Biosensors and Bioelectronics 27 (2011) 192–196

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

A lateral flow biosensor for rapid detection of DNA-binding protein c-jun

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ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 13 June 2011
Accepted 13 June 2011
Available online 21 June 2011

Keywords:
Lateral flow assay
Biosensor
DNA-binding protein
c-jun

ABSTRACT

A lateral flow biosensor based on an immuno-chromatographic assay has been developed for the detection of DNA-binding proteins. The biosensor is composed of four parts: a sample pad, a conjugate pad, a strip of nitrocellulose membrane and an absorbent pad. A DNA probe containing a specific protein binding consensus sequence is coated onto gold nanoparticles, while an antibody against the DNA-binding protein is immobilized onto a test zone of the nitrocellulose membrane. The target protein binds to the protein binding DNA sequence that is coated on the gold nanoparticles to form nanoparticle-DNA–protein complexes, and the complexes are then captured by the antibody immobilized on the test zone to form a red line for visual detection of the target protein. This biosensor was successfully applied to a DNA-binding protein, c-jun, and the developed biosensor allows for the rapid detection of down to 0.2 footprint unit of c-jun protein within 10 min. This biosensor was verified using HeLa cells and it visually detected c-jun activity in 100 μg of crude cell lysate protein. The antibody against c-jun used in the biosensor can distinguish c-jun from other nonspecific proteins, with high specificity.

1. Introduction

DNA-binding proteins have DNA-binding domains and thus have specific or general affinity for either single or double stranded DNA. Transcription factors, a group of DNA-binding proteins, have specific affinity for particular DNA sequences and are the key factors in gene expression (Latchman, 1997; Mitchell and Tjian, 1989; Reese, 2003). The determination of interactions between DNA-binding proteins and their counterparts is pivotal to our understanding of cellular functions. Since a high proportion of transcription factors are encoded by proto-oncogenes or tumor suppressor genes which are usually dysregulated in cancer progression, they represent highly desirable and logical targets for disease diagnosis, therapeutical interference, and drug development (Libermann and Zerbini, 2006; Oikawa, 2004). Thus, the development of rapid and simple methods for DNA-binding protein detection has great potential in the area of diagnosis and biomedicine research.

Commonly used methods for studying sequence-specific DNA-binding proteins are gel electrophoretic mobility shift assays (EMSA) (Garner and Revzin, 1981) and DNase footprinting assays (Galas and Schmitz, 1978). These assays are based on migration retardation of protein and DNA complexes through gel electrophoresis, but are laborious, as they require complex instrumentation and laboratory operations. Furthermore, they are time-consuming and require radioisotope or fluorescence labeling. Several novel methods have been developed for the detection of DNA-binding proteins such as chromatin immunoprecipitation chips (Bulyk, 2006b; Viggiani et al., 2009; Zhu et al., 2009), protein-binding microarrays (Bulyk, 2006a), and electrode DNA–protein detection (Boon et al., 2002). But the precise immobilization of DNA on chips or electrodes hinders their application. Recently, gold nanoparticle (AuNP) based colorimetric (Ou et al., 2010), fluorescence resonant energy transfer (FRET) (Knoll and Heyduk, 2004; Xu et al., 2008), and surface-enhanced Raman spectroscopy based detection assays (Joshi et al., 2010) were established. But the requirement of digestion by exonuclease III needs additional procedures and increases the detection time. The chromatographic lateral flow assay represents a fast and convenient method that offers a new approach for DNA–protein interaction studies.

The chromatographic lateral flow strip is a one-step test for rapid identification of various analytes. We have previously developed different types of lateral flow biosensors for nucleic acids (Mao et al., 2009), proteins (Xu et al., 2009), and cancer cells (Liu et al., 2009). In this study, we describe a simple and easy-to-use biosensor for rapid detection of DNA-binding proteins. The unique property of AuNPs renders visual detection of target proteins without the need of nuclease or fluorescence labeling. Qualitative analysis is performed by observing the presence of a red test line and semi-quantitative analysis is performed by

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doi:10.1016/j.bios.2011.06.014

reading the optical density of the test line with a portable "strip reader".

C-jun was chosen to evaluate the performance of this biosensor because the dysregulation of c-jun kinase-activated pathway signaling was reported to contribute to the development of many diseases, especially cancer (Jiao et al., 2010; Maeda and Karin, 2003; Schutte et al., 1989; Xin et al., 2004; Zhang et al., 2007).

The biosensor was composed of four parts: a sample pad, a conjugate pad, a strip of nitrocellulose membrane, and an absorbent pad. A 5′-thiol-modified 40-mer-DNA probe (probe 1, SH-5′-AAAAAAAAAAAAAAAAAAAATCCGGCTGAATCATCAAGCG-3′) containing the c-jun binding site (underlined) was coated onto 15 nm AuNPs. Another 5′-biotin-modified 20-mer DNA probe (probe 2, biotin-5′-CGCTTGATGATTCAGCCGGA-3′) was mixed with probe 1 coated AuNPs to form a double stranded DNA probe. A monoclonal antibody against c-jun protein and streptavidin (SA) were immobilized onto the nitrocellulose membrane at test zone and control zone, respectively. The target c-jun protein binds to the double stranded DNA probe to form c-jun-probe-AuNPs complexes. The c-jun protein on the AuNPs is then captured by the antibody on the test zone, to form a red test line. The remaining probe-AuNPs complexes continue to migrate towards the absorbent pad and are captured by streptavidin through streptavidin–biotin interaction on the control zone (Fig. 1). The red band on the control zone indicates that the biosensor works properly, and the red band on the test zone indicates the presence of active c-jun protein.

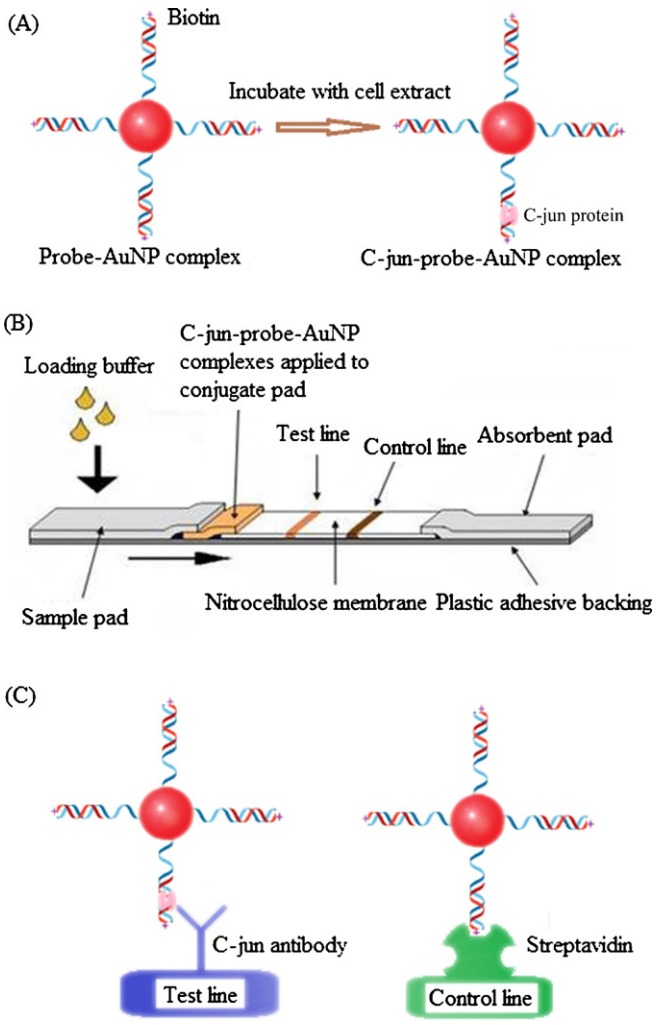

Fig. 1. Schematic of the biosensor design. (A) Probe-AuNP complexes are incubated with cell lysate, and the c-jun protein is captured by the DNA-binding sequence on the AuNPs. (B) Schematic of the lateral flow biosensor. (C) C-jun-probe-AuNP complexes are captured by antibody on the test zone and the excess complexes are captured by streptavidin on the control zone.

2. Experimental

2.1. Materials

Sodium citrate, bovine serum albumin (BSA), ovum albumin (OVA), and streptavidin were purchased from Sigma–Aldrich (Steinheim, Germany). All DNA probes were synthesized by Shanghai Sangon Biological Engineering Technology (Shanghai, China). Human recombinant c-jun protein was purchased from Promaga (Madison, WI, USA). Rabbit monoclonal antibody against c-jun was purchased from Cell Signaling Technology (Beverly, USA). Nitrocellulose membrane was purchased from Shantou ealon (Shantou, China). Fiberglass and absorbent paper were purchased from Shanghai Kinbio (Shanghai, China). All buffer solutions used in this study were prepared in our lab. Other chemicals were purchased from standard commercial sources and were of analytical grade purity.

2.2. Preparation of AuNPs–DNA probe conjugates

AuNPs with an average diameter of 15 nm were prepared using citrate reduction method (Mao et al., 2009). Briefly, 4 mL of 1% sodium citrate was added to 100 mL of a rapidly stirred and boiling aqueous solution of 1 mM $HAuCl_4$. After turning red, the solution was boiled for 10 additional minutes then cooled to room temperature. The AuNPs were collected and concentrated 4 times by centrifugation (12×10^3 rpm, 20 min). One hundred microliters of ultra-pure water (18.2 MΩ/cm, Millipore, Billerica, MA, USA) containing 1 OD of thiolated DNA probe 1 (SH-5′-C6-AAAAAAAAAAAAAAAAAAAATCCGGCTGAATCATCAAGCG-3′) was added to 1 mL of the AuNP solution (4 times concentrated) and shaken gently overnight at 4 °C. The DNA coated AuNPs (1.1 mL) were subjected to "aging" by adding 110 μL 100 mM phosphate buffer (pH 7.0) with 1% sodium dodecyl sulfate and 1.5 M NaCl. The solution was kept at 4 °C for 12 h. After "aging", 1 OD (100 μL) of complementary probe 2 (biotin-5′-CGCTTGATGATTCAGCCGGA-3′) was added. The mixture was shaken gently for 2 h at room temperature. Particles were centrifuged (12×10^3 rpm, 20 min) and rinsed 3 times with rinsing buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20, 10% sucrose, and 0.1% NaN_3) to remove any unbound DNA. The red pellet was re-suspended in 150 μL of rinsing buffer and then stored in a refrigerator at 4 °C until use.

2.3. Construction of lateral flow biosensor

Rabbit monoclonal antibody against c-jun protein (0.67 mg/mL, 30 μL) and streptavidin (0.5 mg/mL, 30 μL) were dispensed onto a strip of nitrocellulose membrane (25 mm in width) simultaneously, to form a test zone and a control zone with a lateral flow dispenser (Shanghai Kinbio, Shanghai, China). The membrane was then dried at room temperature for 12 h and stored at 4 °C until use.

Strips of fiberglass (16 mm in width) were used as sample pads after being soaked in sample pad buffer (1% Triton, 1% BSA, 2% glucose, 50 mM boric acid, pH 8.0). The sample pads were dried and stored in low-humidity at room temperature. Conjugate pads were strips of fiberglass of 6 mm in width and absorbent pads were strips of thick absorbent paper of 17 mm in width. A strip of sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were attached along the long axis of an adhesive plate with an overlap of

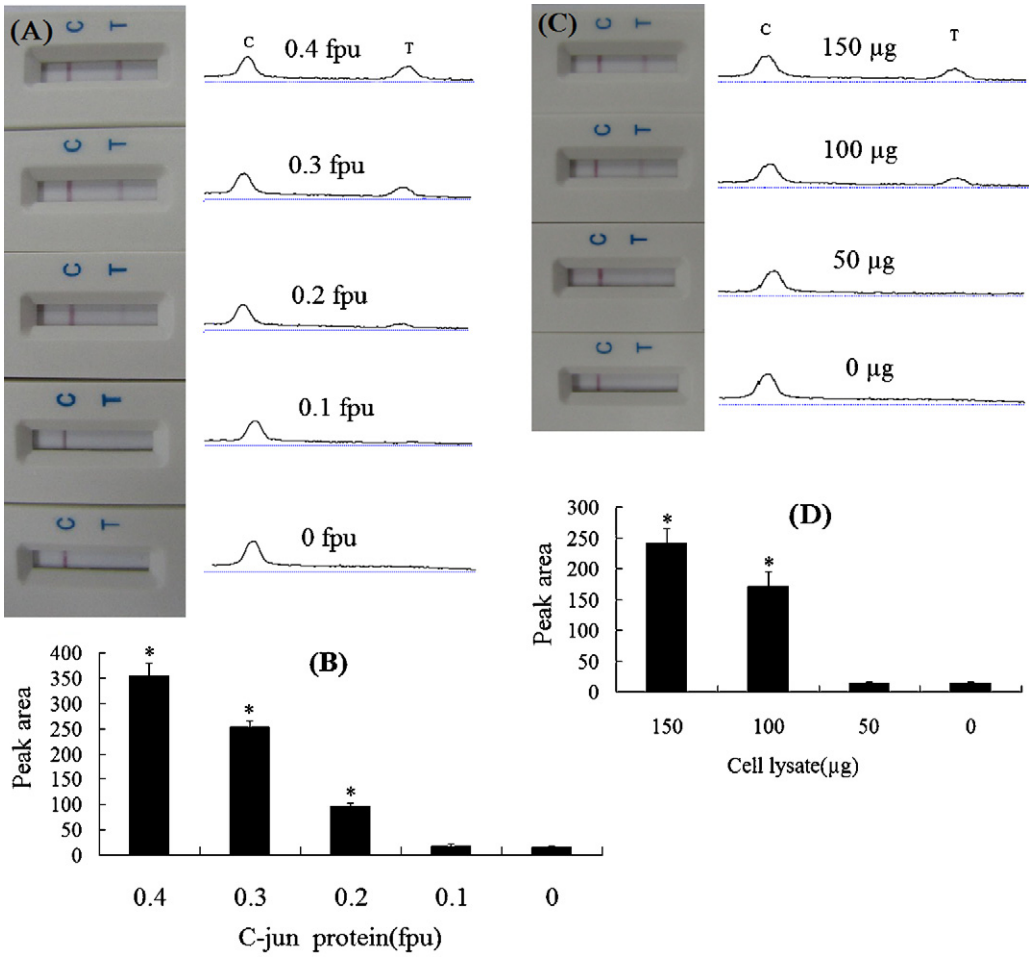

Fig. 2. (A) Photo images (left) and corresponding intensities (right) of the biosensors loaded with different amounts of purified c-jun protein. (B) Histogram presenting the peak areas of the test lines responding to different amounts of purified c-jun protein. The peak area of the test line responding to 0.4 fpu, 0.3 fpu, or 0.2 fpu of c-jun protein is much higher than that without c-jun protein ($^*p < 0.05$, $n = 3$). (C) Photo images (left) and corresponding intensities (right) of the biosensors loaded with crude cell lysate. (D) Histogram presenting the peak areas of the test lines responding to different amounts of cell lysate. The peak area with 150 μg or 100 μg of cell lysate is higher than that without cell lysate ($^*p < 0.05$, $n = 3$).

2–3 mm in the order as shown in Fig. 1B. The plate was then cut into 0.4 cm-wide strips using a paper cutter (Programmed high speed cutter, Shanghai Kinbio, shanghai, China).

2.4. Cell culture

HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were cultured at 37 °C with 5% CO_2.

2.5. Preparation of crude cell lysate

Cells were washed 3 times with cold PBS (supplied with 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM PMSF). Then 200 μL of non-denaturing cell lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mM DTT, and 1 mM PMSF) was added into a 10 cm dish containing the cells. The mixture was kept on ice for 30 min with intermittent mixing and centrifuged for 30 min at 4 °C (12×10^3 rpm). The supernatant was collected as crude cell lysate. Protein concentration in crude lysate was determined by Bradford protein assay (Supplemental information, Fig. S1). In order to test the effect of other cell components (nucleic acids, proteins, cell matrices, etc.) in the cell lysate on the performance of the biosensor, crude cell lysate was inactivated by heating at 50 °C in a water bash for 30 min.

2.6. Detection of c-jun protein by lateral flow biosensor

To detect c-jun by lateral flow biosensor, 1.5 μL of DNA probe conjugated AuNPs was mixed with different amounts of cell lysate or purified c-jun protein in 20 μL of binding buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 4% glycerol) and incubated for 20 min at room temperature before loading onto the conjugate pad of the biosensor. Then, 40 μL of loading buffer (20 mM Tris–HCl (pH 8.0), 150 mM NaCl) was loaded to the sample pad. The biosensor was scanned using a handheld "strip reader" (Shanghai Kinbio, Shanghai, China) 20 min later.

2.7. Statistical analysis

SPSS 10.0 software (SPSS Inc., IL, US) was used for statistical analysis. The results were expressed as means ± SD of three independent experiments. Individual comparisons were made by Student's *t*-test for paired data and *p*-values less than 0.05 were considered to be significant.

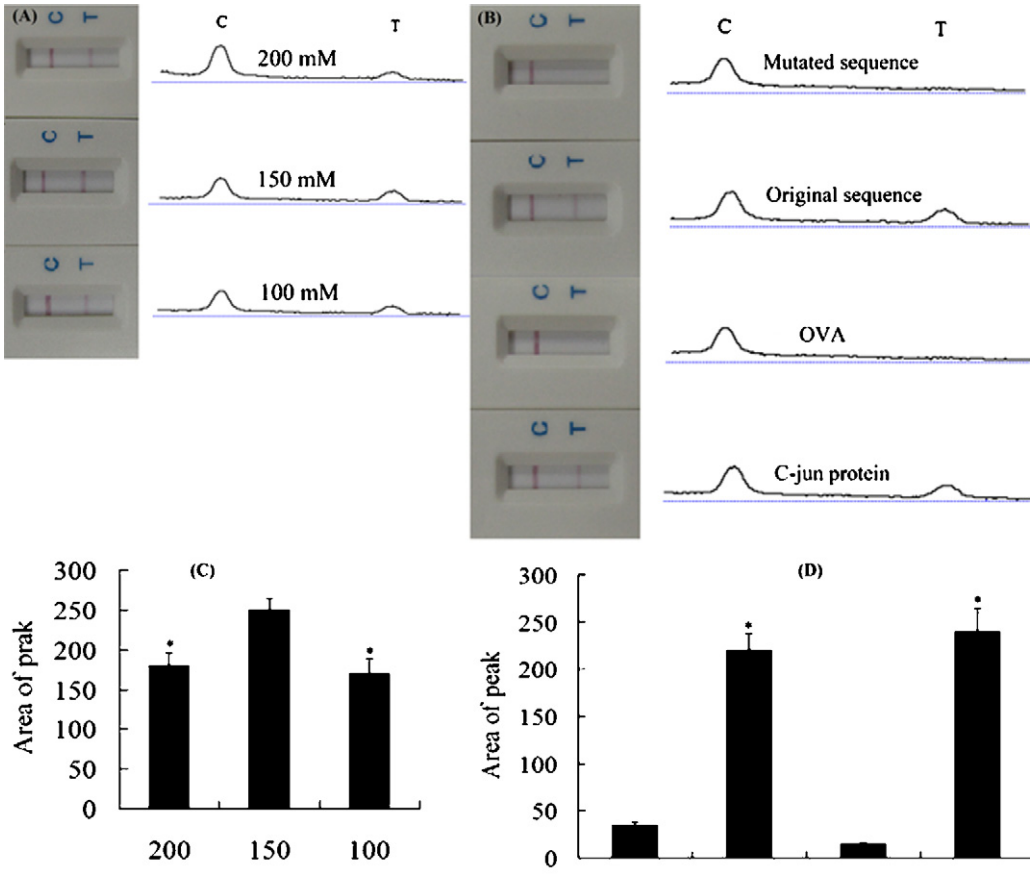

Fig. 3. (A) Photo images (left) and corresponding intensities (right) of the biosensors loaded with 150 μg of cell lysate with three concentrations of NaCl: 200 mM (top), 150 mM (middle), 100 mM (bottom). (B) Histogram representing peak areas of the test lines responding to different concentrations of NaCl. The test line with 150 mM NaCl is higher than others ($^*p < 0.05$, $n = 3$). (C) Photo images (left) and corresponding intensities (right) of the biosensors loaded with c-jun protein and cell lysate with different treatments. (D) Histogram representing peak areas of the test lines responding to 150 μg of inactivated cell lysate, 150 μg of untreated cell lysate, 150 μg of inactivated cell lysate spiked with 0.3 fpu of c-jun protein, and 0.3 fpu of c-jun protein. The intensity of test line responding to inactivated cell lysate is significantly lower than the others, and the intensities of test lines responding to c-jun protein and inactivated cell lysate spiked with c-jun protein are not significantly different ($^*p < 0.05$, $n = 3$).

3. Results and discussion

3.1. Sensitivity of the lateral flow biosensor

The sensitivity of the biosensor was tested using purified human recombinant c-jun protein. Fig. 2A shows the typical images along with the corresponding optical responses of the biosensors loaded with different amounts of purified c-jun protein. There was no red band observed on the test zone of the biosensor in the absence of c-jun protein. The test line was visible at 0.2 footprint unit (fpu, a measurement unit for the activity of DNA-binding proteins defined as the amount of protein required to yield a complete footprint (major and minor protected site) within the 72 bp repeat of the SV40 early promoter) of c-jun protein, which can be considered as the threshold for visual detection (Fig. 2A). The intensities of the bands on the test zones (peak areas) increased with the concentration of c-jun protein (Fig. 2B).

The biosensor was also shown to detect c-jun protein in cultured cell samples. Different amounts of crude cell lysate (0, 50, 100, 150 μg) were tested and the results are shown in Fig. 2C. No test line was observed in the absence of cell lysate. A visible red line was observed at the test zone of the biosensor when loaded with crude cell lysate containing 100 μg or more of total protein. The intensities of the test lines increased with the amount of cell lysate loaded (Fig. 2D). The activities of c-jun protein in the same four samples were confirmed by the conventional method, EMSA. Except for the negative control, all the other three samples had shifted bands, and the trend of band intensity change with the amount of cell lysate is the same as that observed using the biosensor (Supplemental information, Fig. S2).

This test provides an extremely rapid method for detecting activity of transcription factors. It can be performed within 10 min, which is much less time required for EMSA, DNase footprinting assay, and other methods. Although the biosensor is less sensitive than EMSA as a shifted band was observed in the lane loaded with 50 μg of cell lysate (Supplemental information, Fig. S2), it is less time-consuming and provides an alternative method for the detection of transcription factors. By using the chromatographic assay as a substitute for the electrophoretic mobility assay, we can avoid the usage of electrophoretic apparatuses and simplify the detection procedure. Furthermore, the usage of AuNPs, instead of fluorescent dye, enables visual detection of the results.

3.2. Specificity of the lateral flow biosensor

Two important types of molecular interactions contribute to the success of this assay. One is hybridization between AuNPs-probe 1 and biotinylated probe 2, which creates a binding site for c-jun protein. The other is association between the DNA-binding site and c-jun protein, through which the target protein is captured to the AuNPs. The binding buffer used for the two types of molecular interactions was based on that used in EMSA. Different concentrations of NaCl in the binding buffer were tested and 150 mM was shown

to be optimal (Fig. 3A and B). This binding buffer was used through out this study.

To determine if the presence of other cell components (nucleic acids, proteins, cell matrices, etc.) in the cell lysate affect the detection, the biosensor was tested with heat inactivated cell lysate, untreated cell lysate, inactivated cell lysate spiked with purified c-jun protein, and purified c-jun protein. As shown in Fig. 3C, no test line was observed when 150 μg of inactivated cell lysate was loaded. A red test line was observed when the biosensor was loaded with 150 μg of untreated cell lysate, 0.3 fpu of purified c-jun protein, or 150 μg of inactivated cell lysate spiked with 0.3 fpu of purified c-jun protein. The intensities of the test lines responding to 0.3 fpu of purified c-jun protein and inactivated cell lysate spiked with 0.3 fpu of purified c-jun were not significantly different (Fig. 3D). The results show that cellular components in the cell lysate do not affect the performance of this biosensor. The results also show that the biosensor detects only active c-jun protein, as the biosensor did not respond to inactivated cell lysate.

Specificity of the biosensor was also investigated by introducing one mutation into the protein-binding site. Adenine deoxynucleotide in the middle of the c-jun protein binding sequence (TGAaTCA, small case) was changed to T (TGTtTCA), and a new biosensor was constructed by using the mutated probes. The two biosensors with original and mutated binding sequences were compared. As shown in Fig. S3, Supplemental information, a clear red test line was observed with the biosensor containing the original probes, when 150 μg of cell lysate was loaded, but no test line was observed with the biosensor containing the mutated probes, when 150 μg of cell lysate was loaded. The specificity of the biosensor was further tested by applying 300 μg of ovum albumin to the biosensor, resulting in no observable band on the test zone (Supplemental information, Fig. S3). These results demonstrate that the biosensor has high specificity.

EMSA and other assays that do not employ antibodies have specificity problems due to nonspecific binding of DNA-binding proteins. There are usually smears in gels following electrophoresis as in EMSA or high background as observed in FERT-based assays. The present biosensor has selectivity with no background, because a specific monoclonal antibody is used in the assay. Even if there are nonspecific proteins bound to the DNA-binding probe, the antibody in the test zone can distinguish c-jun from other proteins, which maintains specificity. Furthermore, other antibody-based or aptamer-based assays cannot differentiate active DNA-binding proteins from inactive proteins, in contrast the current biosensor specifically detects active DNA-binding proteins. Commercialized antibodies or aptamers recognize certain epitopes of target proteins, i.e., the specific polypeptide sequences of the proteins. They capture proteins whether they are active or not. As only active protein can bind to the consensus protein binding DNA sequence, the DNA probe conjugated AuNPs in the current assay captures only active DNA-binding proteins and the test result is a direct measure of the activity of the transcription factor. Gold nanoparticle-based colorimetric assays (Ou et al., 2010) also detect active DNA-binding proteins as well as provide a visual detection method, but the need for exonuclease III treatment in this format makes the assay more laborious. In addition, it is difficult to discriminate small color changes at low concentration of target protein.

4. Conclusion

In the present work a rapid and easy-to-use biosensor for detecting DNA-binding proteins was developed and successfully applied for the detection of c-jun activity in purified protein and cell samples. It has several advantages over other published methods. First, this biosensor is rapid and easy-to-use. It takes less than 10 min to visually detect the results. Second, it has high specificity as no background was observed on the test zone. Third, the biosensor can distinguish between active and inactive DNA-binding proteins, and only detects active DNA-binding proteins. Therefore it can be used to detect and measure the activity of transcription factors in cell samples. It is easy to apply this assay to detect different DNA-binding proteins by changing the consensus protein binding sequence and the corresponding antibody. Furthermore, this biosensor can be modified to design two or more test zones on a single strip of nitrocellulose membrane for multiplex assays. Multiplex biosensors can be used to study interactions between two or more different DNA-binding proteins simultaneously.

Acknowledgements

This work was financially supported by the Foundation of Special Research Program of Science and Technology Bureau of Guangzhou (no. 2008Z1-E581, no. 2009Ss-P030) and the Ministry of Science and Technology (no. 2008ZX10004-004). We thank Dr. Scott Spillman and Dr. Micky Tortorella for their comments on this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.06.014.

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