Sensors and Actuators B 209 (2015) 478–485

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Polymer-modified microfluidic immunochip for enhanced electrochemical detection of troponin I

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ARTICLE INFO

Article history:
Received 16 October 2014
Received in revised form
29 November 2014
Accepted 2 December 2014
Available online 11 December 2014

Keywords:
Immunochip
Immunoassay
Electrochemical detection
Polyethylenimine
Troponin I

ABSTRACT

We report enhanced electrochemical detection of a cardiac biomarker using a combination of a disposable microfluidic immunochip fabricated in Vacrel® 8100 photoresist film and a highly effective surface functionalization employing polyethylenimine (PEI). The use of the photoresist film enables fast prototyping and low-cost production without the need for a cleanroom. We use the surface carboxylates of the photoresist to biofunctionalize the microchannel on the chip using direct amine-specific coupling and modification via adsorbed and immobilized PEI in both linear (LPEI) and branched (BPEI) form. Characteristics of each immobilization strategy are assessed by a sandwich immunoassay for troponin I quantification in serum. The best assay performance is achieved using the immunochip modified with immobilized BPEI: the antigen was routinely detected at concentrations of 25 pg ml^{-1} in 4 min read-out time and 5 μl serum sample, representing an 18-fold improvement of the detection limit and 2.5-times faster read-out time in comparison to the assay implemented over amine-reactive esters without the PEI coating. We demonstrate that immobilized BPEI represents a stable and tunable scaffold that enhances biomolecule immobilization up to 60-times and stability 2-fold while lowering non-specific binding by factor of three. This system provides a versatile and nonaggressive means for incorporation of biological material to virtually any platform design.

1. Introduction

The development of a versatile immunosensing platform that can effectively and economically detect a broad range of different molecular targets is highly desirable. Performance characteristics of the vast majority of immunosensors, and biosensors in general, are directly linked to the choice of materials and applicable immobilization methods to attach the immunoreactants onto solid surfaces. The ideal immobilization strategy should employ mild chemical conditions and allow large quantities of biomolecules to be immobilized while retaining their biological activity. The immobilization surface should provide a large area for antibody–antigen interaction within a small total volume and should limit the non-specific protein adsorption. In terms of practical use, immobilization methods should extend the shelf life, which is a necessary criterion for commercial biosensors such as point-of-care (POC) diagnostics.

Without question, polystyrene, silica and polysaccharides are the three most common substrates for biomolecule attachment. Polystyrene is the traditional substrate for enzyme-linked immunosorbent assays (ELISA) in the form of microtiter plates or beads [1,2], but has been employed in biosensors as well [3]. Silica can be fabricated into a number of different forms including particles, wafers and thin films deposited by plasma chemical vapor deposition. Silica particles are employed in both chromatography and immunoassays [4,5], while silica wafers and thin films are increasingly being used in biosensors [6,7].

The same issues arise in both traditional immunoassays and biosensors: the decreased surface activity of the immobilized antibodies (or biomolecules in general) and non-specific binding (NSB) of various sample components to the solid phase. Antibodies attached by passive adsorption to polystyrene undergo conformational changes and remain only marginally active (<10%) [8,9]. The covalent immobilization used for silica and for PDMS relies predominantly on silanization protocols [10], which is a time consuming procedure and requires aggressive conditions that are not always compatible with non-silica parts of the packaging or housing [11]. These can have a negative impact on both the assay sensitivity and NSB – the former because relatively few active capture molecules are available to bind the analyte, and the latter because sample components are inclined to interact nonspecifically with the

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http://dx.doi.org/10.1016/j.snb.2014.12.006
0925-4005/

preponderance of inactive antibodies, which may be partially denatured.

To tackle these problems, various polymers have been used for biosensor applications. Instead of attaching proteins onto a two-dimensional solid surface, biomolecules can diffuse into a porous matrix formed by polymer membranes or surface coatings [12]. Polysaccharides were employed in a number of different types of chromatographic supports and immobilization matrices in biosensors such as carboxymethyl dextran in Biacore systems [13,14]. The traditional membrane material for the vast majority of lateral flow assays is nitrocellulose; nylon and polyvinylidene fluoride membranes had only limited success due to various factors such as high cost and limited utility [12,15].

Based on the aforementioned considerations, we merged the above approaches into a single platform to simultaneously take advantage of our microfluidic immunochip [16] and a novel immobilization strategy. We combined an microfabrication strategy using the flexible dry film photoresist, Vacrel 8100®, which offers good wetting properties for passive fluid control and easy biofunctionalization, together with an immobilization strategy in which the surface is first coated with a thin film of polyethylenimine that acts as a matrix for immobilization of biomolecules. We will refer to such a polymer coating as a "passivating" layer since it limits the interaction of proteins and other sample components with the surface – a fitting term used by Herron at al. [9].

Polyethylenimines are polycationic aliphatic polymers that can be produced either in linear form (LPEI) or with varying degrees of branching (BPEI). PEI has a high density of amino groups – every third atom in the polymer chain is a nitrogen atom. BPEI contains primary, secondary and tertiary amino groups with a ratio of 1:2:1, while LPEI mostly contains only secondary amines. In general, PEIs are both highly basic and water soluble polymers available in molecular weights ranging from 700 Da to 1000 kDa. They have been extensively studied predominantly in medicinal chemistry [17], but also for non-pharmaceutical use [18,19]. Importantly for our application, the polymer has been shown to be effective for immobilization of biocatalysts to solid supports by adsorption [20,21] and to have a positive effect on enzyme activity and stability [22] while having unique protein-resistant properties [23].

In our previous report, we demonstrated the high level of performance of our immunosensing platform using a competitive ELISA for substance P (an analyte of low molecular weight) and simple carbodiimide-based coupling chemistry [24]. To assess the benefit of the PEI modified immunosensor, we present here rapid detection with clinically relevant sensitivity of troponin I (cTnI) in a sandwich assay format.

cTnI is the "gold standard" for diagnosis of cardiac muscle cell damage and death [25]. cTnI (23.8 kDa) is a component of the troponin ITC complex (77.2 kDa), further comprising troponin T (35 kDa) and troponin C (18.4 kDa), a heteromeric protein that plays an important role in the regulation of skeletal and cardiac muscle contraction [26,27]. cTnI is expressed only in myocardium and there are no examples known of cTnI expression in healthy or injured skeletal muscle or in other tissue types [28,29], hence it is currently widely used for the diagnosis of acute myocardial infarction, unstable angina, post-surgery myocardium trauma, as well as several other diseases related to cardiac muscle injury [30].

2. Material and methods

2.1. Materials and chemicals

Pyralux® AP, Vacrel® 8100 and Teflon® 1600 AF were purchased from DuPont™. Monoclonal capture and detection antibodies (mAb); troponin ITC complex and cTnI-free serum were purchased from HyTest Ltd., Finland. Serum and blood samples were obtained from University Medical Center Freiburg. Glucose oxidase avidin conjugate (GOx-avidin) was obtained from Biomol, Germany. All other chemicals were purchased from Sigma–Aldrich or otherwise as stated in the text.

2.2. The chip fabrication

A detailed description of the wafer fabrication process utilizing Vacrel® 8100 can be found in our previous report [16].

2.3. Microtiter plate-based troponin I ELISA

See S1, Supplementary material for further information.

Every step of the immunoreactions was followed by three washing cycles on a commercial ELISA microplate washer using wash buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, 0.005% Tween 20, pH 7.4). Standard coating volume of 100 μl per well was used for all immunoreactants, 200 μl was used for blocking. Plates (Nunc-Immuno™ LockWell™ Modules, Nunc GmbH & Co. KG, Germany) were incubated sealed on a plate shaker preheated to 25 °C and at 450 rpm. Immunoreactants were diluted in PBS buffer (10 mM PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4).

Microtiter plate was coated with capture antibody combination, 16A11 and 19C7, to a final concentration of 10 μg ml^{-1} diluted in PBS. After 2 h incubation, the plate was washed. To saturate any binding sites not already occupied by the capture antibody, incubation with 200 μl of blocking solution was carried out for 30 min at 25 °C on a microplate shaker followed by overnight incubation at 4 °C without shaking. After subsequent washing, the plate was ready to use.

Troponin ITC complex was spiked into cTnI-free serum to concentration range 0.02–12.5 ng ml^{-1} using a 5-fold serial dilution. The calibrators were incubated for 2 h followed by a washing step and 1 h incubation with 4 μg ml^{-1} of biotinylated detection antibody, MF4b. 100 μl of a solution of GOx conjugated avidin diluted to 1 μg ml^{-1} in reagent diluent was added and the plates were incubated for 30 min at 25 °C. After subsequent washing, 50 μl of ABTS diluted in phosphate-citrate buffer (200 mM Na_2HPO_4, 100 mM citric acid, pH 5.6) to concentration 2.2 mg ml^{-1} were pipetted into each well. Solutions containing 160 mM of glucose and 0.8 mg ml^{-1} of horseradish peroxidase both in phosphate-citrate buffer were mixed in 1:1 ratio. A volume of 50 μl of this solution was pipetted into each well. The plate was incubated without shaking for 1 h at 25 °C and then immediately measured on an absorbance microplate reader (SpectraMax 340PC384, Molecular Devices GmbH, Germany) preheated at 25 °C at 405 nm. Software SoftMax Pro (Molecular Devices GmbH, Germany) was used to collect the data.

2.4. Chip-based troponin I ELISA

For every incubation step, a 5 μl of (immuno)reagents was pipetted on the chip inlet to fill the chip immobilization capillary. Chips were incubated in closed Petri dishes at 25 °C. Every step of the immunoreactions was followed by a washing step with a custom made vacuum pump using 200 μl of a wash buffer. PBS buffer and wash buffer were identical to those used for microtiter plate ELISA.

Methods for all immobilization strategies tested are described in S2, Supplementary material.

2.4.1. Direct immobilization strategy

The immobilization area of the chip was pretreated for 10 min with 10% Na_2CO_3 and washed with 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0. The