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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.

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 inmunomagnetic 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 24,000*g* 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

for 1 h. This process was followed by 3 rinses with fresh methanol and dried under N_2. This stage induces amine groups formation on the surface [24]. After that, glutaraldehyde solution (0.21 mol/l) in 0.1 mol/l sodium phosphate buffer (PBS, pH 8) was pumped to induce the formation of aldehyde groups at 25 °C for 2 h. Then, the CC was exposed to a washing step with deionized water at 25 °C for 1 h. As soon as, aldehyde groups were obtained on the glass surface, the immobilization of AgNPs-PVA was performed at 25 °C for 12 h according to the similar procedure proposed by Yu et al. [25].

Later, CC was washed with 0.1 mol/l PBS (pH 7.2) at 25 °C for 1 h. Once AgNPs-PVA were covalently attached to CC, anti-EpCAM recombinant antibodies (10 μg/ml 0.01 mol/l PBS, pH 7.2) were immobilized on their surface through the use of glutaraldehyde solution (0.21 mol/l) in 0.1 mol/l PBS (pH 8). In this case this cross-linker allowed the binding of the antibodies amino groups with those residual amino moieties present on the surface of AgNPs-PVA (Fig. 1). Finally, the CC was rinsed with 0.1 mol/l PBS (pH 7.2) to remove the unbound anti-EpCAM recombinant antibodies and stored in the same buffer at 4 °C. The overall time required for the immobilization procedure was 16 h. The immobilized antibody preparation was stable for at least 1 month.

2.6. Control preparations and patients recruitment

Before to use samples from oncology patients was necessary to make the controls to identify and correlate the number of circulating tumor cells (CTCs) with the protein EpCAM concentration obtained. We take epithelial colon cancer culture cell line HT29, realized a cell spike in blood from healthy voluntaries and blood samples from healthy voluntaries without cells added were used as negative control.

2.7. Spiking

To check whether our microfluidic immunosensor can efficiently detect EpCAM from CTCs, we have developed a spiking of epithelial breast cancer cells with colon cancer cells line HT29. The cells were counted in a Neubauer chamber, an average of 1000, 100, 50, 10 and 5 cells, were added to each 10 ml tube of blood from 20 healthy volunteers recruited with informed consent. The samples were made for quadruplicate, analyzing two by microfluidic immunosensor and two by classic inmunomagnetic determination of CTCs. This immunomagnetic CTCs method (Miltenyi Biotec) positively selects CTCs from the whole blood by using magnetic beads coupled with monoclonal antibodies that recognize epithelial markers such as EpCAM. Once the CTCs bind to magnetic beads, they can be separated from leukocytes by using magnetic field. After isolation of CTCs in the magnetic field, CTCs are subjected for further analysis after isolation.

2.8. Determination on healthy individuals

To determine that EpCAM is not circulating in healthy individuals, 20 healthy volunteers were enrolled. All individuals were over 18-years-old, without apparent inflammatory disease, drug treatment or history of cancer. Four 10 ml tubes of blood were extracted in CellSave® (Veridex) tubes and within 72 h after collection; the blood sample was processed in the same way as with the previously mentioned spiking.

2.9. Cancer patient recruitment

We selected 15 patients with metastatic advanced colon cancer enrolled in a clinical trial for CTCs determination in peripheral blood. After the informed consent sign, two CellSave® tubes with 10 ml of blood were obtained. Samples were processed within 72 h of extraction. Enumeration of $CTCs^{CK\,(+)}$ [26,27] was compared with EpCAM concentration obtained through of proposed microfluidic immunosensor. Any discrepancies between cell counting were resolved by discussion between two independent reviewers.

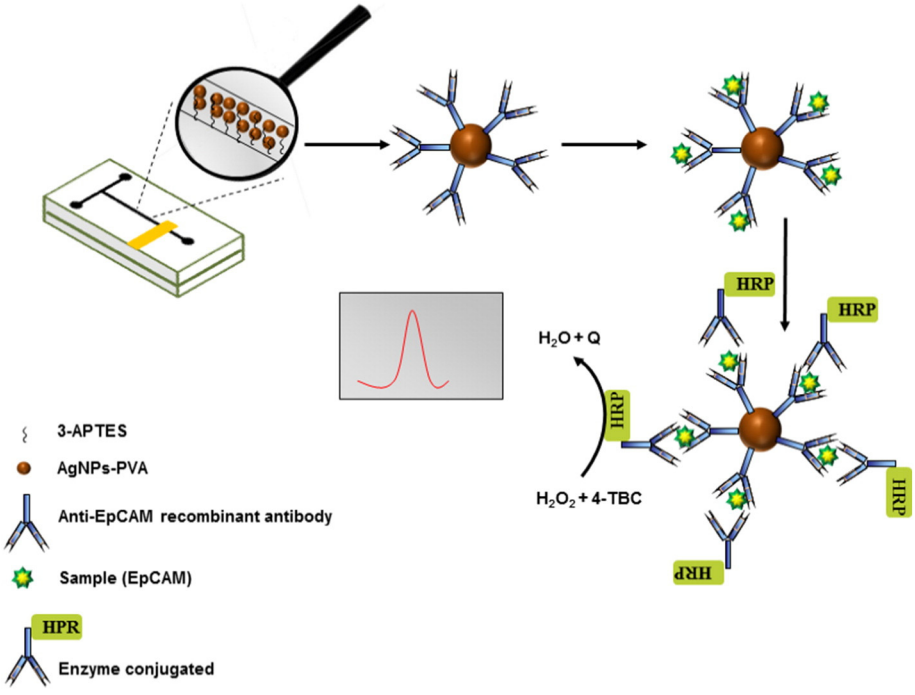

Fig. 1. Representation of the glass microfluidic surface modification and the immunological reaction. Anti-EpCAM recombinant antibodies were covalently bounded onto AgNPs covered by polyvinyl alcohol (AgNPs-PVA), which were covalently attached over 3-aminopropyl triethoxysilane (3-APTES) modified glass microfluidic surface. EpCAM biomarker present in the sample reacted immunologically with anti-EpCAM recombinant antibody immobilized on AgNPs-PVA-3-APTES-modified glass microfluidic immunosensor. The bound EpCAM was quantified by HRP-conjugated anti-EpCAM antibody using 4-tertbutylcatechol (4-TBC) as enzymatic mediator, which was oxidized on the electrode surface at −0.10 V. The current magnitude was directly proportional to the level of EpCAM biomarker.

2.10. Biological sample preparation for microfluidic immunosensor

Ten milliliters of peripheral blood were mixed with 5 ml of PBS (pH 7.2), this mixture was deposited on 10 ml of histopaque 1119 solution (Sigma-Aldrich) and centrifuged at 700*g* for 30 min at room temperature without brake. Then, the superior phase to the histopaque solution was aspired and put in 15 ml plastic tube, then a wash with PBS (pH 7.2) and centrifugation to 350 g during 10 min was performed. After that, the supernatant was eliminated and the pellet was incubated during 5 min with 50 μl of lysis buffer (Cell Signaling). Finally the sample was centrifuged at 4 °C 14000*g* during 30 min and the supernatant was removed for use.

2.11. Analytical procedure for EpCAM biomarker determination

The electrochemical determination of EpCAM biomarker was performed on: controls, samples and blank (0.01 mol/l PBS, pH 7.2). To accomplish it, the solutions employed were injected using syringe pumps at a flow rate of 2 μl/min. As a first step of each sample analysis, the microfluidic immunosensor was exposed to a desorption buffer (0.1 mol/l glycine-HCl, pH 2) for 5 min and then washed with PBS, pH 7.2 for 4 min. This treatment was carried out in order to desorb the immune-complex and start with a new analysis. After that, unspecific bindings were avoided by 5 min treatment with 1% bovine albumin in 0.01 mol/l PBS, pH 7.2 and washed with 0.01 mol/l PBS buffer (pH 7.2) for 4 min. Later, samples were injected into the PBS carrier for 10 min and then the sensor was washed with 0.01 mol/l PBS, pH 7.2 for 4 min. Once the EpCAM biomarker was recognized and captured by anti-EpCAM recombinant antibody on AgNPs-PVA, the HRP-conjugated anti-EpCAM-antibody (dilution of 1/1000 in 0.01 mol/l PBS, pH 7.2) was added in the 0.01 mol/l PBS (pH 7.2) for 5 min followed by a washing procedure with 0.01 mol/l PBS, pH 7.2 for 4 min. Finally, the substrate solution (1×10^{-3} mol/l H_2O_2 and 1×10^{-3} mol/l 4-TBC in 0.01 mol/l phosphate–citrate buffer, pH 5) was pumped and the enzymatic product was detected at −0.10 V (Fig. 1).

In order to start with the next determination of EpCAM biomarker, the microfluidic immunosensor was exposed to a desorption procedure (0.1 mol/l glycine–HCl, pH 2) at a flow rate of 2 μl/min for 5 min and then washed with PBS, pH 7.2. The purpose of this treatment is to have immobilized capture recombinant antibodies with free active sites before each sample analysis. The proposed device could be used with no significant loss of sensitivity for 15 days, whereas its useful lifetime was one month with a sensitivity decrease of 10%. The storage of the microfluidic immunosensor was made in 0.01 mol/l PBS (pH 7.2) at 4 °C.

3. Results and discussion

3.1. Characterization of synthesized AgNPs-PVA

AgNPs-PVA obtained by a chemical reduction method have been characterized by UV–visible spectroscopy, SEM, EDS, TEM and XRD. UV–visible absorption spectroscopy is widely being used technique to examine the optical properties of synthesized nanoparticles. Surface plasmon resonance (SPR) band at 400–450 nm in the UV–visible spectra indicates the formation of AgNPs-PVA [28]. Fig. S1(a) (Supplementary material) shows the characteristic SPR band at 420 nm indicating the formation of AgNPs-PVA [27]. Fig. 2(a) represents the SEM picture of AgNPs-PVA. This fig. substantiates the approximate spherical shape to the nanoparticles, and also can be seen that the size of the nanoparticle is $<25 \pm 5$ nm [28]. The elemental composition was disclosed by EDS analysis in which strong signals of Ag were observed at 3 keV, while signals from C and O were also recorded confirming the presence of AgNPs-PVA (Fig. 2(b)) [27]. Peaks of C and O reflecting the presence of elements constituting PVA [28]. TEM image of AgNPs-PVA is showed in Fig. 2(c), revealing that the primary morphology of the nanoparticles is spherical. It can be seen that the nanoparticles are well dispersed, without aggregation and have small particle size in the range 20–30 nm. Small nanoparticles can be observed in Fig. 2(c), indicating good stabilization by the PVA. The structures and compositions of the AgNPs-PVA were characterized by XRD. X ray diffraction pattern was recorded for AgNPs-PVA in the range $2\theta = 10°$ to 90° using Cukα ($\lambda = 1.54056$ Å) radiation (Fig. S1(b), Supplementary Material). By the observation of X-Ray pattern and calculations it is clear that AgNPs-PVA are having face-centered cubic structure and the peaks corresponding to (111), (200), (220) and (311) [28]. The estimated parameters for this phase is $a = 4.071$ Å that is quite close to the lattice parameter of bulk silver $a = 4.086$ Å [28]. The average size of the crystalline structure of the AgNPs-PVA was calculated according to the Scherrer equation ($t = K\ x\ \lambda / B\ x\ \cos\ \theta$) and the obtained value was approximately 25 nm. No characteristic peaks of any impurities were detected, suggesting that high-quality AgNPs-PVA were synthesized [29].

3.2. Optimization of experimental variables

Several studies of experimental variables that affect the performance of microfluidic immunosensor for EpCAM biomarker determination in biological samples were done. For this purpose an EpCAM control of 500 pg/ml was employed. One of the parameters evaluated was the optimal flow rate, which was determined by employing different flow rates and evaluating the current generated during the immune reaction. As shown in Fig. S2 (Supplementary material), flow rates from 1 to 2.5 μl/min had little effect over immune response and over signals obtained, whereas when the flow rate exceeded 3 μl/min the signal was dramatically reduced. Therefore, a flow rate of 2 μl/min was used for injections of samples, reagents and washing buffer.

The optimum reaction time was also evaluated, due to the fact that it affects the performance of the assay and consequently the obtained results. In order to optimize this factor, we analyzed different reaction times between the capture antibody and EpCAM in the developed microfluidic immunosensor through electrochemical signal. This first immunological stage was followed by adding HRP-conjugated anti-EpCAM-antibody. After washing, 4-TBC was incorporated. This enzymatic substrate suffered an oxidation process whose signal was measured as a function of reaction time. Fig. 3 shows the obtained signals for 125, 500 and 2000 pg/ml EpCAM control concentrations. The signal grew with increasing of the EpCAM concentration. As we estimated, the intensity of the electrochemical signal increased with the reaction time. However, the intensity of the signal did not markedly increase after 10 min for the higher EpCAM control concentration, which corresponds to the saturation of the antigen–antibody reaction in the glass microfluidic immunosensor. As a result, the reaction time used in all sample measurements was 10 min.

The determination of the optimum concentration of capture antibody to be employed in the immobilization procedure was also considered, due to the fact that the amount of this antibody affects the sensitivity of the immunoassay. The optimum value of *anti*-EpCAM recombinant antibody was 10 μg/ml (Supplementary Material).

Finally, the rate of enzymatic response using an EpCAM control of 500 pg/ml under flow conditions was analyzed in the pH range of 4–7 and reached a maximum at pH 5. The pH value used was 5 in phosphate-citrate buffer (Fig. S3, Supplementary material).

3.3. Quantitative determination of EpCAM biomarker in the microfluidic immunosensor

The determination of EpCAM biomarker was performed in 15 biological samples with the proposed microfluidic immunosensor and under the optimized conditions. The calibration curve of the proposed device (Fig. S4, Supplementary material) and commercial ELISA were constructed using different EpCAM control samples. The linear regression equation was $i\ (nA) = 13.63 + 0.13 \times C_{EpCAM}$ ($p = 0.05$), with

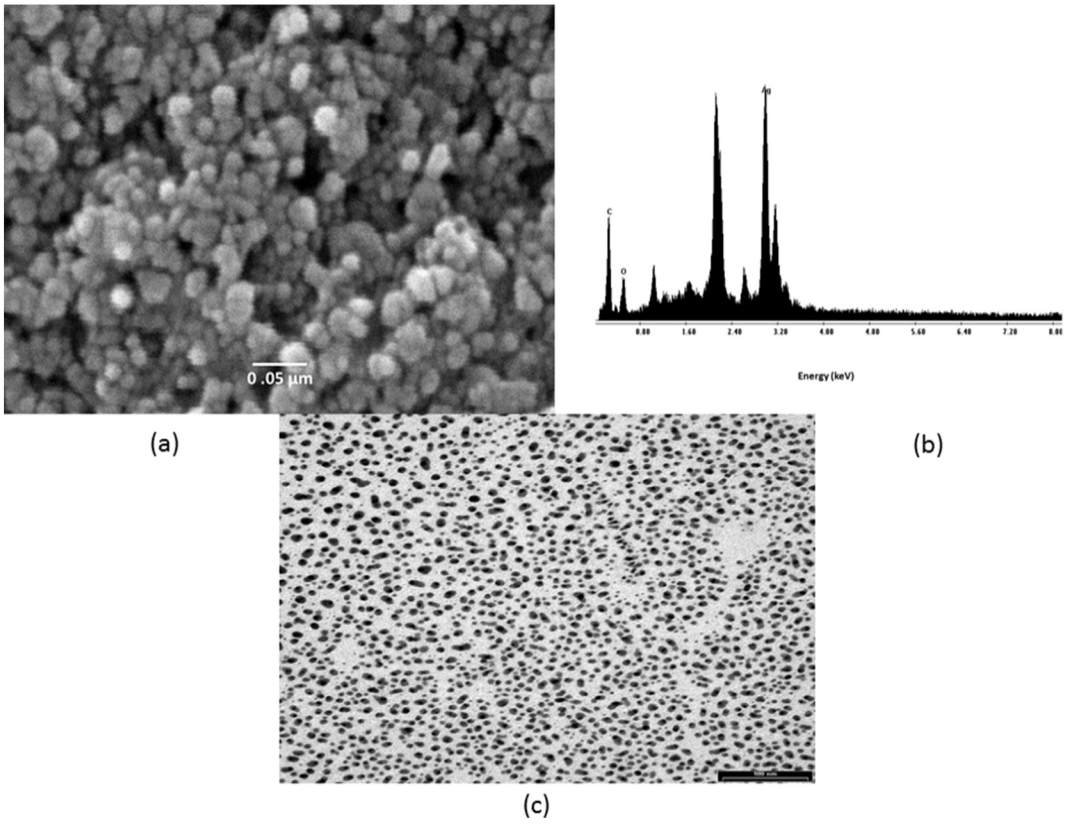

Fig. 2. Characterization of AgNPs-PVA nano-platform. (a) SEM image of AgNPs-PVA. This image confirmed the formation of spherical nanoparticles with a size $<25 \pm 5$ nm. (b) EDS spectra for AgNPs-PVA. Peak of Ag were observed at 3 keV, while signals from C and O were also recorded confirming the presence of AgNPs-PVA. Peaks of C and O reflecting the presence of elements constituting PVA. (c) TEM image of synthesized AgNPs-PVA.

the linear regression coefficient $r = 0.998$. A linear relation was observed between the concentration range 2–2000 pg/ml. The coefficient of variation (CV) for the determination of 2000 pg/ml EpCAM biomarker was 3.83% (5 replicates). An ELISA procedure was also carried out plotting absorbance changes against the corresponding EpCAM concentration then, a calibration curve was constructed. The linear regression equation was $A = 0.20 + 0.001 \times C_{EpCAM}$ ($p = 0.05$), with the linear regression coefficient $r = 0.996$, and the CV for the determination of 2000 pg/ml EpCAM biomarker was 6.58% (5 replicates). The detection limit (LOD) was considered to be the concentration that gives a signal 3 times the SD of the blank. For microfluidic immunosensor and commercial ELISA, the LODs were 0.8 pg/ml and 13.9 pg/ml respectively. This result shows that electrochemical detection was more sensitive than the spectrophotometric method. The precision of the proposed method was tested employing EpCAM controls of 15, 250 and 2000 pg/ml. The within-assay precision was tested with 5 measurements in the same run for each control. These series of analyses were repeated for 3 consecutive days to estimate between-assay precision. The results obtained are summarized in Table 1. The microfluidic immunosensor showed good precision; the CV within-assay values were below 3.95% and the between assay values below 6.45%.

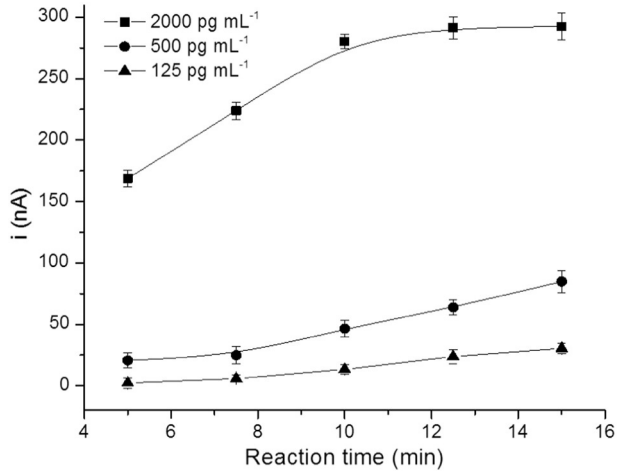

Fig. 3. Study of reaction time effect using EpCAM controls of 125, 500, and 2000 pg/ml; 0.01 mol/l phosphate–citrate buffer, pH 5, containing 1×10^{-3} mol/l H_2O_2 and 1×10^{-3} mol/l 4-TBC were injected into the carrier stream at different flow rates, and the enzymatic product was measured on the electrode surface at −0.10 V. Each value of current is based on 5 determinations.

The accuracy was tested with a dilution test which was performed with a EpCAM control of 2000 pg/ml which was serially diluted in 0.01 mol/l PBS, pH 7.2. The linear regression equation was $i\ (nA) = 0.94 + 293.20 \times C_{EpCAM}$, with the linear regression coefficient $r = 0.998$ (Fig. S5, Supplementary material).

Moreover, the proposed method was compared with a commercial ELISA procedure for the quantification of EpCAM in biological samples.

Table 1
Within-assay precision (5 measurements in the same run for each control sample) and between-assay precision (5 measurements for each control sample, repeated for 3 consecutive days).

[a]Control sample	Within-assay		Between-assay	
	Mean	CV %	Mean	CV %
15	15.16	2.82	15.77	4.84
250	250.12	3.95	250.46	5.63
2000	1999.28	3.83	1999.88	6.45

[a] pg/ml EpCAM.

The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Fig. 4). For this comparison, 5 high level and 10 low level EpCAM biological samples were analyzed. These samples were previously confirmed using the commercial ELISA, which is currently used in clinical diagnostics. The high level samples were later analyzed by our proposed quantitative method, which revealed high concentrations of EpCAM in all of them. The low levels EpCAM biological samples and blanks were also confirmed by our proposed microfluidic immunosensor.

Also, in the present work, 20 samples from healthy voluntaries, 10 spike controls and 15 patients with metastatic colon cancer were analyzed ($n = 45$) with the aim of correlate number of CTCs with EpCAM concentration. These samples were previously confirmed using the commercial immunomagnetic CTCs detection Kit (Miltenyi Biotec), which is currently used in the CTCs field research. The EpCAM levels were later determined by our proposed microfluidic immunosensor, which revealed high correlation between number of CTCs and EpCAM concentration in all of them ($p < 0.001$) (Fig. 5, Table 2). All results obtained in spike, patients and controls where added to the same table (Table 2) in order to correlate EpCAM concentration (by our microfluidic immunosensor) and CTCs number. There were not significant differences between patients and spikes. Therefore, our method is suitable to detection of blood samples of patients.

The increased sensitivity of our microfluidic immunosensor permit determine the EpCAM biomarker levels in a little number of CTCs (≥ 4CTCs) thus being able to correlate the number of CTCs patient with the EpCAM concentration in the lisate of the leucocitrarie fraction. Recent studies show that serum free EpCAM have not clinical significance, for other hand it is known that CTCs determination have great clinic value [26,27]. According with the sample preparation, several blood fractions can be analyzed. When a density gradient enrichment is performed; red cells, white cells, serum and platelets are separated in different fractions. Our immunosensor has been employed to detect EpCAM in white cell fraction of the blood, but not in serum (density gradient was performed as was described in sample preparation in material and methods). EpCAM is a specific marker of Circulating Tumor Cells (CTCs) that is not present in circulating cells of healthy donors [30]. The detection of these cells in peripheral blood has been considered as bad prognosis marker in cancer [30]. We can say that according with the kind of fraction employed, EpCAM concentration is related, or not; with CTCs which are an important marker to disease monitoring.

Moreover, the detection limit of proposed method is 5 CTCs which is way high as this is used for clinical prognosis and monitoring. The experts in the field consider that in the clinical practice ≥5 CTCs in 7.5 ml of blood have prognostic value [31].

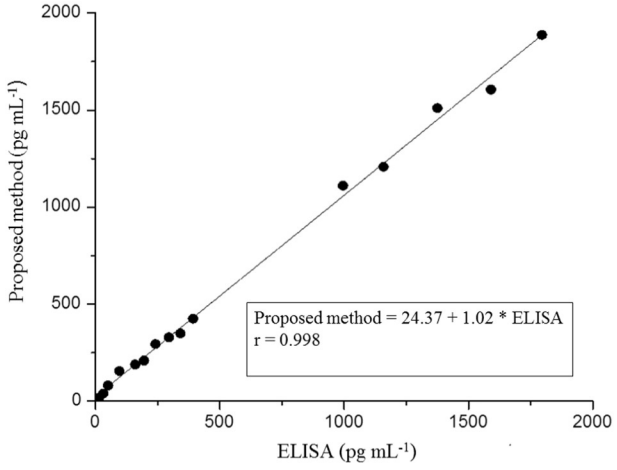

Fig. 4. Correlation between proposed method and commercial ELISA.

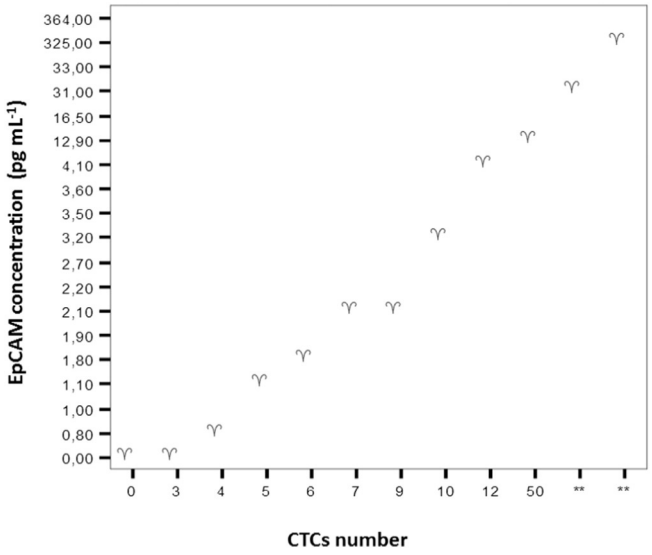

Fig. 5. Correlation between EpCAM concentration determined from proposed microfluidic immunosensor and number of CTCs immune magnetically determined. The samples contained 10 ml of blood from healthy voluntaries ($n = 20$), spikes ($n = 10$) and patients ($n = 15$).

Also, PVA is known to be a good stabilizer of small metal particles [22,32,33] and has been frequently used as particle stabilizers in chemical synthesis of different metal nanoparticles. Besides, this degradable polymer, is easily dissolved in water, and their combination with AgNPs can be easily integrated into microfluidic devices as nano-platform for biomolecules immobilization [32–38].

Furthermore, our electrochemical microfluidic immunosensor is based on recombinant antibodies for specifically recognize, capture and detection of the epithelial cancer biomarker EpCAM present in biological samples. Recombinant antibodies technologies have many attractive attributes compared to the traditional polyclonal and monoclonal antibodies [8]. First, they can be overexpressed and easily purified in a range of common eukaryotic and prokaryotic hosts, therefore they are easier to obtain and are more specific. Second, the genotype and phenotype of the antibody can be linked through various display technologies, allowing for the easy recovery of the coding regions of recombinant antibodies. Third, the sequence information allows for subcloning and bioengineering to generate fusions with proteins or enzymes. Fourth, through the process of subtraction, one can develop antibodies that recognize specific epitopes (regions of the protein), post-translational modifications, and conformations. Finally, they can serve as basis for their immobilization on nanostructures for the incorporation and fabrication of immunosensors [8].

In a previous work, our group developed a microfluidic device for EpCAM determination [21] but this novel electrochemical microfluidic

Table 2
Correlates the CTCs number and EpCAM concentration. The quantification is possible from 4 CTCs in 10 ml of blood. *P* value was obtained by Pearson correlation.

	CTCs number												Total	
[a]EpCAM concentration	0	3	4	5	6	7	9	10	12	50	100	1000	0	P
Undetectable	23	1	0	0	0	0	0	0	0	0	0	0	27	
0.8–2.7	0	1	3	2	1	2	0	0	0	0	0	0	7	
2.7–6.2	0	0	0	0	0	0	2	3	1	0	0	0	5	<0.001
17–20.5	0	0	0	0	0	0	0	0	0	2	0	0	2	
31.5–35	0	0	0	0	0	0	0	0	0	0	1	0	1	
35.1–38.5	0	0	0	0	0	0	0	0	0	0	1	0	1	
Over 45.8	0	0	0	0	0	0	0	0	0	0	0	2	2	
Total	23	2	2	2	1	2	2	3	1	2	2	2	45	

[a] pg/ml.

Table 3
Summary and comparison of relevant articles related to EpCAM immunosensors.

Detection system	Sample	LOD	Reference
Electrochemical microfluidic immunosensor	Peripheral blood samples	0.8 pg/ml	Proposed method
Electrochemical microfluidic immunosensor	Peripheral blood samples	2.7 pg/ml	[21]
Laser-induced fluorescence microfluidic immunosensor	biological samples	1.2 pg/ml	[39]
Fluorescent biosensor	cell membranes	qualitative detection	[40]
Electrochemical immunosensor	serum	1 pg/ml	[41]
Eletrochemical immunosensor	MCF-7 cells	1×10^5 cells/ml	[42]

immunosensor has many differences, one of these is that the proposed method is based on the use of recombinant antibodies for the EpCAM biomarker detection, this has been a clear advance in terms of specificity. In addition, the achieved LOD of proposed method (0.8 pg/ml) was lower than that obtained in the previous microfluidic device (2.7 pg/ml) [21]. Finally, this electrochemical microfluidic immunosensor exhibited excellent performances with a better linear response range in concentrations from 2 to 2000 pg/ml compared with the showed in the previous work [21]. Table 3 summarizes and compares the most relevant articles related to EpCAM immunosensors [21,39–42]. It is important to highlight that the proposed method had been designed for sensitive quantification of EpCAM in peripheral blood samples, while the remaining articles mentioned in Table 3 were applied to general EpCAM determination in serum, cell membranes or simply the qualitative detection of this biomarker.

4. Conclusions

We present an electrochemical microfluidic immunosensor based on AgNPs-PVA as nano-platform for immobilization of specific recombinant antibodies applied to sensitive quantification of EpCAM in biological samples. The overall assay time employed (34 min) was shorter than the time reported for commercial ELISA test kit frequently used in clinical diagnosis (270 min), with no reduction on the sensibility and selectivity. Besides, we could correlate with our microfluidic immunosensor the number of CCTs with EpCAM concentration, which is very important to the clinical diagnosis.

Finally, our microfluidic immunosensor with AgNPs-PVA as immobilizing platform of specific recombinant antibodies for EpCAM determination can be applied to assist diagnosis and prognosis of the epithelial cancer, monitor disease, and assess therapeutic effectiveness.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.11.012.

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