Biosensors and Bioelectronics 91 (2017) 721–727

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

On-chip electrochemical immunoassay platform for specific protein biomarker estimation in undiluted serum using off-surface membrane matrix

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ARTICLE INFO

Keywords:
Electrochemical sensor
Off-surface 2D matrix
Poly carbonate, FNAB
TNF-α
Serum

ABSTRACT

The manuscript presents a new biosensor platform using bioreceptors modified porous 2-dimensional (2D) membrane based off-surface matrix for on-chip electrochemical immunoassay. Antibody based bioreceptors modified 2D matrix of porous polycarbonate (PC) membrane with densely packed 20 μm holes as off-surface matrix was incorporated in very close proximity of the sensor surface and integrated with fluidic system for reagent flow and incubation chamber. Covalent attachment of antibodies on 2D PC membrane based off-matrix was achieved using 4-fluoro-3-nitro-azidobenzene (FNAB) cross-linker. Anti-TNF-α/FNAB/PC membrane was integrated over array of micro fingers of gold based sensor chip using double side tape spacer and StartingBlock phosphate buffer saline- Tween-20; (PBS-T20) blocking buffer was utilized to minimize nonspecific binding. Differential pulse voltammetric studies of Anti-Tnf-α/FNAB/PC-Au for protein biomarker (TNF-α) detection and estimation in undiluted serum indicated that the immunosensor system can detect TNF-α linearly in 100 pg/ml to 100 ng/ml range with insignificant interference from other cytokines and serum proteins. Further, immunosensor exhibited high sensitivity of 194 nA/(ng/ml) and 240 nA/(ng/ml), respectively for single and double membrane based system. Thus, use of 2D membrane based off surface matrix may present the new platform to sensitively measure biomarkers electrochemically to pg/ml range with insignificant nonspecific binding and false signal in undiluted serum.

1. Introduction

Proteins, the building blocks of all living organisms, in appropriate range are crucial for healthy life, however their lower or higher level disturbed biological state and health of living being. This lower and higher level of proteins associated with various disorders has provided the opportunity to researcher to use them as biomarkers for prognosis and diagnosis of disease and to monitor effect of medicines during treatment (Arya and Bhansali, 2011; Chikkaveeraiah et al., 2012; Felder et al., 2014; Guo et al., 2015). Various proteins have already been established as biomarkers and have shown the promise of early and accurate prognosis of disease via their rapid and quantitative estimation using various methods (Chikkaveeraiah et al., 2012; Li et al., 2010). Further, the level of protein markers is known to increase with the stage of disease and its measured level can be correlated to disease stage accurately in most cases.

In recent years, immunosensors has shown great promise to be used as effective methods for protein detection and can potentially result in point-of-care (POC) device developments (Corrie et al., 2015; Kaushik et al., 2014; Vasan et al., 2013). Immunosensors can be tailored with newly researched and developed protocols for recognition molecule binding and desired target detection methods to realize improved, reliable and rapid disease diagnosis (Arya et al., 2013, 2012; Bosnjakovic et al., 2012; Liu et al., 2015a; Mazloum-Ardakani and Hosseinzadeh, 2015; Sun et al., 2013). Among various immunoassay detection methods, electrochemical ELISA based detection, which utilizes specificity of optical ELISA and simple fabrication, higher sensitivity, low cost and direct transduction of signal for electrochemical system has gained interest in development of electrochemical immunosensors (Fei et al., 2015; Gan et al., 2011b; Li et al., 2015; Mazloum-Ardakani et al., 2015; Pan et al., 2017; Sadik et al., 2009; Wei et al., 2016). However, fabrication of such biosensors usually involve binding of bio-recognition molecule on the surface of sensing electrode (Kokkinos et al., 2016; Pan et al., 2017; Zhang et al., 2016), which may cause fouling of sensor surface, and degradation of bio-recognition molecule during measurement scan in case of electroche-

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http://dx.doi.org/10.1016/j.bios.2017.01.033
Received 7 December 2016; Accepted 17 January 2017
Available online 18 January 2017
0956-5663/

mical sensing. Also, it has been observed that surface modification or immobilizing non-conducting bioreceptors such as antibodies or enzyme for selectively capturing target molecules decrease the conductivity of sensing electrode or sometime make them insulating, thus may reduce its ability to detect electrochemical signal sensitively (Arya et al., 2014; Arya and Park, 2014). Furthermore, measurement in real samples like serum, which have large number of highly concentrated non-target proteins may cause large background and false signal. Thus, there is an opportunity to design a electrochemical ELISA based system for enhanced signal detection (Li et al., 2015; Pan et al., 2017; Salimi et al., 2014; Zhang et al., 2016), while avoiding other issues and to measure signal sensitively and selectively especially in real samples.

To avoid the issues related to serum proteins, researchers have used the diluted serum (Gan et al., 2011a), however such step also dilute the desired protein concentration thus, make it difficult to be detected. On other hand to avoid the issues due to surface modification in electrochemical sensors, researchers have tried modifying the surface between or adjacent to electrode (Wang et al., 2014), however, such approach also result in exposure of active sensor surface to various modification reagents, which can adsorb on sensor to give false or varying results. Thus, efforts are required to develop new system, which can avoid these issues, while improving the sensor characteristics.

To develop electrochemical immunosensor without above mentioned issues, this paper describe the use of our recent patent on off-surface matrix based on-chip measurement (Arya et al., 2016), for detection of TNF-α in undiluted serum. There are various approaches have been developed for TNF-α detection (Li et al., 2012; Liu et al., 2015b; Pei et al., 2011; Wang et al., 2006; Yin et al., 2011), however in present work, it is taken as model marker to demonstrate off-surface matrix concept. The approach involves the selection and modification of matrix before attaching on sensor surface using spacer. In present work, 2-dimensional (2D) porous membrane of polycarbonate (PC) with densely distributed 20 μm holes has been chosen for its off-site modification with antibody followed by on-chip electrochemical detection of TNF-α (protein marker) in undiluted serum. Off-surface 2D matrix was first modified with bioreceptor using cross linker (4-fluoro 3-nitro azidobenzene (FNAB)) at remote location and then arranged and packaged in near vicinity of sensor surface via few micron thick spacer for fluidic flow and electrochemical sensing in undiluted serum samples with high specificity, and negligible interference from other proteins. Fig. 1 shows the images of PC membrane at various steps of modification with cross-linker and schematic for matrix modification with anti-TNF-α antibody. Results of present study reveal the potential of such approach to replace gold standard of optical ELISA at present.

2. Materials and methods

2.1. Chemicals

4-Aminophenyl phosphate monosodium salt (4-APP) was procured from Santa Cruz Biotechnology (USA). Antibodies and proteins were procured from Biolegend (USA); StartingBlock phosphate buffer saline-Tween 20; (PBST20) and StartingBlock tris base saline-Tween 20; (TBST20) were from fisher scientific, streptavidin-conjugated alkaline phosphate (ALP) solution was from Mabtech AB; Femto TBS procured from G-Biosciences. FNAB was from Setareh Biotech, LLC, polycarbonate membrane filter containing 20 μm holes was procured from sterlitech. Other analytical grade chemicals were utilized as received.

2.2. Gold electrode construction

Gold electrodes patterned in comb shape were created using microfabrication techniques and lithographic on thermally grown oxide layer coated silicon wafers (Pui et al., 2013). 5 μm wide and 3200 μm long comb fingers separated by 25 μm space were patterned over 5500 μm long base and utilized as sensors. The fabricated comb shaped gold electrodes were washed with ethyl alcohol, acetone and sufficient amount of water and then treated with UV-ozone for 30 min before use.

2.3. PC membrane and its modification using cross-linker

Off-surface matrix made of PC membrane was prepared via laser cutting of large filter into smaller matrix of oval shape with longer width of 9 mm and smaller width of 7 mm. Laser cut membrane was first cleaned with water and methanol followed by drying in air (Fig. 1a). Cleaned PC matrix was then modified with FNAB via photochemical reaction. 1 mg of FNAB in 25 μl of methanol was found to be optimal quantity for each PC membrane surface modification as higher concentration resulted in same response. FNAB solution in methanol was dropped on PC matrix and allowed to dry in dark (Fig. 1b). After drying, membranes were UV treated for 10 min using UV curing setup from Dyna tech (DT-UIS 1020) (Fig. 1c) and then washed with methanol to remove extra FNAB (Fig. 1d). FNAB has been used as cross linker to covalently immobilize antibodies and nitrene reaction occurring at azido group of FNAB during UV irradiation results in insertion of FNAB into C–H bond of PC matrix (Bora et al., 2006). Change in color of PC membrane at every step of FNAB treatment confirmed its modification (Fig. 1a-d).

2.4. Immobilization of anti-TNF-α antibody on FNAB modified PC membrane

Stock solution of 10 μg/ml anti-TNF-α antibody was prepared in phosphate buffer saline solution (PBS) (1x). For covalent immobilization, various volume of antibody solutions were tested and 25 μl was found to be optimum as it is found to cover whole membrane and no drying effect was observed during incubation at 37 °C in humid chamber for 2 h binding process. Thus, for antibody binding each membrane was submerged in 25 μl of Anti-TNF-α antibody stock in PBS and incubated at 37 °C for 2 h in humid chamber. During the incubation thermal replacement of active fluoro group occur by nucleophilic attacking amino group of antibody resulting in covalent immobilization of antibody on PC membrane via FNAB cross-linker (Arya et al., 2006, 2007; Bora et al., 2006). After antibody immobilization, membranes were washed with PBS-tween 20 solution and with PBS to remove non-cross-linked antibodies. Anti-TNF-α/FNAB/PC membrane was then integrated on sensor surface followed by blocking free spaces and preventing non-specific binding via 30 min incubation of starting block PBS T20 (SB) blocker. Fig. 1e shows the schematic for matrix modification and antibody binding.

2.5. Assembly of modified PC membrane on chips and development of off surface matrix with on-chip measurement system

Modified PC membranes were assembled on Au electrode chip using cleaned laser cut double sided tapes. For assembly, 9 mm×11 mm size double sided tape was laser cut to create oval shape chamber with longer width of 8 mm and smaller width of 6 mm. Oval shape was selected for better fluidic flow and to avoid dead fluid. Each modified membrane was sandwiched between two double sided tapes in which one or two membranes were used to create the off surface matrix with on-chip measurement system (Fig. 2a). Finally, to make fluidic and incubation chamber, 9 mm x 11 mm size PMMA sheet with two holes was placed on top of membrane using double sided tape. The Anti-TNF-α/FNAB/ PC-Au electrode thus formed were stored at 4 °C in humid chamber when not in use. Fig. 2 shows the schematic for integrated modified PC membrane on sensor chip along with schematic steps for immunoassay.

2.6. Immunoassay process sequence

Schematic in Fig. 2b shows the various steps involved in immu-