination factor is thus indicative of improved specificity for single-base mismatch. From the data shown in Fig. 4A, we calculated that the discrimination factor was in the range of 3.58–4.54 for single-base mismatch (in a 24-base DNA sequence; see Fig. 4B). The best discrimination factor (4.54) was observed for the C-A mismatch, an intermediate value (3.90) for C-C mismatch, and the lowest (3.58) for C-T mismatch. Recently, many sensing strategies such as "triple stem," "gap ligation," and "junction-probe" strategy together with a deoxyinosine probe have been developed to improve the discrimination ability of DNA biosensor [21–23]. These strategies need complicated and time-consuming sample-labeling or fabrication procedure as well as enzyme-aided reactions. In this study, by employing a microfluidic chip together with electrophoretic driving mode and "sandwich" hybridization strategy, the discrimination ability of the biosensor has been greatly improved. The discrimination factor of 3.58–4.54 for one-base mismatch (in a 24-base DNA sequence) is much better than the 2.0–3.0 of the previously mentioned DNA biosensors [21–23]. Not long ago, a microfluidic chip-based biosensor, which used electrophoretic driving mode and "duplex" hybridization strategy, was developed for the detection of DNA [16]. In comparison with the previous microfluidic chip-based biosensor [16], the proposed biosensor has much better discrimination ability too (the best discrimination factor for one-base mismatch of the proposed biosensor is of 4.54, whereas, that of previous microfluidic chip-based biosensor is 3.04). In addition, the previous microfluidic chip-based biosensor needs complex and time-consuming sample labeling [16]. In view of the easiness of fabrication, our biosensor is obviously of analytical advantage. Of particular note, our biosensor can be used for the genotyping of SNPs in the real sense.

The sensitivity of the DNA biosensor for the target DNA was investigated by detecting a series of different concentrations of target DNA. As shown in Fig. 5, the fluorescence signal (peak area) shows a good linear correlation with the concentration of target DNA in the range of 1.0×10^{-10}–1.0×10^{-8} M. The regression equation is:

$$Y[\mathrm{mV \cdot s}] = 7359.2 \times C\,[\mathrm{nM}] + 5127.9 \quad r^2 = 0.9997$$

The detection limit ($3\sigma/S$, where σ is the SD of the blank solution, $n = 5$) was calculated to be 5.6×10^{-11} M for the complementary target DNA. The RSD of the sensor for the detection of 5.0×10^{-10} M target DNA is 4% ($n = 5$).

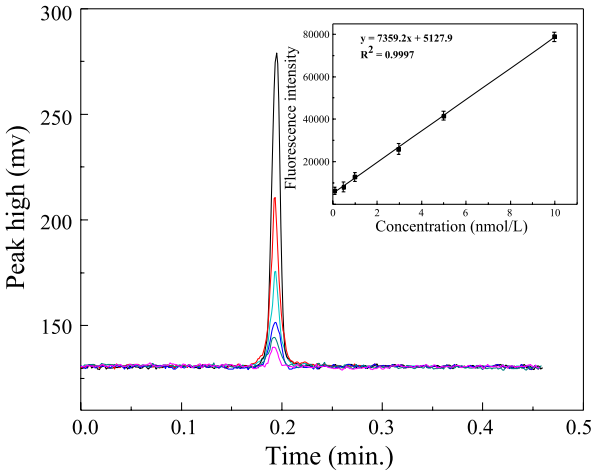


Figure 5. The relationship between fluorescence intensity and target DNA concentration. Inset shows the plot of the fluorescence intensity as a function of the target DNA concentration. Other conditions are the same as that of Fig. 1.

Table 2. Determination of target DNA in artificial saliva and serum samples

	Target DNA added (nmol/L)	Detected results (nmol/L)	Recovery	RSD ($n = 5$)
Saliva sample	0.5	0.46	92%	2%
	1.0	1.04	104%	4%
	5.0	4.74	95%	3%
Serum sample	0.5	0.49	98%	2%
	1.0	0.94	94%	4%
	5.0	5.30	106%	5%

3.5 The resistibility of the DNA biosensor to complex matrix of real samples

The applicability of our biosensor to complex matrix of real samples was demonstrated by analyzing artificial saliva and serum samples. The artificial saliva sample was made up by directly adding an amount of target DNA to an unstimulated saliva sample, which was collected from a normal person with previously established protocols [24]. The artificial serum sample was made up by directly adding an amount of target DNA to serum sample, which was obtained from Fujian Provincial Hospital. The concentrations of target DNA in the artificial saliva and serum samples were then determined with our biosensor according to the procedure of 2.4. From the results shown in Table 2, it was clearly observed that the DNA biosensor has strong resistibility to the complex matrix of saliva and serum, and can be used to detect target DNA in saliva and serum samples directly without sample labeling and any preseparation or dilution.

So far, many high-sensitivity electrochemical and optical DNA biosensors have been developed for the DNA determination. However, all the biosensors previously reported have poor resistibility to complex matrix and cannot be directly used to determine DNA in real samples without preseparation or dilution [25–28]. The poor resistibility to the complex matrix has become one key factor that hampers the commercialization of these sensors. As shown in Scheme 1, our DNA biosensor was fabricated on a microfluidic chip and the target DNA or sample was transported by the capillary electrophoresis mode. When the sample was transported to the C_p-modified MBs for hybridization from sample reservoir, the target DNA had been preliminarily separated from complex matrix of samples by capillary electrophoresis during the transporting process. This is the main reason why our biosensor has a robust resistibility to the complex matrixes of real samples.

The electrophoretic driving mode is also favorable to improve the hybridization rate of the target DNA and probe DNA [29], and finally results in a much shorter analysis time and better specificity for single-base mismatch [30, 31]. The fabrication of the biosensor only takes 10 min (not include the time of fabricating microfluidic chip) and the determination of one sample only takes 15 min, is much shorter than the up to 10 h required by other biosensors such as electrochemical DNA biosensors [32–34] and optical DNA biosensors [35–37] previously reported although the sensitivity of our biosensor is relatively low.

4 Concluding remarks

A microfluidic chip-based fluorescent DNA biosensor was developed for the specific detection of single-base mismatch DNA in this study based on the electrophoretic driving mode and MB-based "sandwich" hybridization strategy. The DNA biosensor has robust resistibility to complex matrix of real saliva and serum samples, short analysis time, and high discrimination ability for the genotyping/detection of single-base mismatch. These features, as well as its easiness of fabrication, operation convenience, stability, better reusability, and low cost, make it a promising alternative to the SNPs genotyping/detection in clinical diagnosis. By using the biosensor, we have successfully determined DNA related to oral cancer in saliva and serum samples without sample labeling and any preseparation or dilution with a detection limit of 5.6×10^{-11} M, an RSD ($n = 6$) < 5% and a discrimination factor of 3.58–4.54 for one-base mismatch.

The authors gratefully acknowledge the financial support of NSFC (41240035, J1103303), Fujian Provincial Department of Science and Technology (2012J01035, 2011Y0016), Ministry of Education, Science and Technology Development Center (20123514110001), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1116), and National Key Technologies R & D Program of China during the 12th Five-Year Plan Period (No. 2012BAD29B06).

The authors have declared no conflict of interest.

5 References

[1] Goode, E. L., Ulrich, C. M., Potter, J. D., *Cancer Epidemiol. Biomarkers Prev.* 2002, *11*, 1513–1530.

[2] Ieiri, I., Higuchi, S., Sugiyama, Y., *Expert Opin. Drug Metab. Toxicol.* 2009, *5*, 703–729.

[3] Nasirizadeh, N., Zare, H. R., Pournaghi-Azar, M. H., Hejazi, M. S., *Biosens. Bioelectron.* 2011, *26*, 2638–2644.

[4] Basuray, S., Senapati, S., Aijian, A., Mahon, A. R., Chang, H. C., *ACS Nano* 2009, *3*, 1823–1830.

[5] Xu, Q. H., Chang, K., Lu, W. P., Chen, W., Ding, Y., Jia, S. R., Zhang, K. J., Li, F. K., Shi, J. F., Cao, L., Deng, S. L., Chen, M., *Biosens. Bioelectron.* 2012, *33*, 274–287.

[6] Fan, C. H., Plaxco, K. W., Heeger, A. J., *Trends Biotechnol.* 2005, *23*, 186–192.

[7] Myers, F. B., Lee, L. P., *Lab Chip* 2008, *8*, 2015–2031.

[8] Choi, S., Goryll, M., Sin, L. Y. M., Wong, P. K., Chae, J., *Microfluid. Nanofluidics* 2011, *10*, 231–247.

[9] Gijis, M. A. M., Lacharme, F., Lehmann, U., *Chem. Rev.* 2010, *110*, 1518–1563.

[10] Chen, W., Peng, C. F., Jin, Z. Y., Qiao, R. R., Wang, W. Y., Zhu, S. F., Wang, L. B., Jin, Q. H., Xu, C. L., *Biosens. Bioelectron.* 2009, *24*, 2051–2056.

[11] Lei, K. F., Butt, Y. K. C., *Microfluid. Nanofluidics* 2010, *8*, 131–137.

[12] Ferguson, B. S., Buchsbaum, S. F., Wu, T. T., Hsieh, K., Xiao, Y., Sun, R., Soh, H. T., *J. Am. Chem. Soc.* 2011, *133*, 9129–9135.

[13] Fan, Z. H., Mangru, S., Granzow, R., Heaney, P., Ho, W., Dong, Q.-P., Kumar, R., *Anal. Chem.* 1999, *71*, 4851–4859.

[14] Goral, V. N., Zaytseva, N. V., Baeumner, A. J., *Lab Chip* 2006, *6*, 414–421.

[15] Kwakye, S., Goral, V. N., Baeumner, A. J., *Biosens. Bioelectron.* 2006, *21*, 2217–2223.

[16] Wang, Z. W., Chen, J. H., Fan, Y. W., Wang, W. J., Fu, F. F., *Microfluid. Nanofluidics* 2013, *14*, 145–452.

[17] Wu, Y. Y., Lin, J. M., Su, R. G., Qu, F., Cai, Z. W., *Talanta* 2004, *64*, 338–344.

[18] Kwon, S. J., Bard, A. J., *J. Am. Chem. Soc.* 2012, *134*, 10777–10779.

[19] Zhang, J., Song, S. P., Zhang, L. Y., Wang, L. H., Wu, H. P., Pan, D., Fan, C. H., *J. Am. Chem. Soc.* 2006, *128*, 8575–8580.

[20] Gassner, A.-L., Abonnenec, M., Chen, H.-X., Morandini, J., Josserand, J., Rossier, J. S., Busnel, J.-M., Girault, H. H., *Lab Chip* 2009, *9*, 2356–2363.

[21] Martin, F. H., Castro, M. M., Aboul-ela, F., Tinoco Jr, I., *Nucleic Acids Res.* 1985, *13*, 8927–8938.

[22] Kawase, Y., Iwai, S., Inoue, H., Miura, K., Ohtsuka, E., *Nucleic Acids Res.* 1986, *14*, 7727–7736.

[23] Zhang, J., Wu, X. Y., Chen, P. P., Lin, N. T., Chen, J. H., Chen, G. N., Fu, F. F., *Chem. Commun.* 2010, *46*, 6986–6988.

[24] Navazesh, M., *Ann. N. Y. Acad. Sci.* 1993, *694*, 72–77.

[25] Chen, J. H., Zhang, J., Wang, K., Lin, X. H., Huang, L. Y., Chen, G. N., *Anal. Chem.* 2008, *80*, 8028–8034.

[26] Peng, Y. H., Wang, X. H., Xiao, Y., Feng, L. Y., Zhao, C., Ren, J. S., Qu, X. G., *J. Am. Chem. Soc.* 2009, *131*, 13813–13818.

[27] Topkaya, S. N., Ozkan-Ariksoysal, D., Kosova, B., Ozel, R., Ozsoz, M., *Biosens. Bioelectron.* 2012, *31*, 516–522.

[28] Lee, S. J., Youn, B. S., Park, J. W., Niazi, J. H., Kim, Y. S., Gu, M. B., *Anal. Chem.* 2008, *80*, 2867–2873.

[29] Edman, C. F., Raymond, D. E., Wu, D. J., Tu, E., Sosnowski, R. G., Butler, W. F., Nerenberg, M., Heller, M. J., *Nucleic Acids Res.* 1997, *25*, 4907–4914.

[30] Sosnowski, R. G., Tu, E., Butler, W. F., O'Connel, J. P., Heller, M. J., *Proc. Natl. Acad. Sci.* 1997, *94*, 1119–1123.

[31] Cheng, I. F., Senapati, S., Cheng, X. G., Basuray, S., Chang, H. C., Chang, H. C., *Lab Chip* 2010, *10*, 828–831.

[32] Yang, K., Zhang, C. Y., *Anal. Chem.* 2010, *82*, 9500–9505.

[33] Liu, Z. Y., Zhang, W., Zhu, S. Y., Zhang, L., Hu, L. Z., Parveen, S., Xu, G. B., *Biosens. Bioelectron.* 2011, *29*, 215–218.

[34] Abi, A., Ferapontova, E. E., *J. Am. Chem. Soc.* 2012, *134*, 14499–14507.

[35] Johnson, R. P., Richardson, J. A., Brown, T., Bartlett, P. N., *J. Am. Chem. Soc.* 2012, *134*, 14099–14107.

[36] Mun, K. S., Alvarez, S. D., Choi, W. Y., Sailor, M. J., *ACS Nano* 2010, *4*, 2070–2076.

[37] Nakano, S., Karimata, H. T., Kitagawa, Y., Sugimoto, N., *J. Am. Chem. Soc.* 2009, *131*, 16881–16888.

