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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).

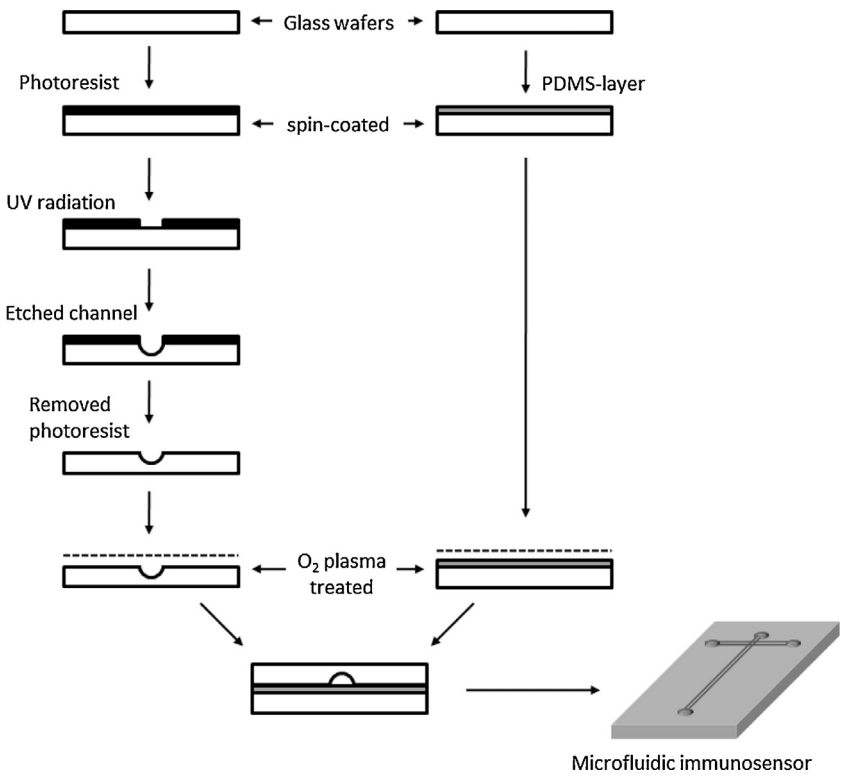
Absorbance was detected by Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 general UV/VIS spectrophotometer.

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



Scheme 1. Microfabrication process of glass microfluidic immunosensor with sealing based on the use of a thin layer of PDMS. Wet chemical etching of glass with 20% HF for 4 min under continuous stirring: spin coating of a PDMS and photoresist layer over flat glass surface at 3000 rpm during 15 s; plasma-oxidized treatment during 1 min.

and an immediate color change from pale yellow to brown indicating the formation of AgNPs was noted. This suspension was stirred for 1 h more, then the obtained AgNPs-Cts were finally achieved for their further characterization [25].

2.4. Microfluidic immunosensor fabrication

The construction of microfluidic immunosensor was carried out according to the procedure proposed by Segato et al. with the following modifications [28].

Scheme 1 shows the developed microfluidic immunosensor, whose design consisted of a T-type format with a CC (60 mm length and 100 μm diameter) and accessory channels (15 mm length and 70 μm diameter). The main body of the sensor was made of glass.

Details of microfluidic device fabrication are shown in Supplementary Material Section.

2.5. Central channel functionalization of microfluidic immunosensor

The CC of glass microfluidic immunosensor was exposed to a cleaning protocol, in which the solutions were pumped at flow rate of 2 $\mu L\,min^{-1}$ 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-aminopropyl triethoxysilane (3-APTES) in methanol for 1 h. This process was followed by three rinses with fresh methanol and dried under N_2. This stage induces amine groups formation on the surface [29]. After that, glutaraldehyde solution (0.21 M) in 0.1 M sodium phosphate buffer (pH 8.0) 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-Cts was performed through reactions between obtained aldehyde groups and amine groups of Cts coated on nanoparticles surface at 25 °C for 12 h. Later, CC was washed with 0.1 M sodium phosphate buffer (pH 7.2) at 25 °C for 1 h. Once AgNPs-Cts were covalently attached to CC, anti-EpCAM antibodies (10 $\mu g\,mL^{-1}$ 0.01 $mol\,L^{-1}$ PBS, pH 7.2) were immobilized on their surface through the use of glutaraldehyde solution (0.21 M) in 0.1 M sodium phosphate buffer (pH 8.0). 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-Cts (Fig. 1). 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.

2.6.1. Spiking

To check whether our microfluidic immunosensor can efficiently detect EpCAM from CTCs, we have developed a spiking of epithelial breast cancer cells with HT29 cells. The cells were

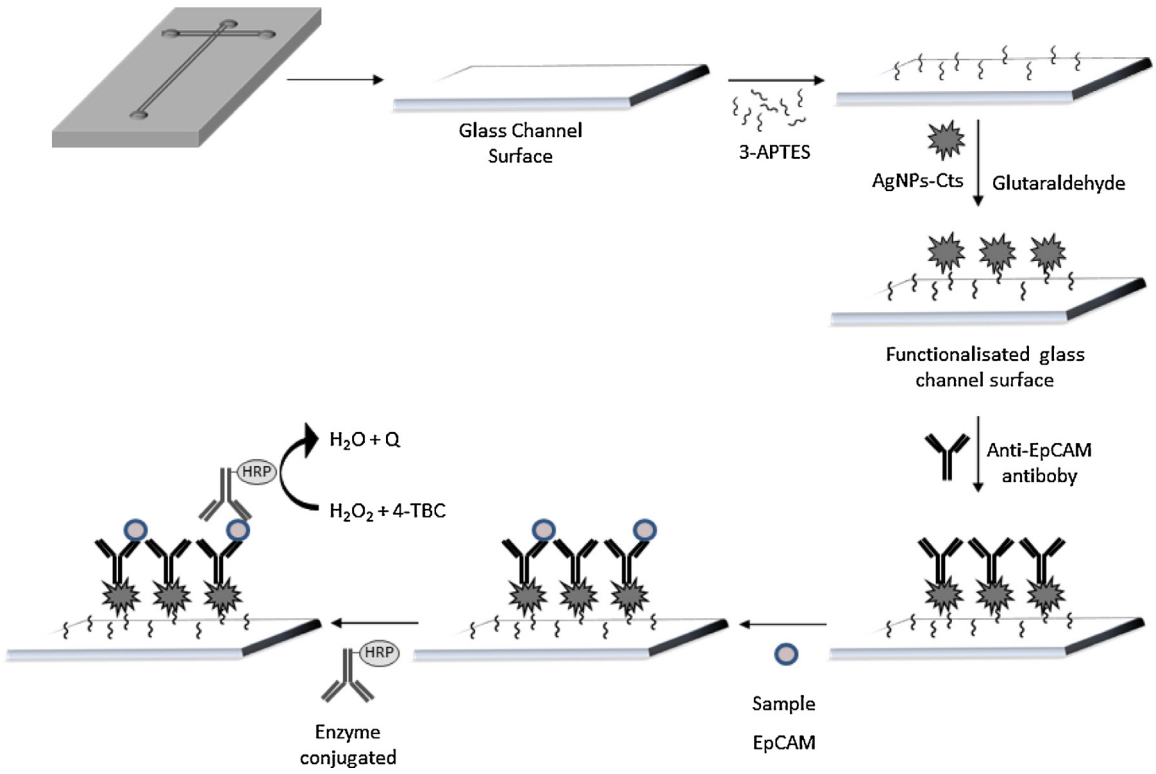


Fig. 1. Representation of the glass microfluidic surface modification and the immunological reaction. Anti-EpCAM antibodies were covalently bounded onto AgNPs covered by chitosan (AgNPs-Cts), which were covalently attached over 3-aminopropyl triethoxysilane (3-APTES) modified glass microfluidic surface. EpCAM present in the sample reacted immunologically with anti-EpCAM immobilized on AgNPs-Cts-3-APTES-modified glass microfluidic sensor. 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.

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 analyzed for duplicate, using two for microfluidic immunosensor and two for classic inmunomagnetic determination of CTCs.

2.6.2. Determination on healthy individuals

To determine that mesenchymal-like or EMT tumor cells are not circulating in healthy individuals, 20 healthy adult women were enrolled. All individuals were over 18-years-old, without apparent inflammatory disease, drug treatment or history of cancer. Two 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.

2.6.3. 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 $CTCs^{CK(+)}$ [30,31] was compared with EpCAM concentration obtained through of proposed microfluidic sensor. Any discrepancies between cell counting were resolved by discussion between two independent reviewers.

2.7. Sample preparation for microfluidic device

10 mL of peripheral blood were mixed with 5 mL of PBS, 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 putted in 15 mL tube, after a wash with PBS and centrifugation to 350 × *g* during 10 min. The supernatant was eliminated and the pellet was incubated during 5 min with 50 μL of lysis buffer (Cell Signaling). To finish the sample was centrifuged at 2 °C 14,000 × *g* during 10 min and the supernatant was removed for use.

2.8. Analytical procedure for EpCAM determination

The electrochemical measurement of EpCAM was performed on: controls, samples and blank (0.01 M PBS, pH 7.2). To accomplish it, the solutions employed were injected using syringe pumps at a flow rate of 2 μL min^{-1}. As a first step of each sample analysis, the microfluidic immunosensor was exposed to a desorption buffer (0.1 M 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 M PBS, pH 7.2 and washed with 0.01 M 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 M PBS, pH 7.2 for 4 min. Once the EpCAM biomarker was recognized and captured by anti-EpCAM antibody on AgNPs-Cts, the HRP-conjugated anti-EpCAM-antibody (dilution of 1/1000 in 0.01 M PBS, pH 7.2) was added in the 0.01 M PBS (pH 7.2) for 5 min followed by a washing procedure with 0.01 M PBS, pH 7.2 for 4 min. Finally, the substrate solution (1×10^{-3} M H_2O_2 and 1×10^{-3} M 4-TBC in 0.01 M 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, the microfluidic immunosensor was exposed to a desorption procedure (0.1 M glycine–HCl, pH 2) at a flow rate of 2 μL min^{-1} for 5 min

Table 1
Analytical procedure required for the EpCAM determination.

Sequence	Condition	Time
Blocking solution	1% bovine albumin in a 0.01 M phosphate buffer saline (PBS), pH 7.2	5 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	4 min
Samples	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	10 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	4 min
Enzyme conjugated	HRP-conjugated (dilution of 1/1000) 2 μL min^{-1}	5 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	4 min
Substrate	2 μL 1 × 10^{-3} M H_2O_2 and 1 × 10^{-3} M 4-TBC (phosphate-citrate buffer, pH 5, 0.01 M)	1 min
Signal analysis	LC-4C amperometric detector, −0.10 V	1 min

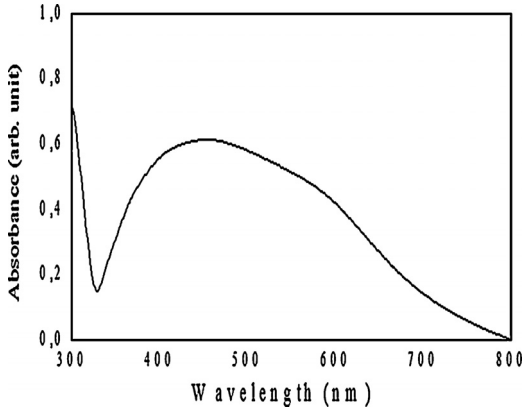


Fig. 2. UV–visible spectra of synthesized AgNPs-Cts.

and then washed with PBS, pH 7.2. The purpose of this treatment is to have immobilized capture 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 M PBS (pH 7.2) at 4 °C. Table 1 summarizes the complete analytical procedure required for the EpCAM determination.

3. Results and discussion

3.1. Characterization of synthesized AgNPs-Cts

AgNPs-Cts obtained by a chemical reduction method have been characterized by UV–visible spectroscopy, SEM, EDS and XRD. Surface plasmon resonance (SPR) band at 400–432 nm in the UV–visible spectra indicates the formation of AgNPs-Cts [32]. Fig. 2 shows the characteristic SPR band at 420 nm indicating the formation of AgNPs-Cts [30].

Fig. 3 shows the typical scanning electron micrograph image of the AgNPs-Cts, from which it can be seen that spherical particles were formed with a size of the nanoparticle less than 30 ± 5 nm [33].

The elemental composition was disclosed by EDS analysis in which strong signals of Ag were observed at 3 keV, while signals from C, O and N were also recorded confirming the presence of

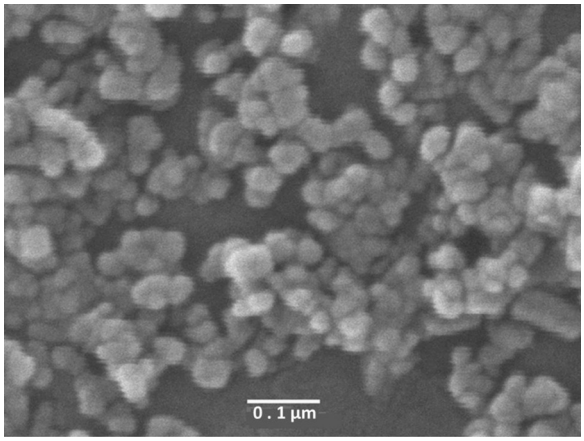


Fig. 3. SEM image of AgNPs-Cts. This image confirmed the formation of spherical AgNPs-Cts with a size <30 ±5 nm.

AgNPs-Cts (Fig. 4) [31]. Peaks of C, O and N reflecting the presence of elements constituting chitosan [33].

The structures and compositions of the AgNPs-Cts were characterized by XRD. X ray diffraction pattern was recorded for AgNPs-Cts in the range $2\theta = 10–90^\circ$ using Cukα (λ = 1.54056 Å) radiation (Fig. 5). By the observation of X-ray pattern and calculations it is clear that AgNPs-Cts are having face-centered cubic structure and the peaks corresponding to (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2) [34,35]. 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 [32,33]. The average size of the crystalline structure of the AgNPs-Cts was calculated according to the Scherrer equation ($t = Kx\lambda/Bx\cos\theta$) and the obtained value was approximately 30 nm. No characteristic peaks of any impurities were detected, suggesting that high-quality AgNPs-Cts were synthesized [34,35].

3.2. Optimization of experimental variables.

Several studies of experimental variables that affect the performance of microfluidic immunosensor for EpCAM determination in peripheral blood samples were done. For this purpose an EpCAM control of 500 pg mL^{-1} 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. 6, flow rates from 1 to 2.5 μL min^{-1} had little effect over immune response and over signals obtained, whereas when the flow rate exceeded 3 μL min^{-1} the signal was dramatically reduced. Therefore, a flow rate of 2 μL min^{-1} was used for injections of 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. 7 shows the obtained signals for 125, 500 and 2000 pg mL^{-1} 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

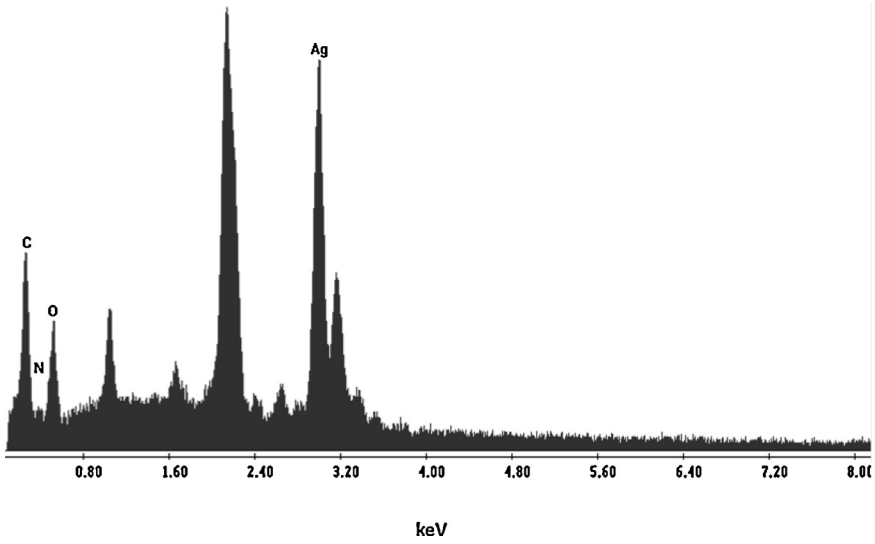


Fig. 4. EDS spectra for AgNPs-Cts. Peak of Ag were observed at 3 keV, while signals from C, O and N were also recorded confirming the presence of AgNPs-Cts. Peaks of C, O and N reflecting the presence of elements constituting chitosan.

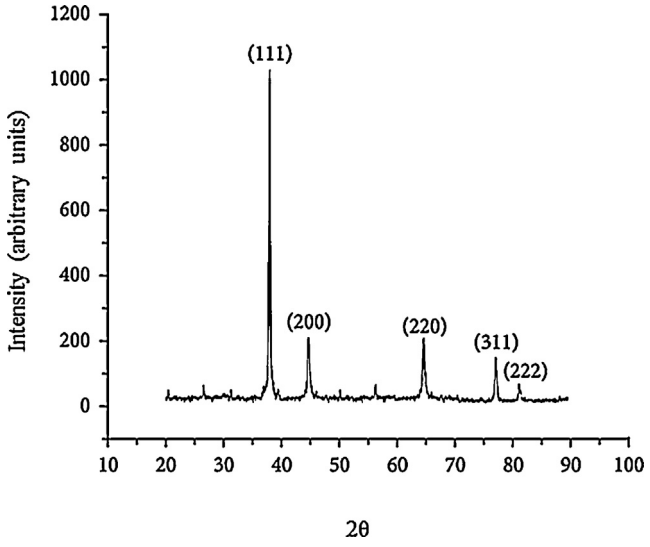


Fig. 5. X-ray diffraction (XRD) pattern for AgNPs-Cts. No characteristic peaks of any impurities were detected, suggesting that high-quality AgNPs-Cts were synthesized.

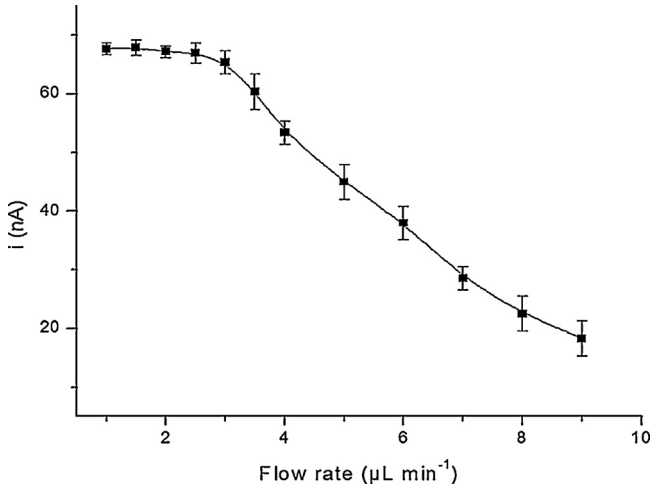


Fig. 6. Study of flow rate effect using an EpCAM control of 500 pg mL^{-1}; 0.01 M phosphate–citrate buffer, pH 5, containing 1×10^{-3} M H_2O_2 and 1×10^{-3} M 4-TBC were injected into the carrier stream at different flow rates, and the enzymatic product was oxidized on the electrode surface at −0.10 V.

microfluidic sensor. 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 antibody was 10 μg mL^{-1} (Supplementary material).

Finally, the rate of enzymatic response 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. S1, Supplementary material).

3.3. Quantitative determination of EpCAM biomarker in the microfluidic immunosensor

The determination of EpCAM biomarker was performed in 15 human peripheral blood samples with the proposed microfluidic immunosensor and under the optimized conditions. The linear regression equation was $i\ (nA) = 10.09 + 0.11 \times C_{EpCAM}$ ($p = 0.05$), with the linear regression coefficient $r = 0.998$. The coefficient of variation (CV) for the determination of 2000 pg mL^{-1} EpCAM biomarker was 3.81% (five replicates). An ELISA procedure was also carried out plotting absorbance changes against the corresponding EpCAM concentrations 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^{-1} EpCAM biomarker was 6.58% (five replicates). The detection limit (LOD) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For microfluidic immunosensor and commercial ELISA, the LODs were 2.7 pg mL^{-1} and 13.9 pg mL^{-1} 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 31.2, 250 and 2000 pg mL^{-1}. The within-assay precision was tested with five measurements in the same run for each control. These series of analyses were repeated for three consecutive days to estimate between-assay precision. The results obtained are summarized in Table 2. The microfluidic immunosensor showed good precision;

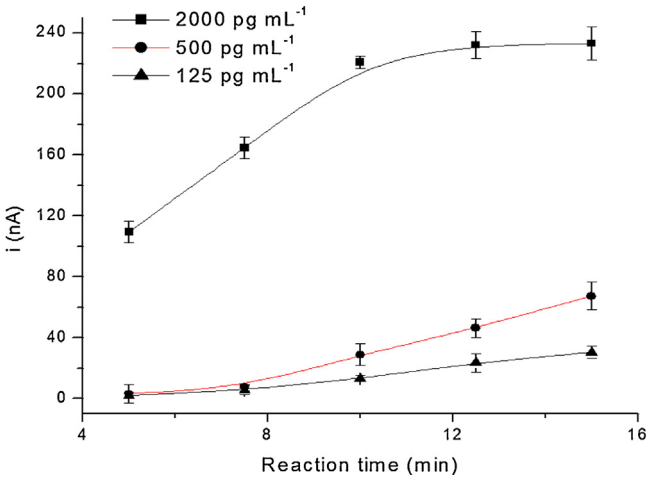


Fig. 7. Study of reaction time effect using EpCAM controls of 125, 500, and 2000 pg mL^{-1}; 0.01 M phosphate–citrate buffer, pH 5, containing 1×10^{-3} M H_2O_2 and 1×10^{-3} M 4-TBC were injected into the carrier stream at different flow rates, and the enzymatic product was measured on the electrode surface at −0.10 V.

Table 2
Within-assay precision (five measurements in the same run for each control sample) and between-assay precision (five measurements for each control sample, repeated for three consecutive days).

[a]Control sample	Within-assay		Between-assay	
	Mean	CV %	Mean	CV %
31.2	31.12	2.81	31.82	4.88
250	250.10	3.98	250.41	5.60
2000	1999.25	3.81	1999.84	6.37

[a] pg mL^{-1} EpCAM.

the CV within-assay values were below 3.98% and the between assay values below 6.37%.

The accuracy was tested with a dilution test which was performed with a EpCAM control of 2000 pg mL^{-1} which was serially diluted in 0.01 M PBS, pH 7.2. The linear regression equation was $i(nA) = 3.01 + 236.90 \times C_{EpCAM}$, with the linear regression coefficient $r = 0.997$ (Fig. S2, Supplementary material).

Moreover, the proposed method was compared with a commercial ELISA procedure for the quantification of EpCAM in peripheral blood samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Fig. 8). For this, 5 high level and 10 low level EpCAM biological samples

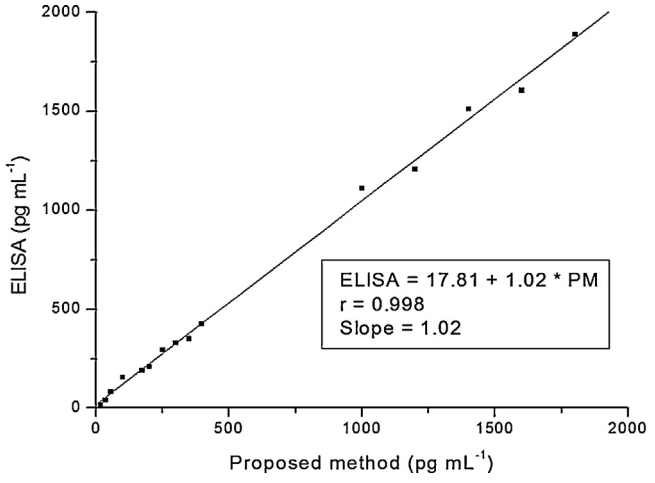


Fig. 8. Correlation between proposed method and commercial ELISA.

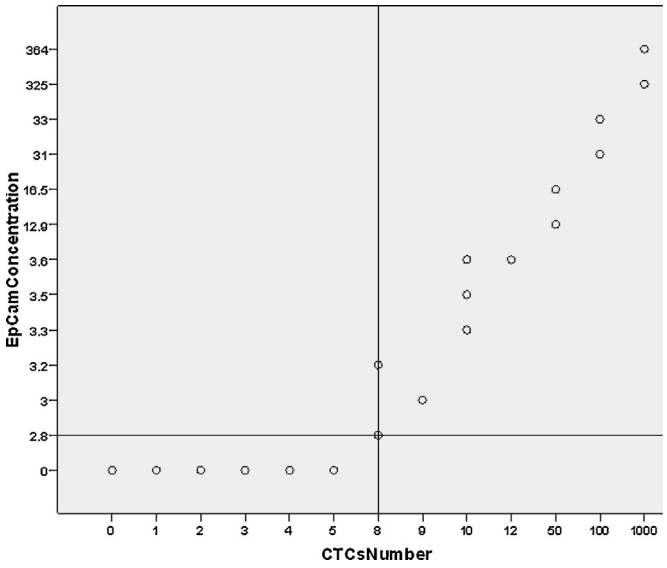


Fig. 9. Correlation between EpCAM concentration determined from proposed method and number of CTCs immunemagnetically determined. The samples contained 10 mL of blood from healthy voluntaries ($n = 20$), spikes ($n = 10$) and patients ($n = 15$).

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 breast cancer were analyzed ($n = 45$) with the aim of correlate number of CCTs with EpCAM concentration. These samples were previously confirmed using the commercial inmunomagnetic CTCs detection Kit (Miltenyi Biotec), which is currently used in the CTCs field research. The EpCAM levels were later determined by our proposed quantitative method, which revealed high correlation between number of CTCs and EpCAM concentration in all of them ($p < 0.001$) (Fig. 9, Table 3).

The elevated sensitivity of our microfluidic immunosensor permit determine the EpCAM protein levels in a little number of CTCs (≥3CTCs) thus being able to correlate the number of CTCs patient with the EpCAM concentration in the lisate of the leucocitrarie fraction. Recent studies show than serum free EpCAM not have clinically significant, for other hand it is known that CTCs determination have great clinic value [30,31]. For this reason a method with high sensitivity, to analyze white cell fraction, generate a concentration of EpCAM with the same clinical significance the determination of CTCs. Moreover, the detection limit of our proposed method is 8 CTCs. The experts in the field consider that in the clinical practice 5 or more CTCs in 7.5 mL of blood have prognostic value [36]. For this reason, we suggest that the blood volume extracted to this determination should to be 12 mL. This way the limit is 8 CTCs in 12 mL, the same as 5 CTCs in 7.5 mL.

On the other hand, chitosan, a natural polymer, has been reported as a polymer based protective agent to stabilize the metal nanoparticles [37,38]. Because of the biocompatibility, biodegradability, nontoxicity and adsorption properties of chitosan [37,38], it was used as a stabilizing agent to synthesize AgNPs. These chitosan protected nanoparticles can be easily integrated into systems relevant for biomedical and biosensor applications.

Furthermore, our proposed method is based on the microfluidic technology which allows to obtain portable and automated microdevices. The extremely small sample volume required by our

Table 3
Correlates the CTCs number and EpCAM concentration. The quantification is possible from 8 CTCs in 10 mL of blood. *P* value was obtained by Chi square test.

[a]EpCAM concentration	CTCs number													Total	P
	0	1	2	3	4	5	8	9	10	12	50	100	1000	0	
Undetectable	26	1	1	1	1	2	0	0	0	0	0	0	0	32	
2.7–6.2	0	0	0	0	0	0	2	1	3	1	0	0	0	7	<0.001
17–20.5	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
20.6–24.1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
38.6–42.1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	
42.2–45.8	0	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	0	2	2	
Total	26	1	1	1	1	2	2	1	3	1	2	2	2	45	

[a] $pg\,mL^{-1}$.

microfluidic immunosensor, the accuracy and the appropriate LOD value achieved compared with commercial ELISA, represent relevant parameters. In addition, our system uses AgNPs-Cts platform for immobilizing biomolecules which provides specificity to the device.

Finally, our device has the potential to answer the growing needs for analytical tools that meet same requirements such as low cost, sensitivity and short analysis time.

4. Conclusions

In this work, we have designed a microfluidic immunosensor with AgNPs-Cts for the 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. Also, we could correlate with our microfluidic immunosensor the number of CCTs with EpCAM concentration, which is very important to the clinical diagnosis. Moreover, the proposed method presents the advantages of low detection limit, speed and simplicity. Besides, chitosan used here is a natural polymer and acts as a very good stabilizing agent; thus, this approach of formation of AgNPs-Ct is proved to be an excellent 'green approach' for the synthesis of metal nanoparticles. These synthesized AgNPs-Cts have their own importance due to their vast area of applications making it to be considered as a candidate for the biomoleculas immobilization process, allowing its incorporation by several strategies in the microfluidic immunosensor production. In conclusion, our proposed method could be well suited for biomedical sensing and clinical applications for diagnosis and prognosis of epithelial origin tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2015.06.066

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