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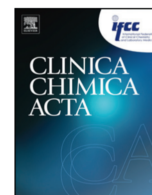

# Integrated bio-affinity nano-platform into a microfluidic immunosensor based on monoclonal bispecific trifunctional antibodies for the electrochemical determination of epithelial cancer biomarker

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ABSTRACT

*Background:* The epithelial cell adhesion molecule (EpCAM) is a biomarker that is highly overexpressed on the surface of epithelial carcinoma cells. In this study, silver nanoparticles covered with polyvinyl alcohol (AgNPs-PVA) were synthesized, characterized and used in a microfluidic immunosensor based on the use of anti-EpCAM recombinant antibodies as a trapping agent.
*Methods:* The concentration of trapped EpCAM is then electrochemically 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 (at a working voltage as low as −0.10 V) is directly proportional to the concentration of EpCAM.
*Results:* Under optimized conditions, the detection limits for the microfluidic immunosensor and a commercial ELISA were 0.8 and 13.9 pg/L, respectively. The within-assay and between-assay coefficients of variation are below 6.5% for the proposed method. The immunosensor was validated by analyzing patient samples, and a good correlation with a commercial ELISA was obtained.
*Conclusions:* The good analytical performance is attributed to the efficient immobilization of the anti-EpCAM recombinant antibodies on the AgNPs-PVA, and its high specificity for EpCAM. This microfluidic immunosensor is intended for use in diagnosis and prognosis of epithelial cancer, to monitor the disease, and to assess therapeutic efficacy.

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## 1. Introduction

The biomarkers for cancer consist of any measurable analyte in a patient that indicates normal or disease-related biological processes or responses to therapy [1–3]. One relevant biomarker of epithelial cancer is the epithelial cell adhesion molecule (EpCAM) which has been widely used for early diagnostic of carcinoma tumors of epithelial origin. This epithelial cancer biomarker is a 40-kDa transmembrane glycoprotein expressed on the surface of most epithelial cells [4]. It is also strongly overexpressed in many types of epithelial carcinomas, including colon and rectum, prostate, liver and esophagus, lung, head and neck, pancreas, and breast [1,4]. Importantly, EpCAM expression in normal epithelia is generally lower than that in carcinoma cells, and most hematopoietic tumors, such as leukaemia and lymphomas, are negative for EpCAM expression [4]. Therefore, EpCAM expression is a hallmark of epithelial tumors, and is widely used in carcinoma diagnostics [1,4]. 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 [5,6]. 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 [5,6]. Therefore, the detection of CTCs may have important prognostic and therapeutic implications [6,7].

In the last years, specific antibodies against different cancer biomarkers have been development by recombinant technology [8]. These antibodies can be used for develop new diagnostic methods and therapy of some types of cancer due to high specificity and affinity to specific cancer biomarkers [8]. The bispecific (*anti*-EpCAM x anti-CD3) trifunctional antibody combines the characteristics of classical monoclonal antibodies and bispecific molecules [9–11]. It is produced via quadroma technology and consists of mouse IgG2a and rat IgG2b. One specific antigen-binding site binds T cells via CD3, the other site binds tumor cells via the EpCAM antigen. The Fc region provides a third functional binding site that is able to selectively bind and activate Fcγ receptor I-, IIa- or III-positive accessory cells [9–11]. These *anti*-EpCAM

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monoclonal bispecific trifunctional antibodies are mainly used for the intraperitoneal treatment of patients with malignant ascites due to epithelial carcinomas [9–11]. In addition, this recombinant antibody is a potential therapeutic option for several primary tumors since the EpCAM molecule is expressed on the majority of epithelial carcinomas [9–11].

On the other hand, these recombinant antibodies can be immobilized on nanomaterials for the developed of novel analytical methods with the purpose of enhancing determination of EpCAM biomarker at low concentrations within complex mixtures such as serum, peripheral blood samples or tissue extracts. Also, the use of technologies of detection as for example the electrochemical technique, can effectively provide a platform for diagnosis of cancer because of the sensitive and wide dynamic range of detection and mainly for its easy adaptation into many miniature formats [8] as such as microfluidic immunosensor devices [8,12] and microarray-based detection methods [13]. Microfluidics technology possesses remarkable features for simple, low-cost, and rapid disease diagnosis, such as low volumes of reagent consumption, fast analysis, high portability along with integrated processing and analysis of complex biological fluids with high sensitivity for health care application [8,12]. Moreover, such devices may soon replace the traditional time consuming ELISAs and Western blots, and deliver rapid, point-of-care diagnostics to market [8]. Because of all these significant features, numerous microfluidic devices have been developed for different cancer biomarkers detection [14–20] but only a microfluidic immunosensor coupled to electrochemical detection has been reported by our group to EpCAM biomarker determination in biological samples [21].

## 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 from Glass Technical. AZ4330 photoresist (PR) and AZ 400 K were obtained from Dow Corning and Clariant Corp. respectively. Glutaraldehyde (25% aqueous solution) and hydrogen peroxide 30% were purchased from Merck. Polyvinyl alcohol (PVA 88% hydrolyzed, Mw = 88,000), 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 from Sigma-Aldrich. The commercial ELISA kit (enzyme immunoassay) for the quantitative determination of EpCAM biomarker was from Uscn Life Science Inc., and it was used according to the manufacturer's instructions. Mouse monoclonal bispecific trifunctional antibody (recombinant antibody) to EpCAM (1 mg/ml) and HRP-conjugated anti-EpCAM-antibody (1 mg/ml) were from Removab® and Abcam® (USA), respectively. Commercial inumunomagnetic CTCs detection kit was from Miltenyi Biotec. All buffer solutions were prepared with Milli-Q water.

### 2.2. Apparatus

Amperometric measurements were performed using the BAS LC 4 C (Bioanalytical Systems). 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.) and the gold thickness electrode was measured using a Quartz Crystal Thickness Monitor model 12161 (Structure).

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

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. Absorbance was detected by Bio-Rad Benchmark microplate reader and Beckman DU 520 general UV/ VIS spectrophotometer. All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc.) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.)

### 2.3. Synthesis of nano-platform: AgNPs-PVA

AgNPs-PVA were synthesized by a chemical reduction method using $NaBH_4$ [22]. $AgNO_3$ (1 mmol/l) was dissolved in 30 ml of ultrapure water (MilliQ-Plus®) in which 30 ml of PVA (1 g/l) was added. AgNPs covered by PVA were synthesized by reduction of $AgNO_3$ solution in $NaBH_4$ (0.1 mol/l) under constant stirring for 2 h. The NPs solution was washed by centrifugation at 24,000g for 1 h to remove the excess of reducing agent. The fresh AgNPs-PVA were sonicated for 30 min before use.

### 2.4. Design of microfluidic immunosensor

The construction of microfluidic immunosensor was carried out according to the procedure proposed by Segato with own modifications [23]. 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 immunosensor was made of glass. Firstly the device layout was drawn using CorelDraw software version 11.0 (Corel Corp.) 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. 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. For bonding of the chip, another glass plate with a thickness of 1 mm was used. The two pieces were thoroughly cleaned to avoid dust particles affecting the yield and they were brought into contact immediately at high temperature (typically above 500 °C) for bonding steps, obtaining a strong irreversible sealing. The bonding resistance of the present device was evaluated under different pressure values by using a high-performance liquid chromatography (HPLC) pump along the modification process. The flow rate ranged from 10 to 300 μl/min.

### 2.5. Surface modification of central channel

The CC of glass microfluidic immunosensor was exposed to a cleaning protocol, in which the solutions were pumped at flow rate of 2 μL/min as well as in all other procedures described in this section. As a first stage, CC was put in contact to 1:1 methanol:HCl solution for 30 min. After this process an additional cleaning step was performed employing concentrated $H_2SO_4$ for 30 min. Each chemical treatment was followed by rinsing with deionized water and drying under $N_2$. The described procedure effectively removes superficial contaminants and permits the homogeneous silanization of the glass surface.

Once the CC was in adequate conditions, the silanization process was carried out by exposing the CC to a 2% solution of 3-APTES in methanol

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