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Nanochannels for diagnostic of thrombin-related diseases in human blood

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ABSTRACT

A high sensitive voltammetric method for rapid determination of thrombin spiked in whole blood by taking advantage of both aptamer-based recognition and the use of a nanoporous membrane has been developed. The nanoporous membrane not only acts as platform for the thrombin recognition but also as filter of the micrometric components such as white and red blood cells, consequently minimizing matrix effects. The protocol involves a sandwich format in the inner walls (200 nm diameter) of an anodized alumina oxide filter membrane (AAO). The analytical signal, by DPV oxidation of $[Fe(CN)_6]^{4-}$, is based on the blockage in the pores which affects the diffusion of $[Fe(CN)_6]^{4-}$ to the screen-printed carbon electrotransducer (SPCEs) modified with the membrane. By labeling the anti-thrombin IgG with AuNPs followed by silver enhancement a greater passive signal enhancement in comparison to the membrane blockage has been observed. The contribution of both electrostatic/steric effects in this blockage due to the subsequent formation of the aptamer-thrombin complex and the final sandwich assay is investigated. The efficiency of the system is also monitored by microscopic techniques. The resulted biosensing system allows detecting thrombin spiked in whole blood at very low levels (LOD 1.8 ng mL^{-1}) which are within the range of clinical interest for the diagnostic of coagulation abnormalities as well as pulmonary metastasis.

1. Introduction

The detection of thrombin in blood is of great importance in clinical analysis. The thrombin–fibrinogen interaction is part of the physiological process of blood clotting and thrombin has also hormone-like properties when it is involved in thrombosis and platelet activation (Wolberg, 2007). The concentration of thrombin in blood can vary considerably: it is not present in blood under normal conditions, but can reach low-micromolar concentrations during coagulation processes (Bichler et al., 1996). Independently of the hemostatic process, thrombin circulates at the high-picomolar level in the blood of patients suffering from diseases known to be associated with coagulation abnormalities. Furthermore, thrombin is considered as a tumor marker useful for the diagnosis of pulmonary metastasis (Nierodzik and Karpatkin 2006). For these both scenarios the development of simple (in comparison to ELISA, Western-blot, etc.) biosensing devices able to rapidly detect thrombin in human blood at picomolar (ng mL^{-1}) levels would be of great interest.

Colorimetric (Chen et al., 2010) and amperometric (Thuerlemann et al., 2009) biosensing strategies that use antibodies as receptors for the detection of thrombin in blood have already been reported. Although the high sensitivity and low detection limits achieved using the mentioned approaches these still do not solve the problems related to real blood sample analysis also the robustness (in terms of the receptor used) while thinking for future in-filed/ point of care applications.

Aptamers are promising bioanalytical tools for rapid and sensitive protein detection, including thrombin analysis, and for developing of protein arrays (Mukhopadhyay, 2005). These synthetic nucleic acid sequences act as antibodies in binding proteins owing to their relatively easy isolation and modification, holding a high affinity and high stability (Hansen et al., 2006). It is well-known that the single-stranded DNA oligonucleotide 5′-GGTTGGTGTGGTTGG-3′ (thrombin aptamer) acts as thrombin inhibitor. The mechanism of interaction consists in the binding of the aptamer to the anion-binding exosite, inhibiting thrombin's function by competing with exosite binding substrates fibrinogen and the platelet thrombin receptor (Paborsky et al., 1993).

This highly specific aptamer/thrombin binding interaction has been extensively approached to develop different biosensors for thrombin, taking advantage of the high sensitivity, selectivity, simple instrumentation, portability and cost effectiveness of the biosensing devices using different transduction techniques such as optical (Pavlov et al., 2005), voltammetric (Suprun et al., 2008) and impedimetric (Zhang et al., 2009).

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The combination of the aptamer-based electrochemical biosensors with the tools provided by the nanotechnology, such as the use of nanoparticle tags (Hianik et al., 2005; Heyduk and Heyduk, 2005) and multi-walled carbon nanotubes (Kara et al., 2010) with final detection by i.e. electrochemical impedance spectroscopy, has given rise to very sensitive biosensing systems for thrombin at even below the picomolar levels. However, in most cases a blood sample pre-treatment is required in order to remove red and blood cells as well as other components which can interfere in the specific thrombin detection.

In this context, nanoporous materials starting from Colter counter (Coulter, 1953) and its evolution from micro- to nano- sized (Bezrukov et al., 1994) sensing pores present outstanding new features for biosensor development (Howorka and Siwy, 2009; Hou and Jiang, 2009; Martin and Siwy, 2007). The pioneered nanopore sensor consisted in a α-hemolysin protein single channel embedded within a lipid bilayer membrane; an ionic current was passed through the channel, and analyte species bound inside the channels were detected as transient blocks in this current associated with translocation of the analyte through the channel-stochastic sensing (Bayley and Cremer, 2001). The drawback related to the fragility of the supported bilayer membrane that houses the nanopore was then solved building artificial nanopores embedded in a mechanically and chemically robust synthetic membranes (Gyurcsányi, 2008), being the anodized alumina oxide (AAO) one of the most commonly used for these purposes.

In addition to their extensive study for DNA sequencing, the blockage of the ion current in AAO nanochannel arrays by a bioreaction has also been approached by our group and others for ssDNA (Vlassiouk et al., 2005; De la Escosura-Muñiz and Mekoçi, 2010a) and protein (De la Escosura-Muñiz and Merkoçi, 2010b) detection, even in blood samples (De la Escosura-Muñiz and Merkoçi, 2011), taking advantage of their properties not only as sensing platforms but also as filters of micrometric components of the blood.

Here we present a novel aptamer-based biosensor for thrombin detection using AAO membranes, containing pores of 200 nm in diameter, and screen-printed carbon electrotransducers (SPCEs). The dual electrostatic/steric blockage in the pores due to the specific binding of thrombin and the further amplification in a sandwich assay using gold nanoparticle tags using the system $[Fe(CN)_6]^{4-/3-}$ as redox indicator is investigated. The nanoparticle-based amplification strategy in combination with the filtering properties of the membranes allows to specifically detect thrombin in whole blood at picomolar levels, which is within the range of clinical interest.

2. Experimental section

2.1. Apparatus and electrodes

Anodized alumina oxide filter membranes (Whatman anodisc AAO filters, 13 mm diameter; 60 μm thick containing 200 or 20 nm pores of $1 \times 10^9\ cm^{-2}$ density) were purchased from Scharlab (Spain). The electrochemical transducers used were homemade screen-printed carbon electrodes (SPCEs) and the measurements were performed using a home-made methacrylate cell connected to an Autolab 20 (Eco-chemie, The Netherlands). See the detailed SPCE fabrication procedure, pictures of the obtained sensors and of the electrochemical cell set-up as well as details of the optical apparatus used at the Supplementary material.

2.2. Reagents and solutions

A 5′ amino modified DNA aptamer, selective to human alpha thrombin: 5′ NH_2-GGTTGGTGTGGTTGG-3′ was purchased from Alpha DNA (Canada). Oligonucleotide solution was prepared in TE buffer, pH 8 (10 mM Tris–HCl buffer solution, 1 mM in EDTA) and maintained at −20 °C. Working solutions were made in 0.1 M Tris, pH 7.2 buffer and conserved at 4 °C.

Thrombin from human plasma (T6884), tyrosinase (T3824) and goat anti-rabbit IgG–FITC (F0382) were purchased from Sigma (Spain). Human alpha thrombin and tyrosinase working solutions were prepared in 0.01 M phosphate buffered saline, pH 7.4 (PBS). Rabbit anti-human thrombin was purchased from Abcam (Spain).

All the chemical reagents were purchased from Panreac, Sigma and Fluka (see Supplementary material).

Lyophilized human whole blood (certified reference material, Community Bureau of Reference-BCR® 635-, preserved in sodium-EDTA anticoagulant) was purchased from Sigma (Spain) and reconstituted in Milli-Q water, following the manufacturer indications. Human alpha thrombin was spiked and homogenized in the resulting blood sample.

Suspensions of AuNPs (80 nm sized) were purchased from BBInternational (UK). The conjugation of AuNPs to anti-human thrombin antibodies was performed according to a previously optimized procedure (see the Supplementary material). Zeta potential of the AuNPs before and after their conjugation with antibodies was determined with a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) according to the manufacturer's recommendations.

Silver enhancement was performed by using an LI Silver Enhancement Kit (Nanoprobes Inc., USA). The two components of the LI Silver Enhancement Kit were stored at 4 °C and a reactive mixture was prepared at 24 °C just before use. The stability (no self-nucleation) is guaranteed at this temperature over 45 min.

2.3. Methods

2.3.1. Zeta potential measurements

A 1 μL suspension of AuNPs (the same as for the anti-thrombin/AuNPs conjugates) was diluted in 1 mL of PBS buffer, vortexed, and transferred into a 4 mL polystyrene cuvette (FB55143, Fisher Scientific). The data were collected and analyzed with the Dispersion Technology software 4.20 (Malvern) producing diagrams for the zeta potential as a distribution versus total counts.

2.3.2. AAO filter membranes functionalization and immobilization of aptamers inside the nanochannels

The aptamer immobilization in the inner walls of the nanochannels was performed following an experimental procedure previously optimized for oligonucleotide sequences (De la Escosura-Muñiz and Mekoçi, 2010a), consisting first in the silanization and generation of carboxyl groups in the walls of the nanochannels. Then the aptamer was bound through the peptide bond by placing 30 μL of 1 mg mL^{-1} solution of the amino-modified aptamer on the filtering side of the membranes and keeping in a moist atmosphere overnight at 4 °C in order to avoid their dryness. To achieve such conditions, the membranes were placed in a glass slide inside a closed plastic box filled with water. The same experimental set-up was used for all the biological incubations.

See the detailed membranes silanization procedure as well as a scheme of the full process at the Supplementary material.

2.3.3. Aptamer/thrombin interaction inside the nanochannels

After thorough washing in PBS buffer, 30 μL of thrombin solution were placed on the filtering side of the membrane and left there for 60 min at room temperature (see the optimization of the reaction time in the Supplementary material).