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Nanostructured platform integrated into a microfluidic immunosensor coupled to laser-induced fluorescence for the epithelial cancer biomarker determination

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ABSTRACT

In the present work, we report a microfluidic immunosensor with a nanostructured platform based in zinc oxide nanoparticles covered by polyvinyl alcohol (ZnONPs-PVA) coupled to laser-induced fluorescence (LIF) for detection of epithelial cell adhesion molecule (EpCAM). EpCAM is a cell surface protein, and is overexpressed by epithelial carcinomas as such as the lung, colorectal, breast, prostate, head, neck, and hepatic origin. The detection limits (LODs) calculated for our method and for a commercial enzyme-linked immunosorbent assay (ELISA) test kit were 1.2 and 13.9 pg mL^{-1}, respectively. The within- and between-assay variation coefficients for the proposed method were below 6.50%. The results correlated well with those obtained with the commercial ELISA method, thus demonstrating that the new nano-platform integrated into a microfluidic device offers a truthful and useful analytical tool to be easily applied in epithelial cancer diagnosis through EpCAM biomarker determination.

1. Introduction

Nanotechnology has contributed to the development of miniaturized immunosensor-based devices with high-throughput analytical properties [1–3]. Nanomaterials, materials with sizes or features ranging from 1 to 100 nm in one or more dimensions, are the core of an emerging technological revolution. They show unique properties not found in conventional materials, such as light absorption and dispersion, which generate interesting immunosensing alternatives [4–6]. In the last years, different nanomaterials have been incorporated into immunosensors as platform for biomolecule immobilization and applied to relevant disciplines such as medicine, biology, food, agronomy and environment [7–12]. Some of the nanomaterials employed as platform are different types of nanoparticles [13–16]. There are many benefits in the use of these nanoparticles. The main advantage is the increase of the surface to volume, whose direct consequence is the increment of the assays sensitivity, because of the higher efficiency of interactions between samples and reagents [14–16]. To the best of our knowledge, a microfluidic immunosensor based in zinc oxide nanoparticles covered by polyvinyl alcohol (ZnONPs-PVA) as platform for biomolecule immobilization has not been reported to date.

Besides, immunosensors use diverse signal transduction pathways to recognize an antibody/antigen binding event. Most applications use a label to increase the sensitivity of detection. The current labels include metals, redox labels, optical labels and enzymes [17,18]. Fluorescence is one of the most sensitive detection methods, and is widely used for immunosensor design [19,20]. Several different setups can be coupled to fluorescence method, such as a microscope focused on the microchannel and connected to a charge coupled device camera or a photomultiplier tube. Some parts of the fluorescence setup, such as the excitation source and detection device, can be miniaturized [21,22]. Fluorescence detection has advantages like: high detection sensitivity (such as single molecule detection); fast response times; localized fluorescence signal; multiplexed assays using multicolor dyes; and the straight forward labeling process, which provides appropriate functional groups [23–25]. Laser-induced fluorescence (LIF) applied for analyte detection is one of the most sensitive detection techniques, which is capable of reaching concentration detection below 10^{-13} mol L^{-1} and a mass detection of less than 10 molecules. Radiation from a laser source can be focused, making it a useful tool for detection in very small volumes [24,25]. This property makes LIF detection a method of choice for detecting analytes on microfluidic devices, where the characteristic length scales are of the order of micrometers or even smaller [26–28].

A relevant area for the development of immunosensors is in cancer diagnosis through specific tumor biomarker determination. These

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biomarkers are one kind of biochemical substances produced by human tumor tissues, which can reflect the existence and growth of tumors in the human body [29,30]. Cancer biomarkers have critical clinical significance in early screening of tumors, assistantly determining the disease phase, selecting the accurate therapy and observing the curative effect. Epithelial cancer biomarker (EpCAM) is a cell surface protein, and is overexpressed by epithelial carcinomas as such as the lung, colorectal, breast, prostate, head, neck, and hepatic origin, and is absent from hematologic cells [31,32]. Besides, circulating tumor cells (CTCs) are cells that have shed into the vasculature from a primary tumor and circulate in the bloodstream. CTCs thus constitute seeds for subsequent growth of disseminated tumor mass (metastasis) in vital distant organs, triggering a mechanism that is responsible for the vast majority of cancer-related deaths [29,30]. Therefore, the detection of CTCs may have important prognostic and therapeutic implications [29,30]. For these reasons, the development of sensitive analytical methods for EpCAM determination would be transformative in the diagnosis and treatment of epithelial origin cancers.

The aim of this work was to develop a sensitive microfluidic immunosensor based in ZnONPs-PVA as platform for biomolecule immobilization coupled to laser-induced fluorescence (LIF) detection for EpCAM determination in biological samples.

2. Experimental

2.1. Materials and reagents

The following materials and chemicals were used as supplied. Soda-lime glass wafers (26 × 76 × 1 mm) were purchased from Glass Technical (São Paulo, SP, Brazil). SYLGARD 184 and AZ4330 photoresist (PR) as well as AZ 400 K were obtained from Dow Corning (Midland, MI, USA) and Clariant Corporation (Somerville, NJ, USA), respectively. Glutaraldehyde (25% aqueous solution), acetone and hydrogen peroxide 30% were purchased from Merck (Darmstadt, Germany). Polyvinyl alcohol (PVA 88% hydrolyzed, Mw = 88.000), zinc nitrate tetrahydrate purum p.a. (crystallized, 99% KT), hydrofluoric acid (HF), 3-aminopropyl triethoxysilane (3-APTES), and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The commercial ELISA kit (enzyme immunoassay) for the quantitative determination of EpCAM biomarker was purchased from USCN Life Science Inc. (USA), and it was used according to the manufacturer's instructions. Mouse monoclonal antibody to EpCAM (1 mg mL^{-1}) and HRP-conjugated anti-EpCAM-antibody (1 mg mL^{-1}) were purchased from Abcam® (USA). Commercial immunomagnetic CTC detection kit was purchased from Miltenyi Biotec (Germany). All buffer solutions were prepared with Milli-Q water.

2.2. Instrumentation

The optical system was constructed using the proposed procedure by Seiler et al. [33] according to the following modification. A 561 nm single-frequency DPSS laser (Cobolt Jive, 561 nm, USA) operated at 25 mW served as the fluorescence excitation source. It was focused on the detection channel at 45° to the surface using a lens with a focal distance of 30 cm. The relative fluorescence signal of ADHP was measured using excitation at 561 nm and emission at 585 nm.

The paths of the reflected beams were arranged so that they did not strike the capillary channels elsewhere and to avoid photobleaching. The fluorescent radiation was detected with the optical axis of the assembly perpendicular to the plane of the device. Light was collected with a microscope objective (10:1, NA 0.30, working distance 6 mm, PZO, Poland) mounted on a microscope body (BIOLAR L, PZO). A fiber-optic collection bundle was mounted on a sealed housing at the end of the lens of the microscope, which was connected to a QE65000-FL scientific-grade spectrometer (Ocean Optics, USA). The entire assembly was covered with a large box to eliminate ambient light.

A syringe pump system (Baby Bee Syringe Pump, Bioanalytical Systems) was used for pumping reagent solutions, sample introduction, and stopping flow.

All solutions and reagent temperatures were conditioned before the experiment using a Vicking Masson II laboratory water bath (Vicking SRL, Buenos Aires, Argentina).

Absorbance was detected by a Bio-Rad Benchmark microplate reader (Japan) and a Beckman DU 520 general UV/VIS spectrophotometer.

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.).

The synthesized ZnONPs-PVA were characterized by UV–visible spectroscopy (UV–visible spectrophotometer model UV-1650 PC — Shimadzu, USA), a scanning electron microscope (SEM) (LEO 1450VP, UK), an energy dispersive spectrometer (EDS) (EDAX Genesis 2000 energy dispersive spectrometer, England), transmission electron microscopy (TEM) (Carl Zeiss CEM902, USA) and X-ray diffraction (XRD) using a Rigaku *D*-MAX IIIC diffractometer with copper radiation (kα = 0.154178 nm) and a nickel filter (Rigaku, Texas, USA).

2.3. Preparation of nanostructured platform: ZnONPs-PVA

ZnONPs-PVA were synthesized by wet chemical method. First, a 0.9 M NaOH aqueous solution was added under high-speed constant stirring and drop by drop to 0.5 M aqueous ethanol solution of zinc ($Zn(NO_3)_2 \cdot 4H_2O$). After addition of NaOH, the reaction was allowed to progress for 2 h. Then, it was kept standing overnight, and the supernatant was carefully separated. The remaining solution was centrifuged for 10 min, and the precipitate was removed. Finally, precipitated ZnONPs were cleaned with deionized water and dried in air atmosphere at about 60 °C. During drying, $Zn(OH)_2$ is converted into ZnO. At the same time, PVA was dissolved in distilled water (3 wt.%) at 80 °C. Then, 0.30 mg of ZnONPs was dispersed in 5 mL of PVA solution. Later, the mixture was stirred for 24 h at room temperature. Finally, the synthesized ZnONPs-PVA were achieved for their further characterization [11].

2.4. Microfluidic device fabrication

The construction of microfluidic immunosensor was carried out according to the procedure proposed by Segato et al. with the following modifications [34]. The microfluidic device design consisted of a T-type format with a central channel (CC) (60 mm length; 100 μm diameter) and accessory channels (15 mm length; 70 μm diameter). The main body of the microfluidic sensor was made of glass. Firstly the device layout was drawn using CorelDraw software version 11.0 (Corel Corporation) and printed on a high-resolution transparency film in a local graphic service, which was used as a mask in the photolithographic step. The printed mask was placed on top of a glass wafer previously coated with a 5 μm layer of AZ4330 (PR). The substrate was exposed to UV radiation for 30 s and revealed in AZ 400 K developer solution for 2 min. Glass channels were obtained employing an etching solution consisted of 20% HF for 4 min under continuous stirring. The etching rate was 8 ± 1 μm min^{-1}. Following the etching step, substrates were rinsed with deionized water and the photoresist layer was removed with acetone. To access the microfluidic network, holes were drilled on glass-etched channels with a Dremel tool (MultiPro 395JU model, USA) using 1-mm diamond drill bits. To achieve the final microfluidic immunosensor format, another glass plate was spin-coated with a thin poly(dimethylsiloxane) (PDMS) layer at 3000 rpm during 10 s. PDMS was prepared by a 10:1 mixture of SYLGARD 184 elastomer and a curing agent. The thickness of this layer was 50 μm. Before sealing, the PDMS layer was cured at 100 °C for 5 min in a hot plate. Glass channels and PDMS-coated glass substrate were placed in an oxygen plasma cleaner (Plasma Technology PLAB SE80 plasma cleaner) and oxidized for 1 min. The two pieces were brought into contact immediately after