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Epithelial cancer biomarker EpCAM determination in peripheral blood samples using a microfluidic immunosensor based in silver nanoparticles as platform

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ABSTRACT

In the present work, we report a microfluidic immunosensor for epithelial cancer biomarker EpCAM (epithelial cell adhesion molecule) determination. It is based on the use of synthetized silver nanoparticles (AgNPs) covered by chitosan (Cts). AgNPs-Cts were covalently attached to the central channel (CC) of the microfluidic immunosensor. These nanoparticles were employed as platform for anti-EpCAM monoclonal antibodies immobilization for specifically recognize and capture EpCAM in peripheral blood samples. Afterwards, the amount of this trapped epithelial cancer biomarker was quantified by HRP-conjugated anti-EpCAM-antibody. HRP reacted with its enzymatic substrate in a redox process which resulted in the appearance of a current whose magnitude was directly proportional to the level of EpCAM in the peripheral blood sample. The structure and morphology of synthetized AgNPs-Cts were characterized by UV–visible spectroscopy, scanning electron microscopy (SEM), energy dispersive spectrometer (EDS) and X-ray diffraction (XRD). The calculated detection limits for microfluidic immunosensor and the commercial ELISA were 2.7 and 13.9 pg mL^{-1}, respectively and the within- and between-assay coefficients of variation were below 6.37% for the proposed method. The microfluidic immunosensor is simple, sensitive, specific and reproducible. It has the potential for reliable point-of-care clinical diagnosis and prognosis of epithelial origin tumors in biological samples.

1. Introduction

Cancer biomarkers are one kind of biochemical substances produced by human tumor tissues, which can reflect the existence and growth of tumors in human body [1,2]. These biomarkers has critical clinical significance in early screening of tumors, assistantly determining the disease phase, selecting the accurate therapy and observing the curative effect. EpCAM antigen (epithelial cell adhesion molecule) is a cell surface proteins, and is overexpressed by epithelial carcinomas as such as lung, colorectal, breast, prostate, head, neck, and hepatic origin, and is absent from haematologic cells [3,4]. On the other hand, 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 additional tumors (metastasis) in vital distant organs, triggering a mechanism that is responsible for the vast majority of cancer-related deaths [1,2]. Therefore, the detection of CTCs may have important prognostic and therapeutic implications [1,2].

Recently, antibodies against EpCAM antigens have widely been used to capture and quantify the tumor cells in patients with cancer of epithelial origin [5]. Thus, the development of sensitive methods based in immunoassays for EpCAM antigen determination would be transformative in the treatment of cancers.

On the other hand, with the development of human genome project and molecular biology, more and more specific cancer biomarkers were developed, in the meantime, the rapid expansion of immunosensors also provides a new approach and effective platform for the identification and detection of tumor markers

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[2]. The basic principle of immunosensors includes two parts, one is the immune conjugation reaction of antigen and antibody and the other is the markers which can generate signal. In last years, electrochemical immunosensors got more and more attention in detection of some cancer biomarkers, owning to its intrinsic advantages of easy operation, low cost and fast analysis [2,6–8]. Moreover, electrochemical immunosensors offers the possibility of being incorporated in a microfluidic system which has been developed to perform a variety of biomedical and chemical analysis [9–11]. Therefore, these methods present important advantages in terms of the small amounts of reagent and sample required, high sensitivity, rapid response, portability, and its simple adaptation to multifunctionality and high-throughput analyses [12].

Recently, some electrochemical microfluidic immunosensors also have been developed for many analytical applications such as for cancer biomarkers [13–15]. Besides, in the last years, different nanomaterials have been incorporated to electrochemical sensors as platform of the specific immunoreactants, which through an appropriate immobilization process enable the fabrication of a biorecognition layer with desirable properties (i.e., large loading, well-preserved bioactivity and good reversibility) [16]. One of the solid supports employed are different types of nanoparticles [17–20]. 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 [16].

Recent publications have focused on the synthesis and characterization of silver nanoparticles (AgNPs). Numerous approaches have been used to prepare AgNPs as biological and physicochemical methods [21,22]. Although these nanofabrication techniques have been demonstrated to be an alternative to solution-solid phase methods, the chemical methods are more versatile [22]. The chemical reduction methods are involved in the preparation of AgNPs with well-controlled size in which silver ions are reduced by reductants and stabilizing or protecting agents to prevent these nanoparticles from agglomeration [22–25]. A variety of chemical approaches have also been utilized to produce AgNPs with different size distribution and different shapes [22–25]. AgNPs have been used in electrochemical immunosensors and they play relevant roles as immobilization of biomolecules, enhancement of electron transfer and labeling biomolecules [26,27]. To the best of our knowledge, no study involving a microfluidic immunosensor with AgNPs as platform for EpCAM determination in biological samples has been reported.

In the present work, AgNPs covered by chitosan (Cts) (AgNPs-Cts) have been synthesized by chemical reduction-based approach and characterized by various techniques. Synthesized AgNPs-Cts were covalently incorporated in a microfluidic immunosensor and then functionalized with anti-EpCAM capture antibodies for EpCAM determination. Initially, the EpCAM biomarker in peripheral blood samples was captured by anti-EpCAM primary antibodies. Afterwards, the bounded cancer biomarker is recognized by specific secondary antibodies. Subsequently, HRP enzyme-labeled secondary antibodies reacted with its enzymatic substrate, generating a product which suffered an oxidation on the electrode surface. This redox process resulted in the appearance of a current whose magnitude was directly proportional to the level of EpCAM in the biological sample. To conclude, the developed system represents an attractive and efficient analytical tool to be applied in the clinical diagnosis and prognosis fields. The microfluidic immunosensor provided a simple, low-cost and sensitive analytical method for detection of tumor EpCAM biomarker.

2. Experimental

2.1. Materials and reagents

The following materials and chemicals were used as supplied. Soda-lime glass wafers ($26 \times 76 \times 1$ mm) were purchased from Glass Technical (São Paulo, SP, Brazil). Sylgard 184 and AZ4330 photoresist (PR) as well also AZ 400 K were obtained from Dow Corning (Midland, MI, USA) and Clariant Corporation (Sommerville, NJ, USA), respectively. Glutaraldehyde (25% aqueous solution), acetone and hydrogen peroxide 30% were purchased from Merck (Darmstadt, Germany). Chitosan (Cts, high purity, M_v 140,000–220,000), silver nitrate ($AgNO_3$, 99.99%), sodium borohydride ($NaBH_4$, 99.99%), hydrofluoric acid (HF), 3-aminopropyl triethoxysilane (3-APTES), and 4-tert-butylcatechol (4-TBC) 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}) was purchased from Abcam® (USA). All buffer solutions were prepared with Milli-Q water. Commercial immunomagnetic CTCs detection kit was purchased from Miltenyi Biotec (Germany).

2.2. Apparatus

Amperometric measurements were performed using the BAS LC 4C (Bioanalytical Systems, West Lafayette, IN, USA). The BAS 100 B electrochemical analyzer Bioanalytical Systems) was used for cyclic voltammetric analysis.

The gold layer electrode was deposited at central channel (CC) by sputtering (SPI-Module Sputter Coater with Etch mode, Structure probe Inc., West Chester, PA, USA) and the gold thickness electrode was measured using a Quartz Crystal Thickness Monitor model 12161 (Structure probe Inc., West Chester, PA, USA). The synthesized AgNPs-Cts were characterized by UV–visible spectroscopy (UV–visible spectrophotometer model UV-1650 PC – Shimadzu, USA), scanning electron microscope (SEM) (LEO 1450VP, Labmen, San Luis, Argentina), energy dispersive spectrometer (EDS) (EDAX Genesis 2000 energy dispersive spectrometer, England) and X-ray diffraction (XRD) using a Rigaku D-MAX IIIC diffractometer with copper radiation ($k\alpha = 0.154178$ nm) and a nickel filter (Rigaku, Texas, USA).

A syringe pumps system (Baby Bee Syringe Pump, Bioanalytical Systems) was used for pumping, 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 Bio-Rad Benchmark microplate reader (Japan) and 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.)

2.3. Preparation of silver nanostructures: AgNPs-Cts

AgNPs were synthesized by a chemical reduction method using $NaBH_4$ [25].

The chitosan suspension was prepared by solubilizing chitosan (1 g) in acetic acid (50 mL, 1 wt%) solution. Then, $AgNO_3$ (50 mL, 0.01 M) was added immediately into the suspension under constant stirring for 2 h for preparation of the $AgNO_3$ in chitosan suspension. $NaBH_4$ (20 mL, 0.04 M) was added to the suspension of $AgNO_3$/Cts