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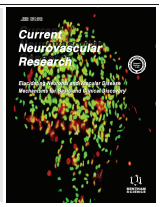
RESEARCH ARTICLE

Point of Care Test Technology Suitable for Early Detection and Monitoring of Ischemic Stroke



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Abstract: ***Background*:** Stroke is one of the leading causes of death and disability in adulthood worldwide. A simple and convenient diagnostic method is needed for monitoring high-risk patients for stroke. Few POCTs are available for stroke diagnosis. Soluble blood P-selectin is known as a biomarker for platelet aggregation. Increased expression of P-selectin is observed in coronary artery disease, acute myocardial infarction, stroke and peripheral arterial disease.

***Objective*:** A simple method that can measure the increased expression of P-selectin in stroke patients is intended to be used for diagnosis or early detection and hospital monitoring of ischemic stroke.

***Methods*:** Plasma proteins in blood were separated using a three-layered filter system. Quantum dot and antibody were conjugated to detect biomarkers present in plasma and then measured with a fluorescence spectrophotometer.

***Results*:** The detection limit of soluble P-selectin confirmed by immunoassay was 1 ng/ul. In order to increase the sensitivity and simplify the reaction, the detection limit was measured to evaluate the sensitivity of the quantum dot labeled anti P-selectin antibody. As a result, P-selectin of 5 ng/ul or more showed saturation signal intensity, indicating the upper limit of detection, and 10 pg/ul was the lower limit of detection.

***Conclusion*:** In this study, we proposed a three-layer filter membrane system that can separate biomarker-rich fractions from whole blood, simplifying the analysis process and improving sensitivity by using quantum dot-labeled antibodies to detect biomarkers. We hope that our system complements the advantages of POCT and can be applied to real clinical applications.

ARTICLE HISTORY

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1. INTRODUCTION

Stroke is one of the leading causes of death and disability in adulthood around the world. Stroke is classified into hemorrhagic and ischemic subtypes [1, 2]. Since stroke attack occurs unexpectedly, monitoring high-risk patients is essential to reduce mortality and morbidity. In the case of hemorrhagic stroke, CT scan is most commonly used for early diagnosis and monitoring of the disease, while MRI is the best choice for ischemic stroke diagnosis. But imaging based diagnosis is not accessible in small medical institutions and patients' homes. Expensive cost and expert dependency are another hindrance to emergent cases. Since rapid diagnosis and monitoring can improve stroke care quality and contribute to better clinical outcomes, the need for simple and convenient diagnostic method is evident. In case of Traumatic Brain Injury (TBI), blood biomarker based point-of-care tests (POCT) have been developed and used for the evaluation of the disease. Blood biomarkers including ubiquitin carboxy-terminal hydrolase isoenzyme L1, glial fibrillary acidic protein, aldolase isoenzyme C and S100 calcium-binding protein B are commonly used for TBI [3-5]. But there are few POCTs that can be used for stroke diagnosis. In spite of many advantages of POCT technology, lower sensi-

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tivity and less reliable results limit its usage as an on-site diagnostic tool. Soluble blood p-selectin is known as a biomarker for platelet aggregation. Increased expression of p-selectin is observed in coronary artery disease, acute myocardial infarction, stroke and peripheral artery diseases [6-8]. It is known to promote the onset of cardiovascular diseases, including myocardial infarction [9]. In this paper, we suggest a simple method which can measure soluble p-selectin in blood. This technology can be used for pre-hospital diagnosis or early detection and in hospital monitoring of ischemic stroke.

2. MATERIALS AND METHODS

2.1. Collection and Preparation of Blood Sample

Blood samples were provided from Kyungpook National University Hospital (KNUH). Experimental procedure was approved by Ethics Committee and Institutional Review Board of KNUH (KNUH 2018-03-013). All experiments were carried out in accordance with relevant guidelines and regulations. Whole blood was collected, transported and stored in EDTA-tread bottles at 4°C when it was used within 48 hours after sampling. Whole blood was used for experiment or fractionated for further use by centrifugation at 3,000g. Plasma or serum was carefully collected from the EDTA-containing or plain tubes, respectively, and was transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were collected. The plasma or serum samples were re-centrifuged at 3,000 g, and the supernatants were collected into fresh polypropylene tubes. The samples were stored at -80°C until further processing.

2.2. Three Layered Filter System for Isolation and Detection of Blood Protein

In order to isolate blood protein enriched fraction from whole blood, we used three-layered filter system, which was proposed in our previous study [10]. To facilitate the detection of target protein, we used nitrocellulose (NC) membrane with a large pore size (10 um, LFNC-C, NUPORE) as a filter layer three instead of a small pore size NC membrane (0.1um, #.10600010, GE Healthcare), which was used at our previous study.

2.3. Separation of Blood Proteins using Three Layered Filter System

Using three-layered filter system, we can obtain target proteins in filter three. Filter one was used to separate blood cells, filter two was used to separate blood proteins with larger size and filter three was used as a reservoir membrane which contains mainly biomarker proteins. By adjusting filter two conditions, we can enrich protein fraction within a specific size range in filter three. When polyethersulfone (PES) membrane (pore size of 0.03 um) was used, filter three contained more large-sized proteins and smaller proteins were enriched by PES membrane (pore size of 0.01 um). Filter three was used for anti-gen-antibody reaction space as well.

2.4. ELISA Assay

Blood samples from stroke patients were used to measure soluble p-selectin using Human SELP/P-selectin/CD62P ELISA assay kit (LS-F475, LSBio). Assay was performed following manufacturer's protocol.

2.5. SDS-PAGE and Western Blot Assay

To confirm the presence and size of target proteins we performed sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot assay. After incubation with primary antibodies (human SELP/P-selectin/CD62P antibody (LS-C296359, LSBio), human IgG antibody (SA1-35470, ThermoFisher Scientific), human IgM antibody (31415, ThermoFisher Scientific), human apolipoproteinA1 antibody (MA5-14732, Invitrogen) for 1 hour at room temperature, horseradish peroxidase (HRP)-linked secondary antibody (#.7074, Cell Signaling) was added for one hour at room temperature. Enhanced chemiluminescence (ECL) reaction was done to visualize the protein band using PicoEPD Western reagent (#.EBP1073, ELPIS-BIOTECH).

2.6. Detection using Color Dye or ECL

We used recombinant human p-selectin/CD62P (AD-P3-050, R&D systems), recombinant human apolipoproteinA1 (3664-AP, R&D systems) or blood proteins harvested by centrifugation or three layered filter system to evaluate the immunoassay using color dye. To measure the limits of detection of the assay, we used serial diluted recombinant proteins. One ul of recombinant protein solution was applied to the filter system. Filter three was blocked with 1% bovine serum albumin (A7906, Sigma) in phosphate buffered saline for 10 minutes at room temperature. After brief washing with 0.1 M Tris buffer solution for 5 minutes, primary antibodies (anti-human SELP/P-selectin/CD62P antibody (LS-C296359, LSBio) and mouse anti-human ApoA1 antibody (MA5-14732, Invitrogen) were used for antigen-antibody reaction. HRP-linked secondary antibody (#.7074, Cell Signaling) was incubated for one hour at room temperature and followed by color reaction with 3,3′,5,5′-tetramethylbenzidine (TMB)-D solution (4600, Kem-En-Tec Diagnostics). When HRP conjugated mouse anti-human IgG antibody (SA1-35470, ThermoFisher Scientific) and HRP linked goat anti-human IgM antibody (31415, ThermoFisher Scientific) were used, color reaction with TMB solution was made directly. Enhanced chemiluminescence (ECL) using PicoEPD Western reagent (EBP1073, ELPIS-BIOTECH) was performed to compare the result with TMB solution.

2.7. Quantum Dot and Antibody Conjugation

To make quantum dot labeled antibody solution, hydrophilic quantum dot (CZM-620W, ZEUS) and p-selectin antibody (Human SELP/P-selectin/CD62P Antibody, LS-C296359, LSBio) were used. After adding 20 ul of concentrated quantum dot solution (5 mg/ml) to 980ul of phosphate buffer solution (10 mM), 10ul of N-ethyl-N'-dimethylaminopropyl-carbodiimide solution (100 ug/ml, 22981, SIGMA)

and 10 ul of N-hydroxysulfosuccinimide solution (200 ug/ml, 24510, SIGMA) was added. After 30 minutes of reaction, centrifugation (5000 rpm, 3 minutes) using ultrafiltration unit (Vi-vaspin 500, Sartorius) was done to remove unreacted materials. By adding phosphate buffer solution to filtered quantum dot solution to make final volume of 1 ml. One hour after mixing 1 ml of quantum dot solution and 20 ul of anti-p-selectin antibody solution (2 mg/ml) at room temperature, 10 ul of bovine serum albumin solution (500 uM) was added and incubated for one hour. The fluorescence intensity of quantum dot-antibody conjugation was measured using a fluorescence spectrophotometer.

2.8. Detection of p-selectin using Quantum Dot Labeled Antibody

To evaluate the performance of quantum dot labeled p-selectin antibody, serially diluted (10 ng/ul to 1 pg/ul) recombinant p-selectin solution was used. After incubation for 30 minutes at room temperature, the fluorescent signal from reaction mixture was observed on third filter (NC membrane, pore size 10 um) in the small dark box under LED light (emission wavelength 365~370 nm). Image was visible with naked eyes and the corresponding fluorescent images were captured using smartphone camera (Samsung Note10). Dark box (width 30 mm, length 90 mm, height 80 mm) is made of black matte cardboard. On top of the dark box, there is one opening (width 10 mm, length 30 mm) for smartphone camera. Inside the box, we placed LED strip on the edge of the roof. LED strip is connected to auxiliary battery (Samsung EB-P1100, 10,000 mAh). Filter three, NC membrane is placed on the bottom plate during and after immunoassay (Fig. **1**).

Fig. (1). Dark box made of matte cardboard is used to detect fluorescent signals using smartphone camera (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3. RESULTS

3.1. Soluble p-selectin ELISA Assay of Blood from Ischemic Stroke Patients

Using blood samples from ischemic stroke patients, level of soluble p-selectin was measured. Even though the number of samples was not big, there was a significant difference between control group (n=12, 20.17±14.47 pg/ul) and ischemic stroke group (n=15, 58.65±10.64 pg/ul). The concentration of soluble p-selectin was similar to others' reports [11, 12].

3.2. Separation of Blood Proteins using Three Layered Filter System

Using three layered filter system, we obtained blood proteins from filter three layer. By adjusting filter two condition, we can enrich protein fraction within specific range. The presence of bigger proteins including immunoglobulin M (IgM, 180 kDa) and G (IgG, 150 kDa) (Fig. **2A**) was identified from filter three when PES membrane with pore size 0.03um was used as filter two. Those proteins were hardly detected when PES membrane with pore size 0.01um was used. ApolipoproteinA1 (apoA1, 28.3 kDa) was detected by both PES membranes. The amount of apoA1 was smaller by PES with pore size 0.01 um compared with 0.03 um (Fig. **2B**).

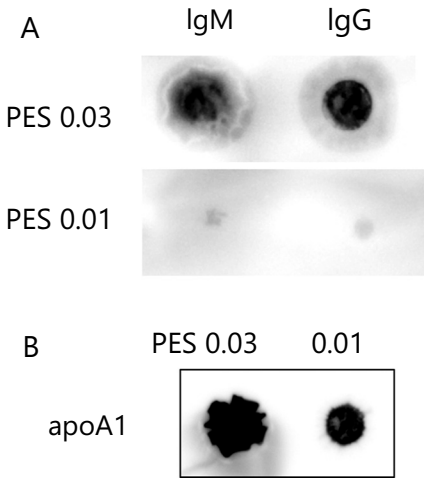

Fig. (2). (**A,B**) Separation of blood proteins using three layered filter membrane system. The presence of Ig M and G and apoA1 was visualized with ECL assay at filter layer three. PES membrane with different pore size (0.03 *vs*. 0.01 um) demonstrated different passage of proteins depending on the particle size of proteins.

3.3. Visualization of Soluble p-selectin using TMB or ECL

To test the limit of detection of soluble p-selectin, we checked the sensitivity of immunoassay using TMB dye. Primary antibody against p-selectin was reacted and then HRP labeled secondary antibody and TMB dye solution was added sequentially for visualization. When recombinant p-selectin was tested the lowest limit of detection was 5 ng/ul (Fig. **3A**). Since normal concentration of soluble p-selectin is around 10 ~ 20pg/ul, we tried more sensitive method using enhanced chemiluminescence (ECL). But the lower limitation of ECL method was 1 ng/ul (Fig. **3B**). To enhance sensitivity and simplify the reaction, we tried quantum dot labeled antibody as a next step.

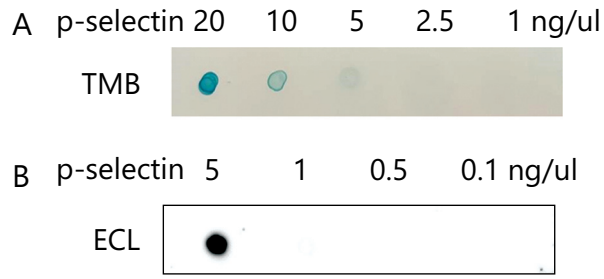

Fig. (3). (**A,B**) Visualization of p-selectin after immunoassay using color dye or enhanced chemiluminescence (ECL). Both methods could not detect the normal level of soluble p-selectin in the blood (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.4. Quantum Dot-antibody Conjugation

We conjugated quantum dot with antibody and measured fluorescence intensity of unlabeled antibody, unlabeled quantum dot and antibody labeled with quantum dot. Both quantum dot and labeled antibody showed the same peak of emission wavelength while unlabeled antibody showed no signal (Fig. **4A**). To confirm the quality of conjugation reaction gel electrophoresis was performed. Pure quantum dot, quantum dot labeled antibody and p-selectin reacted with quantum dot labeled antibody showed different size of bands (Fig. **4B**). To check the stability of quantum dot labeled antibody we measured fluorescent intensity for 4 weeks after conjugation. Intensity dropped during the first two weeks after conjugation and stayed at stable level till 4 weeks (Fig. **4C**).

3.5. Detection of Soluble p-selectin using Quantum Dot labeled Antibody

To evaluate the sensitivity of quantum dot labeled anti-p-selectin antibody, limitation of detection was measured. After the serially diluted recombinant p-selectin solution was applied on the filter system, filter 3 membrane was reacted with antibody. P-selectin over 5 ng/ul was showed saturated signal intensity, suggesting the upper limit of detection while 10 pg/ul was the lower limitation of detection since 5 pg/ul was almost the same as negative control (NC) (Fig. **5A** and **B**).

3.6. Optimization of Quantum Dot Labeled Antibody Assay

To simplify the assay process, we optimized antibody reaction condition to set the threshold of detection limit around 30 pg/ul. Recombinant p-selectin (10 pg/ul and 50 pg/ul) spiked blood samples were tested and 10 pg/ul was not detectable while 50 pg/ul was detected. Using this optimized antibody detection condition, detection of p-selectin concentration over the normal range is visible with naked eye (Fig. **6**).

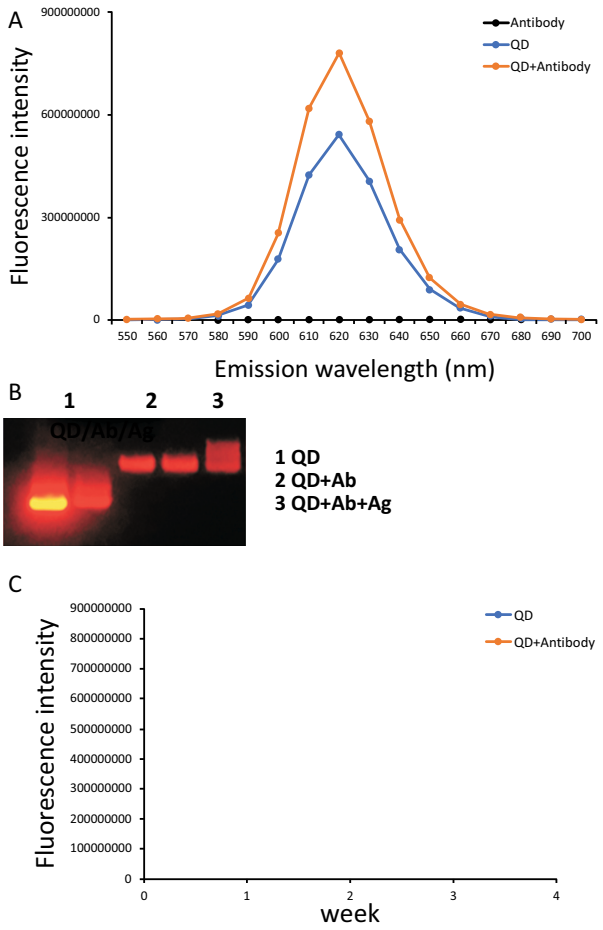

Fig. (4). (**A-C**) Quantum dot-antibody conjugation. After Quantum dot is labeled with p-selectin antibody, its characteristics are evaluated. Peak of emission wavelength, size of conjugated antibody, and stability were measured (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

Detection of early stroke or monitoring of high risk population is medically important and highly required by patients. Even though the concept of POCT or test at home has been developed for decades but practical application has limitations because of some obstacles. To be used by people without medical expertise the assay should be simple, including sampling, process of assay and detection. The assay should provide a meaningful information without using complicated device or process to determine the result. In this study, we suggested three layered filter membrane system which can isolate biomarker rich fraction from whole blood. This system can use only 20ul of finger pricked blood. By using quantum dot labeled antibody to detect biomarker, assay process was simplified and sensitivity was enhanced. After applying a drop of blood to filter system for 1 minute (separation step) and third filter membrane was isolated from the filter system and quantum dot labeled antibody solution was added to the membrane and incubated for 20 ~ 30 minutes at room temperature. To observe the result of assay and

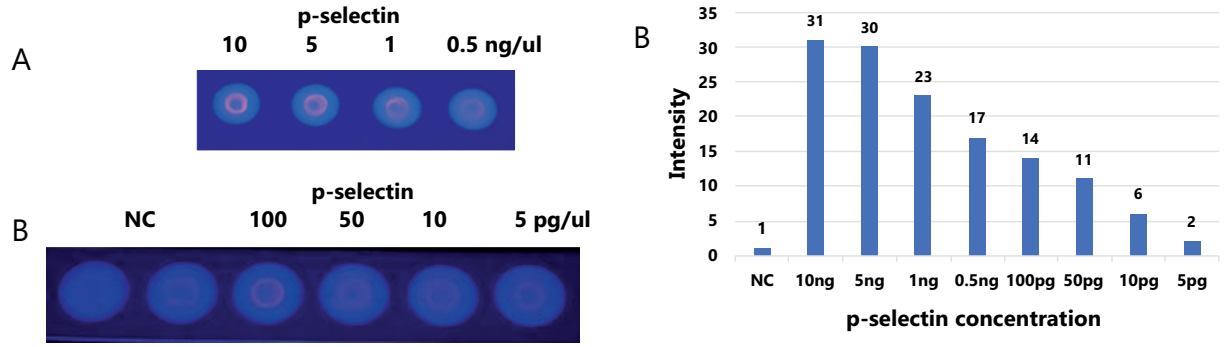

Fig. (5). (**A-B**) Detection of p-selectin using Quantum dot labeled antibody. The upper limit of detection was 5ng/ul and lower limit was 10pg/ul (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

store the record, the image was taken with smartphone camera in a small dark box which hosts third filter membrane inside. Antibody reaction was optimized to demonstrate fluorescent signals when abnormally high range (over 50 pg/ul) of soluble p-selectin while normal blood (less than 10 pg/ul) showed no detectable signals at our experimental condition. Three layered filter membrane system has several advantages. First, it minimizes the need for medical experts by reducing the blood volume suitable for assay. 20 ul of blood can be obtained by finger pricking instead of the intravenous route. Second, vertical flow of blood sample through three layered filter membrane system can reduce the interference by blood cells, large size proteins and other blood components by leaving those components mainly in the first and second layers. Biomarkers in the third layer are used for assay. In addition, by modifying the pore size and features of second layer, biomarkers with different sizes or characteristics can be fractionated in the third layer. Third, the loss of blood sample could be minimized and the amount of sample volume reached to third layer is consistent.

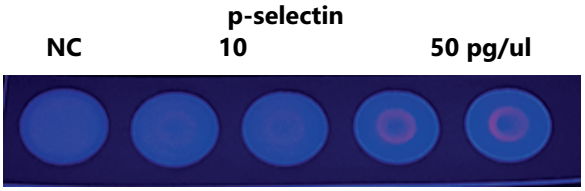

Fig. (6). Detection of p-selectin using quantum dot labeled antibody (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

This feature provides advantage in measuring the biomarker by reducing the error made during sample processing. In this study, we used a quantum dot to visualize the assay result. Quantum dot is an inorganic semiconducting nanoparticle with unique optical properties, such as broad absorption spectrum, narrow emission peak, resistance to photo-bleaching, and high quantum yield. Various functionalization and conjugation strategies have been developed to enable the labeling and detection of various biological analytes including small molecules, proteins, cells, and bacteria [13-15]. Even though quantum dot has several advantages, it has a critical pitfall since use of fluorescence detection system is mandatory. To check whether our assay can be detected with smartphone camera we tried a variety of different conditions and found out that LED (365nm) light placed at the top of the dark box and facing downward to keep the light away from illuminating directly to camera was successfully working. Even though the advantages of POCT compared with conventional technologies are portability, simple and easy to use, quick outcome to user directly, POCT usually demonstrates bad reputation including lower sensitivity, less reliable and inaccurate results. However, our system worked effectively and can be applied to the real site if manufacturing and quality control of the final product issues are well controlled. The future for POCT or test at home will be promising in case of telemedicine, mobile stroke unit and healthcare centers. POCT can be used in the post-hospital setting as well. We hope our study can contribute to implementation of point of care technologies in real clinical environments.

CONCLUSION

POCT has the advantages of being portable, simple and easy to use, and the user can quickly check the results. Therefore, the development of a diagnostic kit capable of early detection and prediction of stroke is medically very important. In our system, quantum dots were used to detect biomarkers in blood. By using a quantum labeled antibody, the analysis process was simplified and the sensitivity was improved. We hope that POCT will be useful for user convenience in clinical settings.

LIST OF ABBREVIATIONS

TBI	=	Traumatic Brain Injury
POCT	=	Point-of-care Tests
NC	=	Nitro Cellulose
PES	=	Poly Ether Sulfone

AUTHORS' CONTRIBUTION

Conceptualization, HSH; methodology, YML, MJB; validation, YSC, EL and MGK; formal analysis, JC; data curation, LMT; writing-original draft preparation, YML; writing-review and editing, GOC, NJYP; visualization, THDN; supervision, HSH; funding acquisition, HSH. All authors

have read and agreed to the published version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Board of Kyungpook National University Hospital, China (KNUH 2018-03-013, 10-05-2018).

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are basis of this research. All the humans procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

As Kyungpook National University Hospital takes e-IRB system, all patient concent forms are processed as electronic document without physical signatures.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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