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A new aptamer immobilization strategy for protein recognition

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ABSTRACT

The interest towards aptamer-based surfaces in the field of bioaffinity assays is constantly growing. A crucial aspect is related to the ability of maintaining the high specificity of these molecules once immobilized on the surface. In this article we compare the immobilization of aptamers on a silanized silicon nitride surface as well as on a soft polymeric layer. An innovative immobilization approach, based on a copolymer, N-dimethylacrylamide-N-acryloyloxysuccinimide-3-(trimethoxysilyl)propylmethacrylate (DMA-NAS-MAPS) coating, able to crosslink itself to the substrate and to bind amino-modified biomolecules is proposed. Comparing this coating with a more classical functionalization process based on silane chemistry, we propose that the better results obtained on the polymeric layer are due to an increased binding efficiency of the aptamers bound to a soft material. The high specificity of immobilized DNA-aptamers is demonstrated using two sequences specific for thrombin detection and a non-sense sequence as negative control. The coating provides higher sensitivity compared to classical self-assembled silane coatings, probably due to a better mobility of bound aptamers. The aptamer immobilization on both surfaces was characterized and optimized using atomic force microscopy, X-ray photoelectron spectroscopy, contact angle and fluorescence microscopy. The comparison between the two different functionalizations highlights the better performances of the copolymer coating in terms of protein recognition, demonstrating thrombin detection down to 0.011 nM in buffer solution and 4.9 nM in complete human serum. Moreover, the localized immobilization of the aptameric sequences, utilized in this work, suggests the possibility of employing this platform also for multianalyte detection.

1. Introduction

The interest in proteomic technologies is growing at a fast pace, thanks to the possibilities they open up in discovering new potential biomarkers useful for the development of new drugs and therapeutic treatments [1]. In this perspective, there is an increasing interest in the development of biosensors able to analyze specific proteins. Different materials have been employed as solid supports for biosensing. Among them, those based on silicon are widely used thanks to their compatibility with microelectronic technologies that allow their integration in microfluidic devices. Silicon nitride is of particular interest for its chemical inertness and optical transparency across the vis-NIR range [2]. The combination of its high refractive index and mechanical properties makes this material extremely attractive in a variety of potential applications, such as optical label-free biosensors [3], nanopores based sensors [4,5], impedimetric sensors [6], and functionalized atomic force microscope tips for biosensing [7–9].

A common way to functionalize silicon nitride substrate relies on organosilane chemistry [6,10,11], that takes advantage of silanol groups exposed on oxidized substrates. A few works report the

Abbreviation: GPTMS, (3-glycidyloxypropyl)trimethoxysilane; DMA-NAS-MAPS, N,N-dimethylacrylamide-N-acryloyloxysuccinimide-3-(trimethoxysilyl) propylmethacrylate; TBA, thrombin binding aptamer; THR, thrombin; BioRecognition Buffer (BRB), Tris 50 mM, EDTA 1 mM, $MgCl_2$ 1 mM, KCl 150 mM, pH 7.4.

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direct immobilization of probes using the amino functionalities present on silicon nitride surface [12,8,13,14]; these approaches offer the possibility to tune the position of functional group in mixed structures, but are laborious and chemically aggressive. Moreover, the etching procedure often adopted to make the amino functionalities available on silicon nitride, are partially efficient: only 17% of surface chemical composition are Si–NH_2 and 37% are composed by Si–OH groups [15]. Therefore, we propose here a simple functionalization protocol based on the adsorption of copoly (DMA-NAS-MAPS), a ter-copolymer composed of N,N-dimethylacrylamide (DMA), N-acryloyloxysuccinimide (NAS) and 3-(trimethoxysilyl)propylmethacrylate (MAPS) which allows the covalent binding of amino functionalized molecules. This copolymer has been successfully used on a wide range of materials: polystyrene [16], polydimethylsiloxane [17], silicon or glass slide [18–20], nitrocellulose [21], and gold [22]. However, its ability to form a coating on silicon nitride was never demonstrated. In this paper, copoly (DMA-NAS-MAPS) creates a highly hydrophilic and soft layer on silicon nitride substrates. When DNA-aptamers are immobilized on such functional layer, their binding efficiency increases compared to more rigid silanized surface and this difference is probably due to a higher mobility of the aptamers bound to a brush polymer.

Aptamers are single-stranded nucleic acid molecules and represent a promising alternative to traditional antibodies in immunoassays and other biotechnological applications. They are a versatile class of capturing molecules that can target a wide variety of analytes [23,24] with high reproducibility. Easily modified by synthetic reactions, aptamers are highly stable even in non-physiological conditions [24]. Moreover, unlike antibodies, they can be regenerated, making the platform reusable. In order to fully exploit the potentialities of these molecules, a crucial aspect is to maintain their specificity once they are immobilized on a surface. Here we demonstrate that the copolymer coating acts as ideal substrate for aptamer immobilization, it allows a better target recognition compared to conventional functionalization based on silanization with (3-glycidoxypropyl)trimethoxysilane (GPTMS) both in terms of amino-modified DNA-aptamer binding accessibility and protein recognition.

Thrombin (factor IIa) has been selected as target protein because of its clinical interest. Thrombin is a highly specific serine protease that converts soluble fibrinogen into insoluble strands of fibrin: its detection and quantification in complex biological samples such as serum or plasma is therefore clinically relevant. In addition thrombin plays an important role in thrombosis and hemostasis and it is a key element in various pathogenesis such as leukemia, arterial thrombosis and liver disease [25].

In the first part of the paper, the two coatings formed either by an organosilane self assembled monolayer or by a thin copoly(DMA-NAS-MAPS) layer are characterized using different surface analysis techniques: XPS (X-ray photoelectron spectroscopy), AFM (atomic force microscopy) and contact angle. In the second part, the DNA-aptamer immobilization is optimized to provide the highest protein recognition; the aptamer immobilization was performed through a spotter deposition enabling the use of this method in microarrays. Finally, in order to characterize the coating performance, the surface was imaged with a fluorescence detector to assess the amount of protein bound to the aptamers. Although the polymer is not new, the immobilization of different aptameric sequences on the surface of miniaturized silicon nitride sensors is of great interest especially in point-of-care applications. This work demonstrates an innovative strategy for the immobilization of aptamer as a proof of principle for the construction of multiplex biosensors for the detection of specific protein markers with application in diagnostic.

2. Materials and methods

2.1. Materials

(3-Glycidyloxypropyl)trimethoxysilane (GPTMS, 98%), toluene anhydrous (99.8%) and toluene are purchased from Sigma-Aldrich. The copolymer DMA-NAS-MAPS is synthesized by ICRM group following the receipt reported in Pirri et al. [18]. All powders for buffered solution are purchased from Sigma–Aldrich. Purified Human Thrombin (0.31mg/ml, Bioultra, THR), bovine serum albumin (BSA) and Dulbecco's Phosphate Buffered Saline (DPBS) are purchased from Sigma–Aldrich. The two Thrombin Binding Aptamer (TBA) sequences utilized in this work [26] are reported in Table 1, together with a non-sense sequence. All the sequences are HPLC purified and purchased from IDT Integrated DNA Technologies (Leuven, Belgium). The monoclonal anti-thrombin antibody produced in mouse is from Abcam (Cambridge, UK), while the anti-mouse AlexaFluor488-conjugated antibody is from Life Technologies. Human blood from healthy donors was kindly provided by the Laboratory and Services Department of the Provincial Agency for Health Services of Trento (S. Chiara Hospital). Serum samples were obtained after clotting by centrifugation of whole blood at 700 g for 10 min and stored at −20 °C until use. The silicon nitride samples are fabricated depositing a 400 nm-thick silicon nitride (SiNx) layer on top of a silicon substrate in a parallel-plate plasma-enhanced chemical vapour deposition (PECVD) chamber at a rate of 22 nm/min. During the deposition process, two plasma frequencies are alternated (13.56 MHz for 50 s and 308 kHz for 10 s) in order to realize a stress-free (26 MPa) layer after each 1 min long deposition cycle (more details in Cazzanelli et al. [27]).

2.2. Substrate functionalization

In order to introduce chemical groups able to bind the amino group at the 5′ end of the aptamer sequence, the silicon nitride surface is functionalized in wet conditions in two different ways, as described in Fig. 1: (i) using the GPTMS silane (hereafter called GPTMS) or (ii) using the copolymer (hereafter called copoly (DMA-NAS-MAPS)). The silicon nitride substrates are cut into 1 cm × 1 cm pieces and cleaned with an argon plasma, applying 6.8 W of power to the RF coil for 1 min to remove organic contaminants and to hydroxylate the surface. For the silanization based functionalization, the substrate is then placed in a GPTMS toluene solution (0.1% v/v) at 60 °C for 10 min. Silane-coated substrates are rinsed several times with toluene and then dried in a nitrogen flow [28,29]. For the copolymer deposition, the procedure developed by Pirri et al. [18] was adopted. Briefly, the substrates are immersed in a 1% DMA-NAS-MAPS w/v ammonium sulfate solution at 20% saturation level for 30 min. After a washing step in MilliQ water, they are put in an oven at 80 °C for 15 min. The substrates modified with either the silane or the copolymer are then ready for the next functionalization step with the aptamers. Aptamers are subjected to thermal shock (95 °C for 1 min, ice for 10 min) in order to unfold the sequence strands and make the amino group available for the immobilization reaction. The aptamer deposition is performed spotting the aptamer solution in 50 mM phosphate buffer pH 8 in different arrays configuration (Biodyssey Calligrapher Spotter, Biorad, 100 μm capillary pin). Different incubation times and values of buffer ionic strength are tested in order to find the optimal parameters. After a washing step, the surfaces are incubated with a 1 mM solution of passivating agent (ethanolamine) in the same buffer as the one used for the aptamer deposition. This allows to block the un-reacted free epoxy and —NHS groups present on the surface of the silane and copoly (DMA-NAS-MAPS) modified samples respectively. Finally, three washing steps in buffer