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Characterization of Capillary Immunosensor for Capillary-Assembled Microchip (CAs-CHIP) Integration

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A capillary immunosensor is developed as a sensing unit of a capillary-assembled microchip (CAs-CHIP) that is geared towards simultaneous multiple immunoassay. It is prepared by subsequently treating the inner surface of a square glass capillary (internal dimension 50 μm) with 3-aminopropyltriethoxysilane -glutaraldehyde-protein A (APTES-GA-Protein A), which significantly reduced the non-specific adsorption. Consequently, accurate quantitative immunoassay measurement is demonstrated using human and chicken IgG as model samples, yielding a detection limit of about 1 ng mL^{-1} for both antigens with a total analysis time of about 60 min. The immunosensor also displayed interesting practical properties like long-term stability of at least a month at 10°C and minimal consumption of secondary labeled-antibody with one-million-fold dilution. It is expected that the integration of this fluorescent immunosensing unit into the CAs-CHIP will enhance the analytical performance of the microchip through simultaneous multiple immunoassay in a single microfluidic device. This type of biochip may have a significant impact in clinical diagnostics and drug-screening applications.

1. Introduction

There has been a rapid development in the area of immunosensors integrated in microfluidic devices.[(1–6)] The fusion of immunosensing and microfluidic technology has resulted in a very attractive range of analytical devices that offers short analysis time and

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minimal sample/reagent consumption. Since microfluidic immunoassay also deals with protein handling and analysis, thorough characterization based on protein orientation and stability is important in enhancing the overall analytical performance of microfluidic devices. These practical analytical attributes of the immunoassay biochip directly complement the need for various bioanalytical applications in the field of proteomics, drug screening and clinical diagnostics.

There are two major considerations in implementing an effective immunosensor inside a microchannel; first, non-specific adsorption must be minimized since it can cause measurement inaccuracy and second, a well-oriented protein must be immobilized so as to deliver an efficient product turn over of the enzymatic reaction. Different surface modification techniques using a wide range of polyelectrolytes or biopolymers have been utilized to minimize the non-specific adsorption thus increasing the sensitivity of the immunosensor on a glass or polymer substrate.(7–9) Alternatively, Cheung and co-workers implemented a supported phospholipid bilayer membrane to significantly passivate the hydrophobicity of a poly(dimethylsiloxane) (PDMS) surface and therefore reduce non-specific adsorption in a microenvironment.(10,11) Moreover, Sebra *et al.* developed whole photografted antibodies bound through solvated, mobile polymer chains that resulted in a blocking step-free immunoassay.(12) Meanwhile, Vijayendran *et al.* emphasized the effect of randomly surface-immobilized proteins using a fiber-optic biosensor that led them to conclude that a uniformly oriented antibody displayed an efficient transport kinetics. To demonstrate the importance of well-oriented immobilized antibodies inside an immunosensor, researchers have usually used protein A/G(14–16) and avidin/streptavidin(11,12,17) that permit favorable interaction between the antibody and antigen.

The aforementioned surface modification methodologies are effective in making the surface unreactive to proteins and allowing the maximum antibody-antigen binding, thus increasing the immunosensor sensitivity. Nevertheless, it would be more practical if a simple glass surface treatment in a silica capillary with an equally effective immunosensor will be used, while minimizing the non-specific adsorption, enhancing surface homogeneity and reducing the analysis time and sample/reagent consumption.

In this paper, we present the development of a facile glass capillary immunosensor that is intended to be the sensing unit of a capillary-assembled microchip (CAs-CHIP) system geared towards multiple immunoassay application using a fluorescence microscope as the detecting unit. The CAs-CHIP is a recently proposed microchip composed of PDMS and square glass capillaries.(18) We have demonstrated various applications of the CAs-CHIP elsewhere.(19–22) Recently, our group has developed a multiple enzyme-linked immunosorbent assay (ELISA) on a CAs-CHIP using a thermal lens microscope (TLM) as the detector.(23) This system has been used to successfully establish the integration of multiple immunoassay and microvalving on a single microchip. However, this limits the simultaneous determination of different antigens since the TLM responses are obtained one by one on each capillary immunosensor. Thus, it is the intention of this paper to demonstrate the development of a simple fluorescence read-out glass capillary immunosensor for CAs-CHIP integration towards the realization of simultaneous multiple ELISA using fluorescence image analysis.

Here, the non-specific adsorption of various surface modifications are investigated:

3-aminopropyltriethoxysilane-glutaraldehyde (APTES-GA), APTES-GA-Protein A and APTES-GA-Avidin. Then, the system with the minimal protein adsorption is further characterized in terms of its analytical performance using chicken and human IgGs as model samples. Also, the long-term stability of the capillary immunosensor is explored. This simple protein-sensing unit that we developed will facilitate the simultaneous immunoassay determination on a single microfluidic device upon CAs-CHIP integration, which can be used for various bioanalytical purposes like clinical diagnostics and drug screening.

2. Materials and Methods

2.1 *Square capillaries and reagents*

Square capillaries having a 300-μm outer widths and a 50 μm inner width were purchased from Polymicro (Phoenix, AZ, USA). The polyimide coating of these capillaries was removed by heating before use. Reagents of the highest grade commercially available grade were used for the preparation of buffer solutions. 3-Amino-propyltriethoxysilane and glutaraldehyde were purchased from Tokyo Chemical Industry (Tokyo, Japan). Bovine serum albumin (BSA), protein A, biotinylated antibody, avidin and alkaline phosphatase-conjugated antibody were acquired from Sigma-Aldrich (Milwaukee, WI, USA). Fluorescein diphosphate tetraammonium salt was purchased from Wako (Osaka, Japan). Human and chicken IgG ELISA kit were purchased from Bethyl Laboratories (Montgomery, TX., USA). All reagents were used without further purification. The distilled and deionized water used had a resistivity of more than 1.7 × 107 Ω cm^{-1} at 25°C.

2.2 *Preparation of functional capillaries*

All the capillaries used in this work were washed with 1 M sodium hydroxide solution (30 min), flushed with pure water, and then acetone, and heated at 70°C for 30 min prior to use. This ensured that surface modifications could be made.

2.2.1 *APTES-GA capillary immunosensor*

The capillary immunosensors were prepared by immobilizing anti-IgGs (human and chicken) onto the inner surface by well-known silanization chemistries. Briefly, 3-amino-propyltriethoxysilane was introduced into the square capillary (internal dimension 50 μm), and left for 40 min to introduce the amino group onto the inner surface. After that, the capillary was washed with methanol and dried at 70°C. A 5% aqueous solution of glutaraldehyde was then introduced and allowed to react for 40 min to attach the aldehyde group. The surface was then reacted with 100-times-diluted primary antibody solution in 50 mM Tris buffer containing 1 mM $MgCl_2$ (pH 8) (Tris buffer 1) and incubated for 40 min. To avoid non-specific adsorption, the antibody-immobilized surface was further coated with 1% BSA in Tris buffer 1 for at least 2 h. Finally, the imine groups were reduced by 0.5% aqueous sodium borohydride ($NaBH_4$) solution for 40 min, followed by washing with Tris buffer 1 before preparing the completed immuno-reaction capillary.

2.2.2 *APTES-GA-Protein A / APTES-GA-Avidin capillary immunosensor*

The preparation of the APTES-GA has already been described above, i.e., the portion until the glutaraldehyde surface treatment. The glutaraldehyde-treated surface was then washed with water followed by 0.5 mg mL^{-1} of either protein A or avidin in 10 mM borate buffer (pH 8) was introduced and incubated overnight. The capillaries were washed thoroughly with distilled water. Then, the imine groups were reduced by 0.5% $NaBH_4$ in aqueous solution for 40 min, followed by distilled water and borate buffer washing. After that, 100-times diluted whole primary antihuman IgG or biotinylated primary antihuman IgG (for avidin surface) solution (Tris buffer 1) was introduced into the protein A- or avidin-treated surface, respectively, and left for 40 min. The capillaries were washed with Tris buffer 1, followed by a blocking step using 1% BSA (Tris buffer 1) for 2 h. This capillary immunosensor was finally washed with Tris buffer 1.

2.3 *Enzyme-linked immonosorbent assay (ELISA) with the capillary immunosensor*

A specific concentration of antigen solution in Tris buffer 1 containing 1% BSA was manually introduced into the capillary immunosensors using a syringe then incubated for 15 min, and subsequently washed with Tris buffer 1. One-million-times diluted alkaline phosphatase-conjugated antihuman IgG (Sigma, A 8542) solution (Tris buffer 1, 1% BSA) was introduced and allowed to bind for 20 min followed by washing with 50 mM Tris buffer (pH 9) containing 10 mM $MgCl_2$ and 10 mM glycine (Tris buffer 2). The fluorescence was detected using 20 µM fluorescein diphosphate (FDP) solution (Tris buffer 2) which was allowed to react with the immobilized antibody labeled with alkaline phosphatase for at least 20 min. Fluorescent images were collected using a 120-W mercury lamp as a light source and a filter pair (excitation filter at 470/40 nm, emission filter at 510 nm) (VB-L11, Keyence, Japan). Fluorescence images were converted to a numerical response using Scion Image software.

3. Results and Discussion

The capillary immunosensors having different types of surface treatments (APTES-GA, APTES-GA-Protein A and APTES-GA-Avidin) were characterized in terms of non-specific adsorption. Figure 1(a) shows the basic concept of the glass capillary immunosensor using the protein A-treated capillary. In the case of APTES-GA, the primary antibody is covalently attached directly to the glutaraldehyde instead of protein A. In contrast, for APTES-GA-Avidin, protein A is replaced by avidin, wherein the biotinylated human antibody forms a stable complex. Although the use of $NaBH_4$ may appear to be inconsistent in terms of the application sequence, we applied the reagent after a protein (primary antibody, protein A or avidin) had been attached to the glutaraldehyde. To characterize these capillary immunosensors, the surface was exposed to alkaline phosphatase conjugated human antibody solution (one-million-times diluted) then incubated for 20 min at room temperature (~25°C). Then, an enzyme substrate, FDP, was manually introduced and allowed to react. As shown in Fig. 1(b), FDP undergoes enzymatic cleavage in the presence of alkaline phosphatase yielding a green

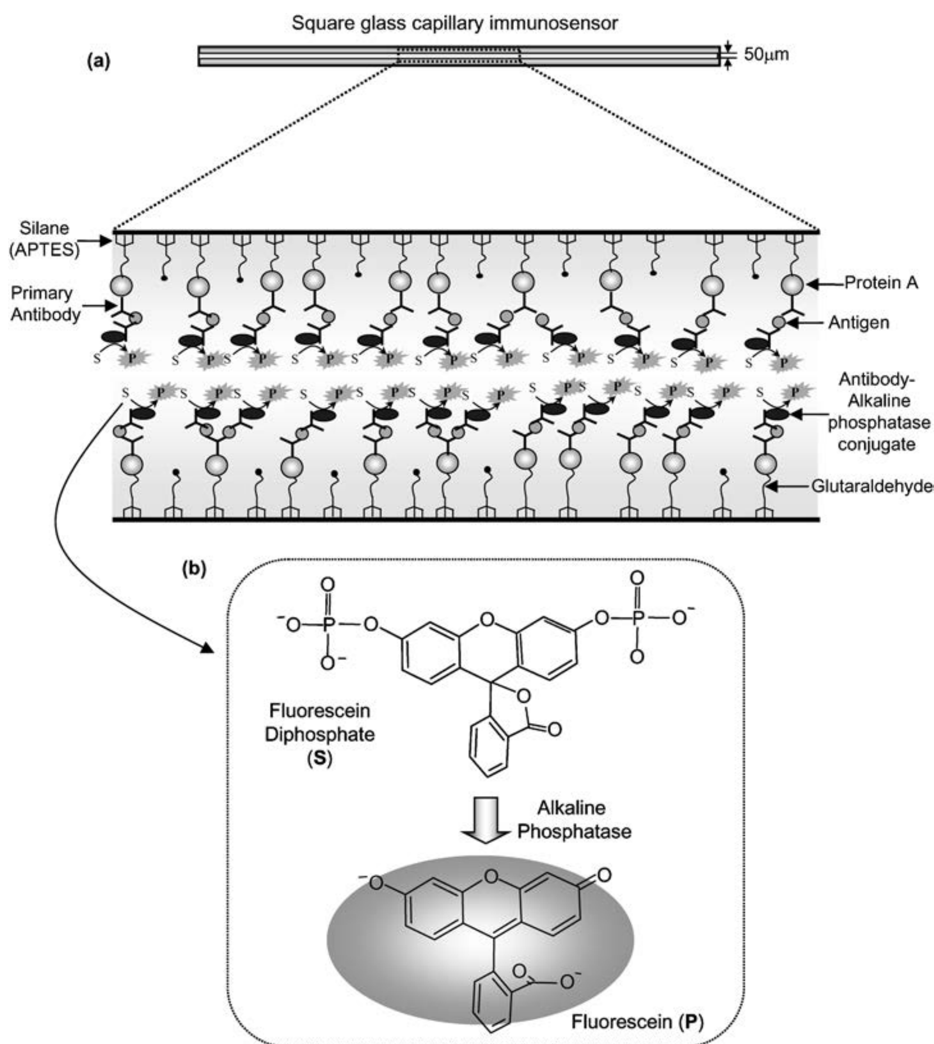

Fig. 1. (a) Basic concept of the capillary immunoassay undergoing the APTES-GA-Protein A surface treatment. (b) Biochemical reaction during immunosensing, where S is fluorescein diphosphate and P is fluorescein.

fluorescent product, fluorescein. Theoretically, the alkaline phosphatase conjugated human antibody should not bind onto the surface because there is no antigen present on the surface. Hence, minimal fluorescence is expected and this is primarily due to the FDP molecules alone. With this in mind, Fig. 2(a) depicts the fluorescence image of the different capillary immunosensors using the APTES-GA-Avidin system that registered the greatest fluorescence response relative to the other two immunosensors. The APTES-

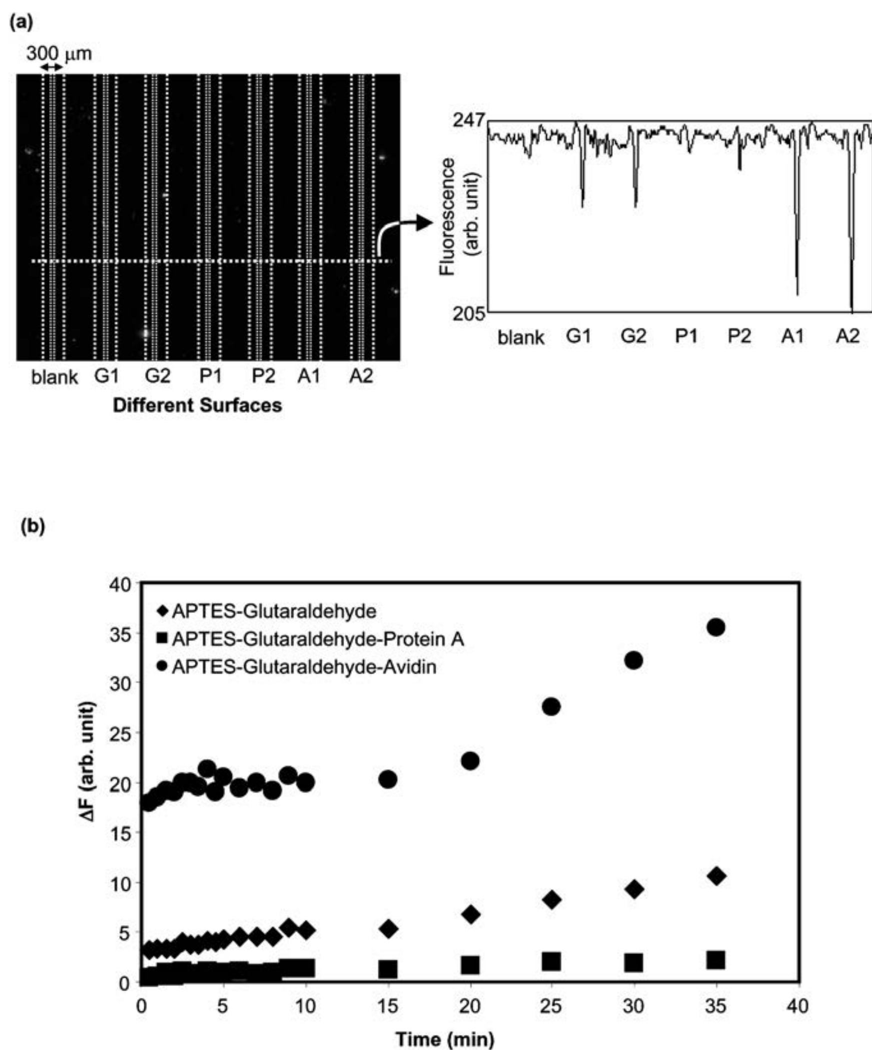

Fig. 2. (a) Fluorescence image and response of various surface treatments for two trials: G is APTES-GA, P is APTES-GA-Protein A, and A is APTES-GA-Avidin. (b) Response curve of the capillary immunosensor for various surface treatments.

GA-Protein A treatment yielded the least fluorescence implying that under this treatment minimum non-specific adsorption can be achieved. A vivid demonstration supporting this result is shown in the graph of alkaline phosphatase activity on the capillary immunosensor's surface, which is depicted in Fig. 2(b). To account for the differences in immunosensor behavior in terms of non-specific adsorption, it is important to consider the surface charge through the isoelectric point (pI) of the immobilized protein. The

pI values of antihuman IgG, protein A and avidin are approximately 7[24], 5[25] and 10.5[26], respectively. This means that if the pH of the working buffer is 9, the APTES-GA-Avidin surface is positively charged whereas the other two surfaces are negatively charged. Thus, it is possible that non-specific adsorption due to electrostatic attraction may occur leading to the cleavage of the FDP molecule and the consequent intensification of the fluorescence signal. APTES-GA yielded a higher fluorescence signal than APTES-GA-Protein A, possibly because the capillary surface was not completely blocked. The addition of protein A or avidin also serves as a blocking agent since they are also proteins. Note that the alkaline phosphatase-conjugated antibody dilution is important in the capillary immunosensor preparation. At about one-hundred-times dilution, the initial fluorescence response in the absence of antigen was clearly greater than that after million-fold dilution (data not shown). On the basis of the results shown in Figure 2(a) and 2(b), the use of protein A for glass capillary treatment can give a sensitive and more accurate analytical measurements due to the minimal non-specific adsorption.

Using the APTES-GA-Protein A system, various concentrations ranging from 0.01 to 1000 ng mL^{-1} of human and chicken antigens were used to test the ability of the capillary immunosensors to conduct analytical measurements. Figure 3(a) exhibits the fluorescence image of a successful response of the capillary immunosensor at various antigen concentrations. It is noteworthy to mention that the capillary which is exposed to 0 ng mL^{-1} of antigen showed minimal fluorescence intensity. This result suggests that an accurate immunoassay can be conducted using this capillary since at 0 ng mL^{-1} of antigen almost no cleavage of FDP was observed. The excellent analytical performance using this capillary was confirmed by the results shown in Fig. 3(b), wherein two different capillaries sensing human IgG and chicken IgG antigens were tested. The detection limit obtained from both capillaries was around 1 ng mL^{-1}. This demonstrates that capillaries sensing different antigens can be embedded onto the CAs-CHIP system for simultaneous multiple antigen measurement. Thus, the preparation of a capillary immunosensor using the APTES-GA-Protein A system is a reliable surface modification method leading to the dependable quantitative determination of antigens.

Another important factor to consider in the area of immunosensors is their long-term stability, since this type of sensor involves the handling and analysis of delicate protein molecules. To evaluate this factor, the capillary containing the primary human antibody was stored at 10°C for 24 h. Then, a normal ELISA run was performed using 8 ng mL^{-1} of human IgG serum and an alkaline phosphatase-conjugated human antibody solution (one-million-times diluted). Since the working concentration of 8 ng mL^{-1} of human IgG serum is almost near the detection limit, a more reliable and sensitive determination of capillary stability can be achieved. The stability was measured relative to the activity of the alkaline phosphatase inside the capillary. This enzyme activity is directly correlated with quality of the immobilized primary antibody. Ideally, if the immobilized primary antibody is of good quality or undenatured, the interaction between the primary antibody and the antigen will be successful. This, in turn, will yield an active complex formed between the antigen and alkaline phosphatase-conjugated human antibody. Figure 4(a) demonstrates the enzyme activity after it was stored at 10°C for 24 h under Tris buffer 1. To clearly show the effect of storage, the slope of the line in Fig 4(a) was obtained, and

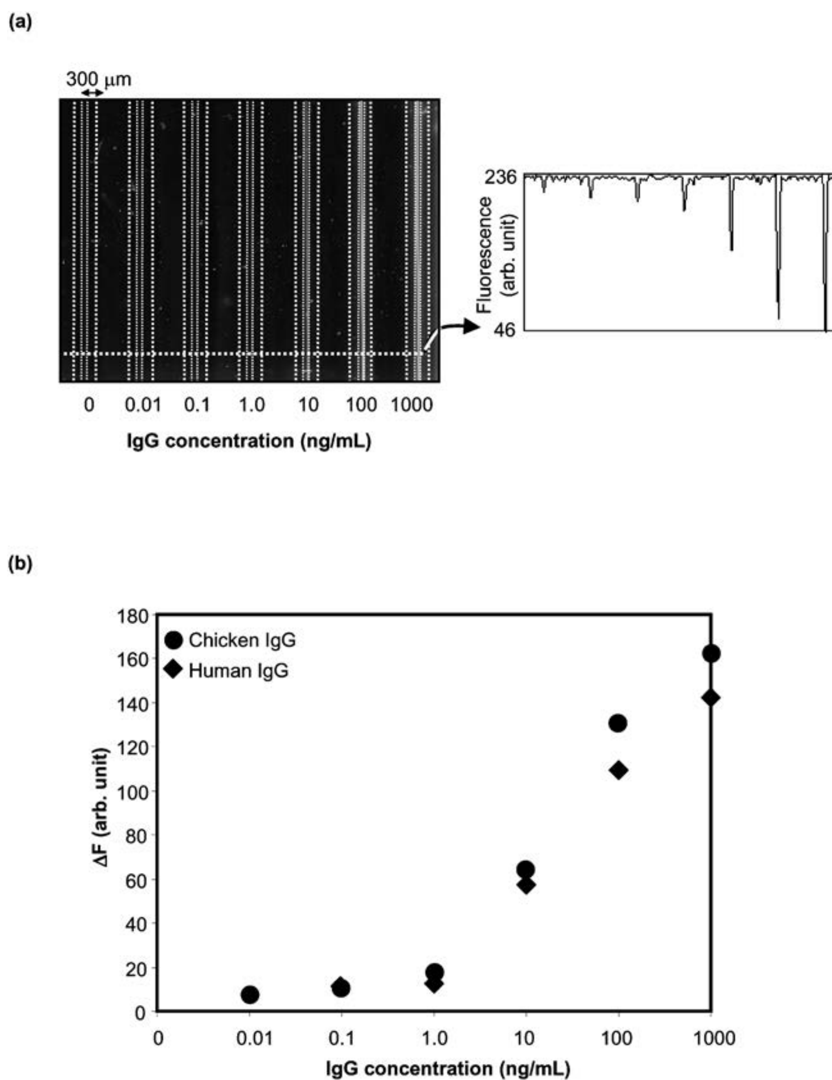

Fig. 3. (a) Fluorescence response of the APTES-GA-Protein A-treated capillary for various chicken IgG concentrations. (b) Typical calibration curves for the capillaries immunosensing human and chicken antigens.

the enzyme activity in terms of ΔF min^{-1} was acquired from the reaction profile shown in Fig. 4(a). The APTES-GA-Protein A-treated capillary containing primary antihuman IgG was also stored for almost one month at 10°C under Tris buffer 1 solution. The capillary was sampled on specific days during the one-month period and the enzyme activity was measured (Fig. 4(a)). This type of sensor appears to be stable for at least a month, yielding an average activity of 0.27 ΔF min^{-1}, though the standard deviation (n=3)

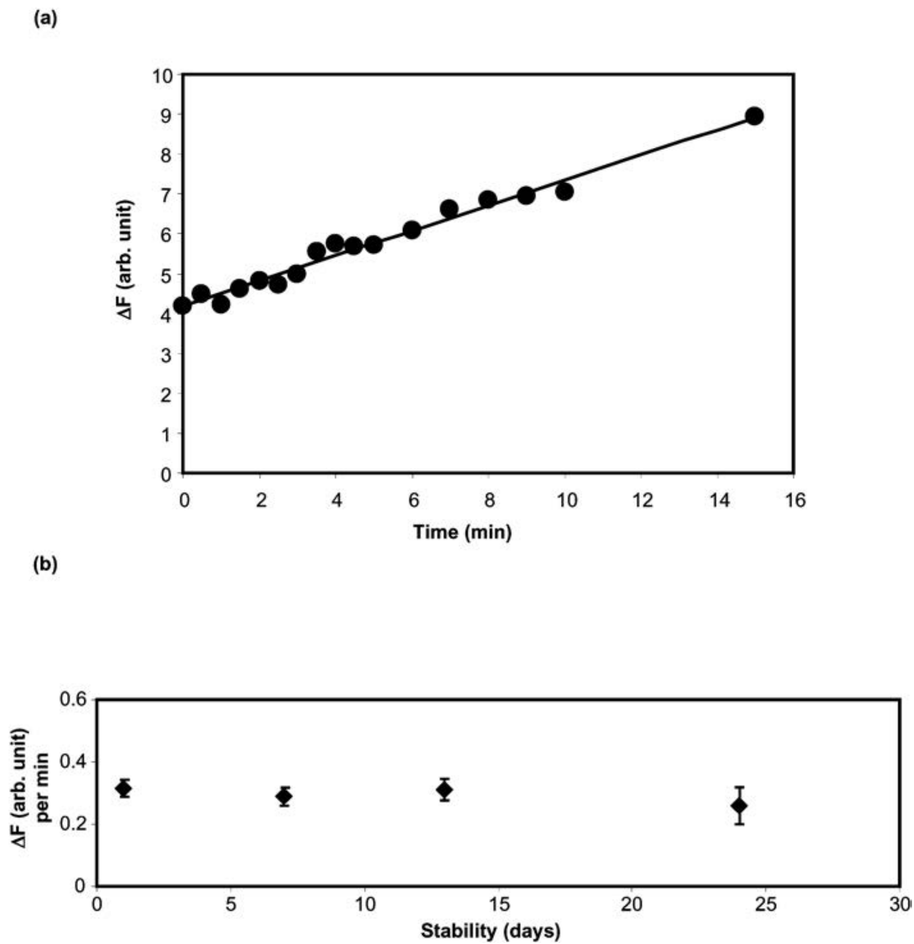

Fig. 4. (a) Capillary immunosensor response after 24 h storage at 10°C. (b) Long-term stability of the capillary immunosensor.

on the 24th day was higher. This result demonstrates the practical use of the capillary immunosensor since it can be prepared in a long, square glass capillary wherein a few millimeters can be cut for use during CAs-CHIP preparation.

4. Conclusions

The ability of the APTES-GA-Protein A-treated square glass capillary to perform accurate quantitative analysis was successfully demonstrated by showing that this method significantly reduced non-specific adsorption, which led to detection limit of

about 1 ng mL^{-1} and that the sensor is stable for at least one month. This capillary immunosensor serving as the sensing unit of a CAs-CHIP system may offer a practical and simple device for simultaneous immunoassay for clinical diagnostics and drug-screening applications.

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