Analytical Biochemistry 440 (2013) 137–141

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Capillary-based enzyme-linked immunosorbent assay for highly sensitive detection of thrombin-cleaved osteopontin in plasma

Shun-ichi Funano [a,1], Terence G. Henares [a,1], Mie Kurata [b,c], Kenji Sueyoshi [a], Tatsuro Endo [a], Hideaki Hisamoto [a,*]

[a] Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, Osaka 599-8531, Japan
[b] Department of Pathogenomics, Graduate School of Medicine, Ehime University, Ehime 791-0295, Japan
[c] Department of Cardiology, Thorax Center of Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 10 March 2013
Received in revised form 1 May 2013
Accepted 16 May 2013
Available online 2 June 2013

Keywords:
Square glass capillary
Enzyme-linked immunosorbent assay (ELISA)
Thrombin-cleaved osteopontin (trOPN)

ABSTRACT

In this study, a highly sensitive capillary-based enzyme-linked immunosorbent assay (ELISA) has been developed for the analysis of picomolar levels of thrombin-cleaved osteopontin (trOPN), a potential biomarker for ischemic stroke, in human plasma. Using a square capillary coated with 8.5 μg/ml anti-human trOPN capture antibody for ELISA, the linear range obtained was 2 to 16 pM trOPN antigen. This concentration range was in the detection window of trOPN antigen in plasma samples. Compared with the conventional microplate-based ELISA, the current capillary technique significantly reduced the amounts of reagent from milliliter to microliter, reduced the analysis time from 8 to 3 h, and had a better sensitivity and detection limit performance from approximately 50 pM down to 2 pM of trOPN antigen. These results indicate that this capillary-based immunoassay is a potential tool for biomarker detection and may be useful in clinical trials and medical diagnostic applications.

According to the World Health Organization, approximately 17 million people worldwide die annually of cardiovascular diseases (CVDs),[2] such as heart attack and stroke, despite awareness of the fact that many CVDs could be prevented by lifestyle changes and annual medical checkups [1]. This is most likely due to the late diagnosis of the disease. Therefore, early diagnosis of such health risks by using novel biomarkers may significantly lower their incidence.

Atherosclerosis or atherothrombosis is the primary pathophysiology of CVDs and involves stenosis of the body's arteries. Clinically, it has been reported that atherosclerotic lesions or plaques secrete an extracellular matrix protein, called osteopontin (OPN), in a manner that correlates with plasma OPN levels [2,3]. These findings suggest that OPN is an important component of plaque formation in the arteries. In the presence of thrombin around the atherothrombotic plaque, OPN is cleaved, released, and detected in the bloodstream as thrombin-cleaved osteopontin (trOPN).

In the case of ischemic stroke, Kurata and coworkers observed that trOPN has a more distinct distribution around the intra-plaque vessels than OPN, which could also be found in macrophages, endothelial cells, lipid cores, and calcified nodules [4]. Based on their study, which involved at least 100 patients, these authors concluded that trOPN could be a biomarker that reflects the atherothrombotic status in ischemic stroke. A common microplate-based enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of trOPN in plasma samples, which is present in picomolar levels. However, in some cases the trOPN levels were below the detection limit of this method. Furthermore, advanced clinical trials may require a very large patient population, for which a microplate-based ELISA might not be practical because it would require a vast amount of expensive reagents, a long analysis time, and low sensitivity due to the large assay volume.

A capillary-based ELISA could circumvent these drawbacks. This format offers nanoliter volume confinement, a short diffusion distance, and a high surface-to-volume ratio. Over recent years, capillary-based immunoassays have been used to analyze various antigens such as C-reactive protein [5,6], digoxin [7], *Escherichia coli* [8], and pesticides [9]. However, most of these capillary immunosensors were done in cylindrical capillaries, which are prone to unwanted reflection or refraction of light that may affect optical measurements [10]. Previously, our group used a square glass capillary to eliminate the unfavorable features of cylindrical capillaries [11]. We previously demonstrated the use of various capillary immunosensors employing a long capillary that is cut into small sections for analysis [12–14]. Moreover, small sections of capillary biosensors, involving immunosensors and other biosensors, could

* Corresponding author. Fax: +81 72 254 9284.
E-mail address: hisamoto@chem.osakafu-u.ac.jp (H. Hisamoto).

[1] These authors contributed equally to this work.

[2] *Abbreviations used:* CVD, cardiovascular disease; OPN, osteopontin; trOPN, thrombin-cleaved osteopontin; ELISA, enzyme-linked immunosorbent assay; FDP, fluorescein diphosphate tetraammonium salt; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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http://dx.doi.org/10.1016/j.ab.2013.05.021

be integrated into a capillary-assembled microchip that can be used for multi-analyte sensing [15–20].

Here, we have developed a highly sensitive capillary-based ELISA for the measurement of trOPN in plasma samples by using a square glass capillary and compared its performance with that of the commonly used microplate ELISA (Fig. 1). We hypothesized that a more sensitive ELISA could be developed by increasing the concentration of the capture antibody immobilized on the inner surface of the square glass capillary and combining this with the nanoliter assay volume of the capillary (Fig. 1B). This capillary format may be a potential platform for biomarker detection for other diseases.

Materials and methods

Materials

Square capillaries with 300-μm outer widths (flat to flat) and 100-μm inner widths were purchased from Polymicro (Phoenix, AZ, USA). The polyimide coating of these capillaries was removed by heating before use. Microplates were purchased from Greiner Bio-One (Frickenhausen, Germany). 3-Aminopropyltriethoxysilane was purchased from Tokyo Chemical Industry (Tokyo, Japan). Fluorescein diphosphate tetraammonium salt (FDP) and glutaraldehyde were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-human trOPN antibody (mouse IgG), anti-human OPN antibody (rabbit IgG), and recombinant human trOPN were purchased from Immuno Biological Laboratory (Gunma, Japan). Alkaline phosphatase-conjugated anti-rabbit IgG antibody (goat IgG) was purchased from Rockland (Gilbertsville, PA, USA). All reagents were used without further purification. Distilled and deionized water used had resistivity values of more than 1.8×10^7 Ωcm at 25 °C.

Plasma samples

The ethics committee of the Ehime University Graduate School of Medicine approved the study. Written informed consent was obtained from all participating patients. Patients with moderate- to high-grade carotid artery stenosis with more than 70% diameter reduction were prospectively included in this study.

Preparation of immunoreaction capillary

All of the fluid handling for capillary cleanup, modification, and immunoassay was done using a glass syringe. The capillaries used in this work were washed with 1 M sodium hydroxide solution (30 min), flushed with pure water, followed by acetone, and heated at 70 °C for 60 min prior to use. This washing procedure ensured that surface modifications could be made. An immunoreaction capillary was prepared by immobilizing IgG to the inner surface by the well-known silanization chemistries. The inside of the capillary was filled with 3-aminopropyltriethoxysilane and left for 30 min to introduce amino groups to the inner surface. Then, the capillary was flushed with methanol and dried at 70 °C for 60 min.

The capillary was subsequently filled with a 5% aqueous solution of glutaraldehyde and left for 40 min to attach an aldehyde group. After flushing with water, 8.5 or 0.85 μg/ml anti-human trOPN antibody solution (50 mM carbonate buffer, pH 9.6) was introduced and left for 60 min. After flushing with water, a 0.5% sodium borohydride solution was introduced at 15 μl/min for 40 min to reduce the imine group. Then, after flushing with water, blocking buffer (10 mg/ml BSA in phosphate-buffered saline [PBS] solution) was introduced and left overnight to avoid nonspecific

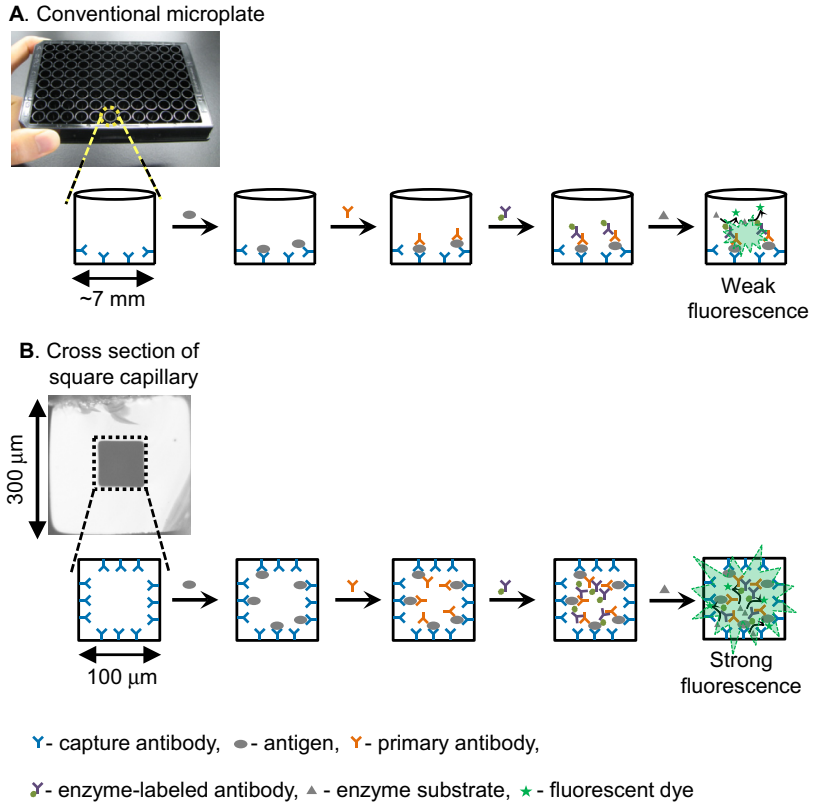

Fig.1. Basic concept of capillary-based ELISA (B) as compared with microplate ELISA format (A).