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Abstract

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

Keywords: Nanostructured platform; microfluidic immunosensor; cancer diagnostics; EpCAM biomarker; epithelial cancer; circulating tumour cells.

1. Introduction

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

Besides, immunosensors use diverse signal transduction pathways to recognize an antibody/antigen binding event. Most applications use a label to increase the sensitivity of detection. Current labels include metals, redox labels, optical labels and enzymes [17, 18]. Fluorescence is one of the most sensitive detection methods, and is widely used for immunosensor design [19, 20]. Several different setups can be coupled to fluorescence method, such as a microscope focused on the microchannel and connected to a charge coupled device camera or a photomultiplier tube. Some parts of the fluorescence setup, such as the excitation source and detection device, can be miniaturized [21, 22].

Fluorescence detection has advantages like: high detection sensitivity (such as single molecule detection); fast response times; localized fluorescence signal; multiplexed assays using multicolor dyes; and the straight forward labeling process, which provides appropriate functional groups [23-25]. Laser-induced fluorescence (LIF) applied for analyte detection is one of the most sensitive detection techniques, which is capable of reaching concentration detection below 10^{-13} mol L^{-1} and a mass detection of less than 10 molecules. Radiation from a laser source can be focused, making it a useful tool for detection in very small volumes [24, 25]. This property makes LIF detection a method of choice for detecting analytes on microfluidic devices, where the characteristic length scales are of the order of micrometers or even smaller [26-28].

A relevant area for the development of immunosensors is in cancer diagnosis through of specific tumor biomarkers determination. These biomarkers are one kind of biochemical substances produced by human tumor tissues, which can reflect the existence and growth of tumors in human body [29, 30]. Cancer biomarkers has critical clinical significance in early screening of tumors, assistantly determining the disease phase, selecting the accurate therapy and observing the curative effect. Epithelial cancer biomarker (EpCAM) is a cell surface 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 [31, 32]. Besides, circulating tumor cells (CTCs) are cells that have shed into the vasculature from a primary tumor and circulate in the bloodstream. CTCs thus constitute seeds for subsequent growth of disseminated tumor mass (metastasis) in vital distant organs, triggering a mechanism that is responsible for the vast majority of cancer-related deaths [29, 30]. Therefore, the detection of CTCs may have important prognostic and therapeutic implications [29, 30]. For these reasons, the development of sensitive

analytical methods for EpCAM determination would be transformative in the diagnosis and treatment of epithelial origin cancers.

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

2. Experimental

2.1. Materials and reagents

The following materials and chemicals were used as supplied. Soda-lime glass wafers (26 × 76 × 1 mm) were purchased from Glass Technical (São Paulo, SP, Brazil). Sylgard 184 and AZ4330 photoresist (PR) as well 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). Polyvinyl alcohol (PVA 88 % hydrolyzed, Mw= 88,000), zinc nitrate tetrahydrate purum p.a. (crystallized, 99 % KT), hydrofluoric acid (HF), 3-aminopropyl triethoxysilane (3-APTES), and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The commercial ELISA kit (enzyme immunoassay) for the quantitative determination of EpCAM biomarker was purchased from Uscn Life Science Inc. (USA), and it was used according to the manufacturer's instructions. Mouse monoclonal antibody to EpCAM (1 mg mL^{-1}) and HRP-conjugated anti-EpCAM-antibody (1 mg mL^{-1}) were purchased from Abcam® (USA). Commercial inmunomagnetic CTCs detection kit was purchased from Miltenyi biotec (Germany). All buffer solutions were prepared with Milli-Q water.

2.2. Instrumentation

The optical system was constructed using the proposed procedure by Seiler et al. (1993) [33] according to the following modification. A 561 nm single-frequency DPSS laser (Cobolt Jive, 561 nm, USA) operated at 25 mW served as the fluorescence excitation source. It was focused on the detection channel at 45° to the surface using a lens with a focal distance of 30 cm. The relative fluorescence signal of ADHP was measured using excitation at 561 nm and emission at 585 nm.

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

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

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

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

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

The synthesized ZnONPs-PVA were characterized by UV-visible spectroscopy (UV-visible spectrophotometer model UV-1650 PC – Shimadzu, USA), 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, USA) and X-ray diffraction (XRD) using a Rigaku D-MAX IIIC diffractometer with copper radiation (kα=0.154178 nm) and a nickel filter (Rigaku, Texas, USA).

2.3. Preparation of nanostructured platform: ZnONPs-PVA

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

2.4. Microfluidic device fabrication

The construction of microfluidic immunosensor was carried out according to the procedure proposed by Segato et al. (2010) with the following modifications [34]. The microfluidic device design consisted of a T-type format with a central channel (CC) (60 mm length; 100 μm diameter) and accessory channels (15 mm length; 70 μm diameter). The main body of the microfluidic sensor was made of glass. Firstly the device layout was drawn using CorelDraw software version 11.0 (Corel Corporation) and printed on a high-resolution transparency film in a local graphic service, which was used as a mask in the photolithographic step. The printed mask was placed on top of a glass wafer previously coated with a 5 μm layer of AZ4330 (PR). The substrate was exposed to UV radiation for 30 s and revealed in AZ 400 K developer solution for 2 min. Glass channels were obtained employing an etching solution consisted of 20% HF for 4 min under continuous stirring. The etching rate was 8±1 μm min^{-1}. Following the etching step, substrates were rinsed with deionized water and the photoresist layer was removed with acetone. To access the microfluidic network, holes were drilled on glass-etched channels with a Dremel tool (MultiPro 395JU model, USA) using 1-mm diamond drill bits. To achieve the final microfluidic immunosensor format, another glass plate was spin-coated with a thin poly(dimethylsiloxane) (PDMS) layer at 3000 rpm during 10 s. PDMS was prepared by a 10:1 mixture of Sylgard 184 elastomer and a curing agent. The thickness of this layer was 50 μm. Before sealing, PDMS layer was cured at 100°C for 5 min in a hot plate. Glass channels and PDMS-coated glass substrate were placed in an oxygen plasma cleaner (Plasma Technology PLAB SE80 plasma cleaner) and oxidized for 1 min. The two pieces were brought into contact immediately after the plasma treatment, obtaining a strong irreversible sealing. The final device format was achieved in less than 30 min. The bonding

resistance of the 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^{-1}.

2.5. Surface modification of microfluidic immunosensor

The CC of glass microfluidic device was exposed to a cleaning protocol, in which the solutions were pumped at flow rate of 2 μL 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 [11]. After that, glutaraldehyde solution (0.21 M) in 0.1 M sodium phosphate buffer (PBS, 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 ZnONPs-PVA was performed at 25°C for 12 h according to the similar procedure proposed by Yu et al. (2007) [35].

Later, CC was washed with 0.1 M PBS (pH 7.2) at 25°C for 1 h. Once ZnONPs-PVA were covalently attached to CC, anti-EpCAM antibodies (10 μg mL^{-1} 0.01 M PBS,

pH 7.2) were immobilized on their surface through the use of glutaraldehyde solution (0.21 M) in 0.1 M PBS (pH 8) (Figure 1) [35]. In this case this cross-linker allowed the binding of the antibodies amino groups with those residual amino moieties present on the surface of ZnONPs-PVA. Finally, the CC was rinsed with 0.1 M PBS (pH 7.2) to remove the unbound anti-EpCAM 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.

Figure 1

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 HT29 cells. 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, Germany) 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 separation 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 voluntaries 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 $^{CK\ (+)}$ [36, 37] was compared with EpCAM concentration obtained through of proposed microfluidic immunosensor. Any discrepancies between cell counting were resolved by discussion between two independent reviewers.

2.10. Biological sample preparation for microfluidic immunosensor

10 mL of peripheral blood were mixed with 5 mL of 0.01 M PBS (pH 7.2), this mixture was deposited on 10 mL of histopaque 1119 solution (Sigma-Aldrich, St. Louis, MO, USA) 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 plastic tube,

then a wash with 0.01 M 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 2ºC 14000 g during 10 min and the supernatant was removed for use.

2.11. EpCAM LIF determination

The EpCAM determination process included several steps in which reactive and washing solutions were pumped at a flow rate of 2 µL min^{-1}. After each reagent solution injection, the CC of the device was exposed to a washing procedure with 0.01 M PBS (pH 7.2) for 5 min in order to remove the reagent excess.

As a first step, a blocking treatment was performed through injecting 1% of bovine serum albumin (BSA) in 0.01 M PBS (pH 7.2) for 5 min in order to avoid unspecific bindings. Once the device was blocked, the biological sample was injected into the PBS carrier stream for 10 min. The anti-EpCAM antibody bounded on the ZnONPs-PVA in the CC wall reacted with EpCAM present in the biological sample. Bound EpCAM was quantified using HRP-conjugated anti-EpCAM-antibody (dilution of 1/1000 in 0.01 M PBS, pH 7.2) injected for 5 min.

For the relative fluorescence measurement, the substrate solution was prepared by dissolving 0.01 M ADHP stock solution in dimethyl sulfoxide (DMSO) and stored at -20°C. The ADHP solution previously obtained and the H_2O_2 solution were diluted to 0.001 M with 0.1 M phosphate–citrate buffer (pH 5) before being used. The substrate solution was injected into the carrier stream for 1 min, and the enzymatic product was measured by LIF. The HRP in the presence of H_2O_2 catalyzed the oxidation of

nonfluorescent ADHP to highly fluorescent resorufin, which was measured using excitation at 561 nm and emission at 585 nm (Figure 1).

After each sample measurement, the device was exposed to a flow of desorption buffer (0.1 M glycine–HCl, pH 2) at a flow rate of 2 μL min^{-1} for 5 min and then washed with PBS (pH 7.2). With this treatment, bound immune-complexes were desorbed, allowing us to start with the next determination. The storage of the device was done in 0.01 M PBS (pH 7.2) at 4°C. The proposed microfluidic immunosensor could be used with no significant loss of sensitivity for 15 days, whereas its useful lifetime was 1 month with a sensitivity decrease of 10%. The storage of the device was done in 0.01 M PBS (pH 7.2) at 4°C. Table S1 (Supplementary Material) summarizes the complete analytical procedure required for the EpCAM determination.

Table S1

3. Results and discussion

3.1. Characterization of synthesized ZnONPs-PVA

ZnONPs-PVA obtained by a wet chemical 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. The absorption spectrum of ZnONPs-PVA is shown in Figure 2 (a). It exhibits a strong absorption band at about 355 nm [38]. An excitonic absorption peak is found at about 258 nm due to the ZnONPs-PVA which lie much below the band gap wavelength of 358 nm (Eg = 3.46 eV). It is also evident that significant sharp absorption of ZnO indicates the monodispersed nature of the nanoparticle distribution [38]. Figure 2 (b) represents the SEM picture of ZnONPs-PVA. This figure substantiates the approximate spherical shape to the nanoparticles, and also can be seen that the size of the nanoparticle is less than 20±5 nm

[38]. The elemental composition was disclosed by EDS analysis in which strong signals of Zn were observed at 1 keV, while signals from C and O were also recorded confirming the presence of ZnONPs-PVA (Figure 2 (c)) [38]. Peaks of C and O reflecting the presence of elements constituting polyvinyl alcohol [39]. The TEM image of synthesized ZnONPs-PVA is also shown in Figure 2 (d). The size of particles ranged from 15 to 25 nm and they showed a relatively spherical geometry [39]. Figure 2 (e) represents the XRD pattern of ZnONPs-PVA. A definite line broadening of the XRD peaks indicates that the prepared material consist of particles in nanoscale range. From this XRD patterns analysis, we determined peak intensity, position and width, full-width at half-maximum (FWHM) data. The diffraction peaks located at 31.84°, 34.52°, 36.33°, 47.63°, 56.71° and 62.96° have been keenly indexed as hexagonal wurtzite phase of ZnO with lattice constants $a = b =$ 0.324 nm and $c = 0.521$ nm [39], and further it also confirms the synthesized ZnONPs-PVA were free of impurities as it does not contain any characteristics XRD peaks other than ZnO peaks. The synthesized ZnONPs-PVA diameter was calculated using Debye-Scherrer formula [39], $d = 0.89\ \lambda/\beta \cos\theta$, where 0.89 is Scherrer's constant, λ is the wavelength of X-rays, θ is the Bragg diffraction angle, and β is the full width at half-maximum (FWHM) of the diffraction peak corresponding to plane 101. The average particle size of the sample was found to be 20 nm which is derived from the FWHM of more intense peak corresponding to 101 plane located at 36.33° using Scherrer's formula. These results are in good accordance with the results obtained from the work of Ahangar et al. (2014) [39].

Figure 2

3.2. Optimization of experimental variables

Several studies of experimental variables that affect the performance of microfluidic immunosensor for EpCAM determination in biological 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 relative fluorescence obtained during the immune reaction. As shown in the picture (Figure S1, Supplementary Material), 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 samples, reagents and washing buffer.

Figure S1

Regarding incubation time, the minimum time required for EpCAM binding is also a critical assay factor, especially when the use of a minimum total analysis time is desired. Figure S2 (Supplementary Material) shows the measured fluorescence for 125, 500 and 2000 pg mL^{-1} EpCAM control concentrations. The fluorescence intensity increased when the EpCAM concentration grew. As expected, the intensity of the fluorescence increased with the reaction time. The intensity of the fluorescence, however, did not increase considerably until 10 min had passed, which was likely due to saturation of the specific antibody sites in the ZnONPs-PVA–3-APTES-modified glass microfluidic device. Therefore, the optimal reaction time was 10 min.

Figure S2

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. For study of optimum concentration of anti-EpCAM antibody to be employed in the immobilization procedure,

different concentrations of anti-EpCAM antibody (1-20 µg mL^{-1}) were immobilized on ZnONPs-PVA. Later, HRP (10 µg mL^{-1}) was adsorbed at unoccupied sites. Finally, the enzymatic activity was studied for the addition of the substrate solution and the enzymatic product was measured by LIF using excitation wavelength 561 nm and emission wavelength 585 nm. The optimum value of anti-EpCAM antibody was 10 µg mL^{-1}.

Finally, the rate of enzymatic response under flow conditions was performed in the pH range of 4-7 and showed a maximum value of activity at pH 5 in phosphate-citrate buffer (Figure S3, Supplementary Material).

Figure S3

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 curves of the microfluidic immunosensor and commercial ELISA were constructed using different EpCAM control samples. The linear regression equation was relative fluorescence units (RFU) = 68.23 + 0.86 x C_{EpCAM} (p = 0.05), with the linear regression coefficient r = 0.998. A linear relation was observed between the concentration range 2.5-2000 pg mL^{-1}. The coefficient of variation (CV) for the determination of 2000 pg mL^{-1} EpCAM biomarker was 3.84 % (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 x 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 1.2 pg mL^{-1} and 13.9 pg mL^{-1} respectively. This result shows that LIF 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 1. The microfluidic immunosensor with LIF detection showed good precision; the CV within-assay values were below 3.92 % and the between assay values below 6.50 %.

Table 1

The accuracy of the LIF microfluidic immunosensor 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 RFU = 19.01 + 1787.68 x C_{EpCAM}, with the linear regression coefficient r = 0.998 (Figure S4, Supplementary Material).

Figure S4

Moreover, the proposed method was compared with a commercial ELISA procedure for the quantification of EpCAM in biological samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Figure 3). For this, 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.

Figure 3

Also, in the present work, 20 samples from healthy voluntaries, 10 spike controls and 15 patients with 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 inmunomagnetic 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) (Figure 4, Table 2).

Figure 4

Table 2

The elevated sensitivity of our proposed immunosensor permit determine the EpCAM protein 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 than serum free EpCAM not have clinically significant, for other hand it is known that CTCs determination have great clinic value [36, 37]. 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 5 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 [40]. For this reason, we suggest that the blood volume extracted to this determination should to be 10 mL.

On the other hand, PVA is known to be a good stabilizer of small metal particles [39] and has been frequently used as particle stabilizers in chemical synthesis of metal and metal oxide nanoparticles. Moreover, this degradable polymer, is easily dissolved in water,

and their combination with ZnONPs results in improved electrical, mechanical and optical properties [39].

According to our detailed search, there are not previously reported articles related to EpCAM determination in biological samples using a microfluidic immunosensor with LIF detection. It is important to highlight that our method is the only LIF microfluidic immunosensor for this cancer biomarker reported to date. Ortega et al. (2015) [41] developed a microfluidic device, coupled to electrochemical detection, with silver nanoparticles coated chitosan as biorecognition platform. In addition, the achieved LOD of LIF proposed method (1.2 pg mL^{-1}) was lower than that obtained by the mentioned electrochemical method (2.7 pg mL^{-1}) [41].

4. Conclusions

In this article, we describe a microfluidic immunosensor with ZnONPs-PVA coupled to LIF detection for the quantitative determination of EpCAM as biomarker for epithelial cancer diagnosis. Moreover, our LIF proposed method is based on the microfluidic technology. This modern technology allowed the miniaturization of devices, which results in a substantial reduction of reactive solutions, shorter analysis time, improved portability, and better detection limits. Also, the LIF detection coupled to microfluidic sensors has received considerable attention due to their inherent simplicity, compatibility with microfabrication techniques, high sensitivity, fast detection, and low cost. In addition, our system uses ZnONPs-PVA platform for immobilizing biomolecules which provides specificity to the device due to they provides a large availability of binding sites. Compared with the commercial ELISA analysis (270 min), our microfluidic immunosensor reduces the total assay time, with our sensor it was less than 40 min. Also,

we could correlate with our microfluidic immunosensor the number of CCTs with EpCAM concentration, which is very important to the clinical diagnosis.

In summary, an alternative analytical method for epithelial cancer biomarker EpCAM determination was developed. The microfluidic immunosensor offers a truthful and useful analytical tool to be easily applied in diagnosis and prognosis of epithelial cancer. Finally, our proposed LIF device presents inherent benefits such as miniaturization, integration, sensitivity and short analysis time.

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Figure captions

Figure 1. Schematic representation of the glass microfluidic surface modification and the immunological reaction. Anti-EpCAM antibodies were covalently bounded onto ZnONPs covered by polyvinyl alcohol (ZnONPs-PVA), 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 ZnONPs-PVA-3-APTES-modified glass microfluidic sensor. The bound EpCAM was quantified by HRP-conjugated anti-EpCAM antibody using ADHP as enzymatic mediator. The highly fluorescent resorufin (HP) generated was measured by LIF using excitation at 561 nm and emission at 585 nm.

Figure 2. (a) UV-visible absorption spectrum of synthesized ZnONPs-PVA. (b) SEM image of ZnONPs-PVA. This image confirmed the formation of spherical nanoparticles with a size <20±5 nm. (c) EDS spectra for ZnONPs-PVA. Peak of Zn were observed at 1 keV, while signals from C and O were also recorded confirming the presence of ZnONPs-PVA. Peaks of C and O reflecting the presence of elements constituting polyvinyl alcohol. (d) TEM image of synthesized ZnONPs-PVA. (e) XRD pattern for ZnONPs-PVA. No characteristic peaks of any impurities were detected, suggesting that high-quality ZnONPs-PVA were synthesized.

Figure 3. Correlation between proposed method and commercial ELISA.

Figure 4. 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).

Tables

Table 1. 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	31.16	2.85	31.78	4.84
250	250.12	3.92	250.37	5.63
2000	1999.19	3.84	1999.89	6.50

[a] pg mL^{-1} EpCAM

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	1	2	3	4	5	8	9	10	12	50	100	1000	0	P
Undetectable	22	2	2	1	0	0	0	0	0	0	0	0	0	27	
1.2-2.7	0	0	0	1	2	2	2	0	0	0	0	0	0	7	
2.7-6.2	0	0	0	0	0	0	0	1	3	1	0	0	0	5	<0.001
17-20.5	0	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	0	1	0	1	
35.1-38.5	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	22	2	2	2	2	2	2	1	3	1	2	2	2	45	

[a] pg mL^{-1}

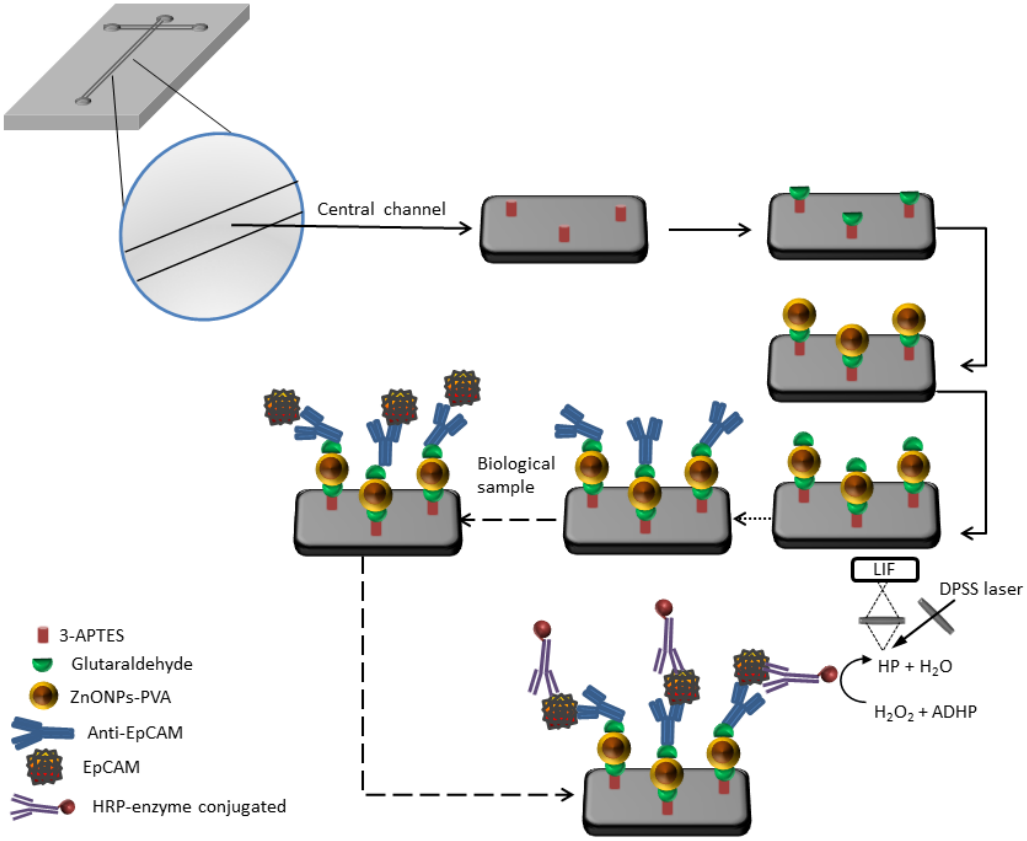


Fig. 1

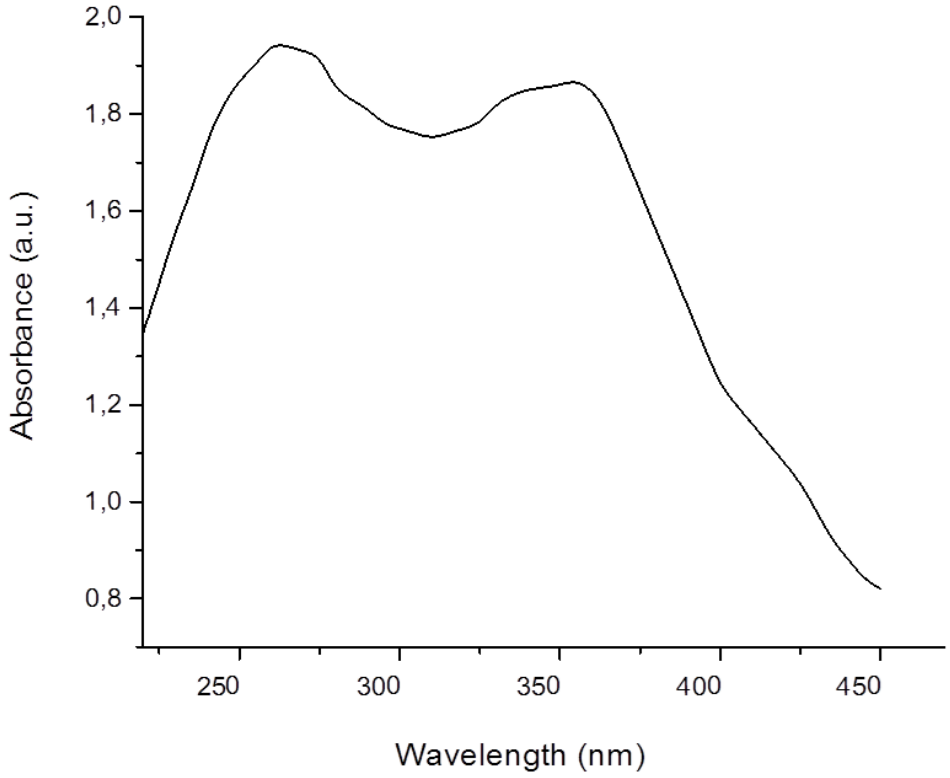
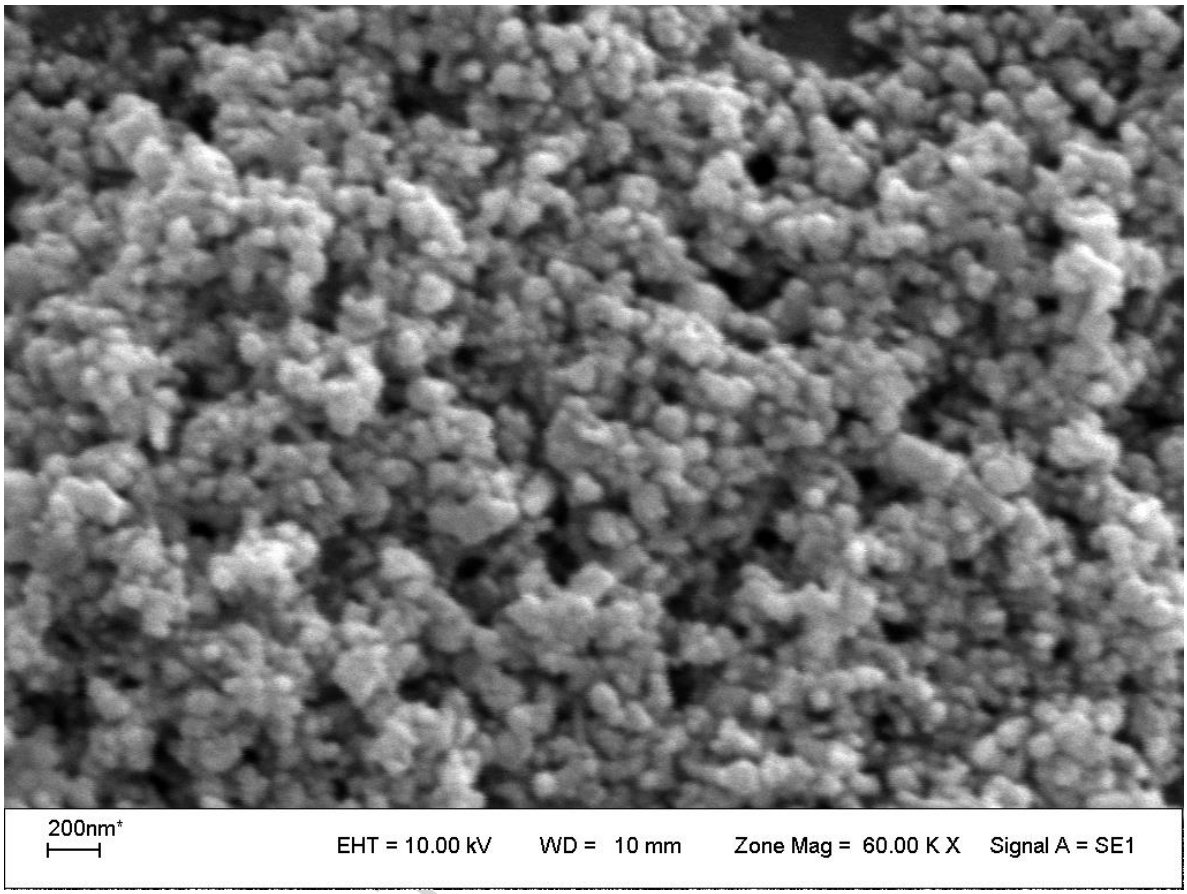


Fig. 2a



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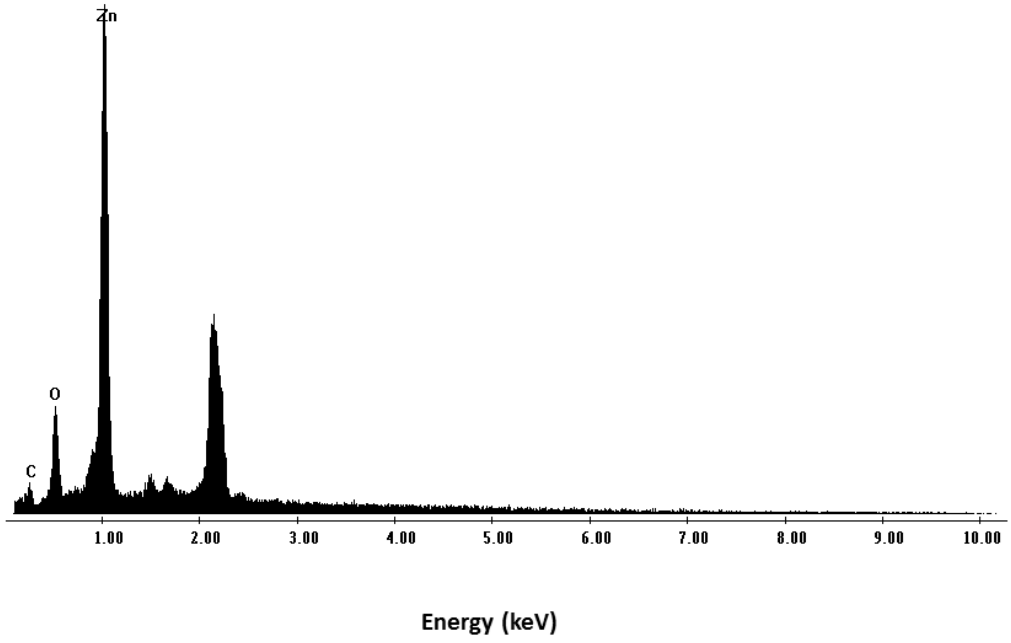

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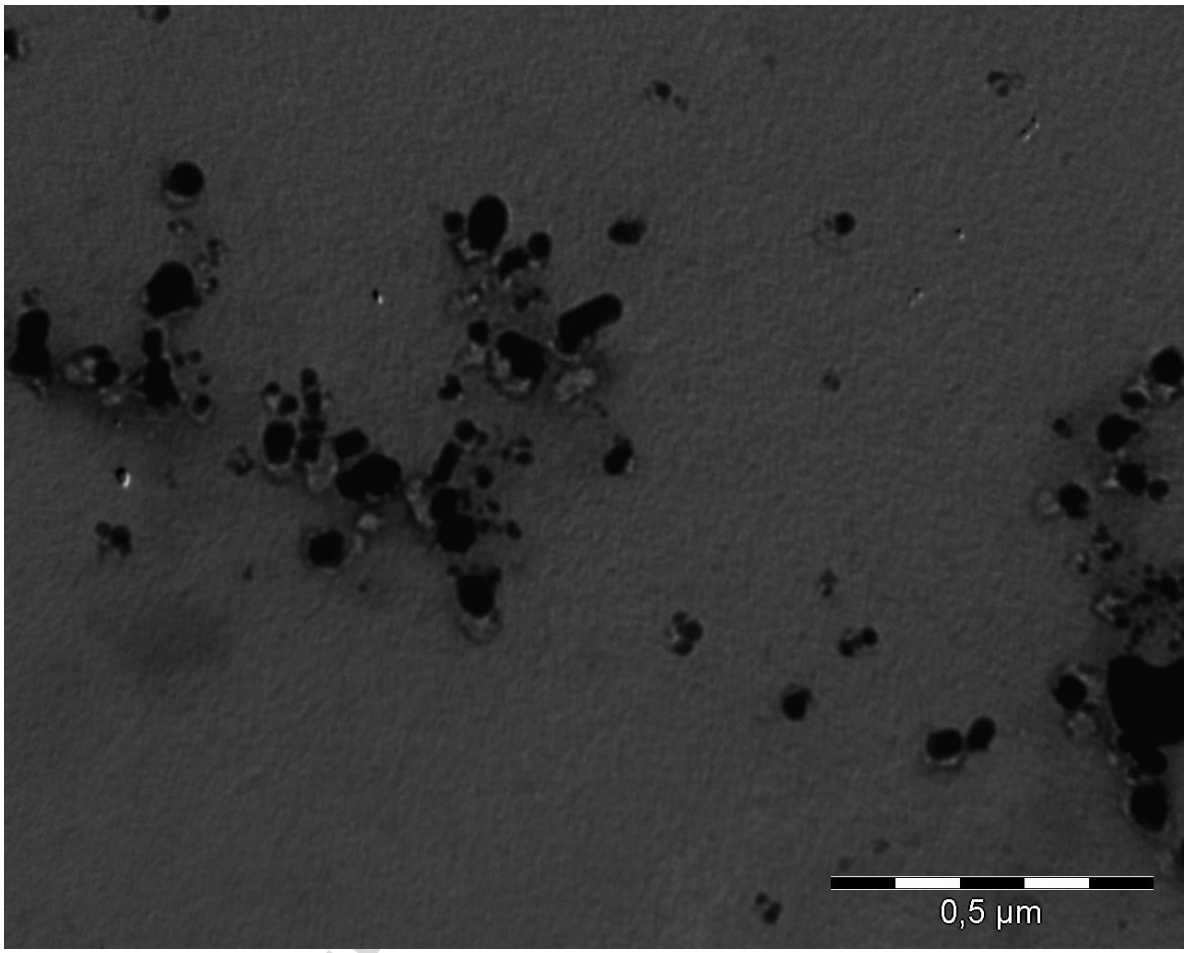
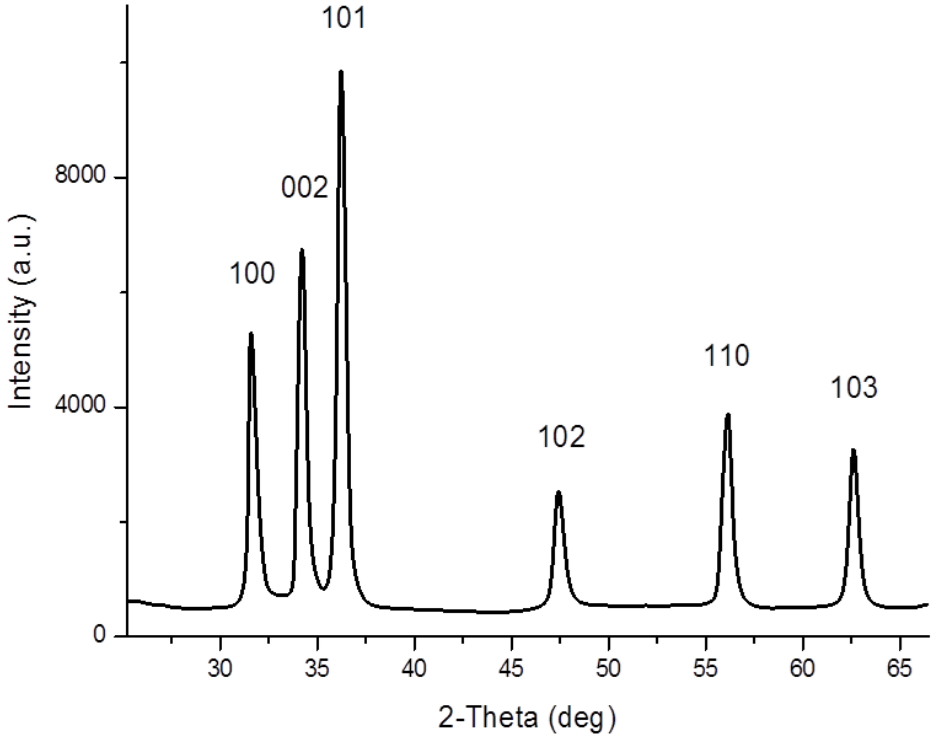


Fig. 2d



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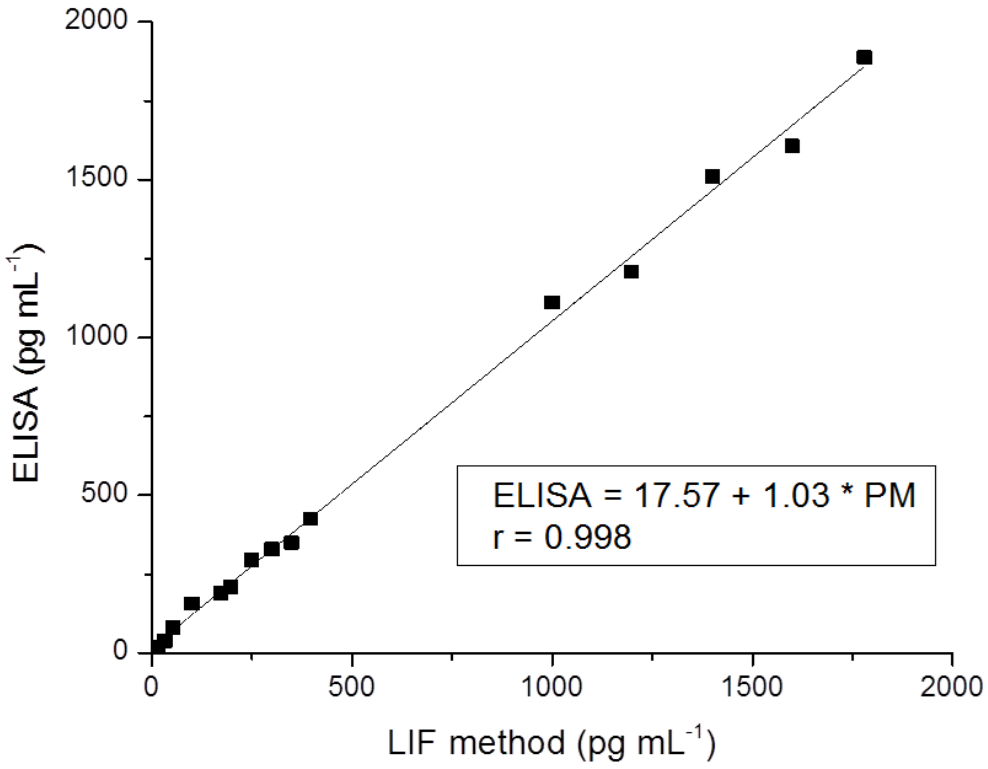

Fig. 3

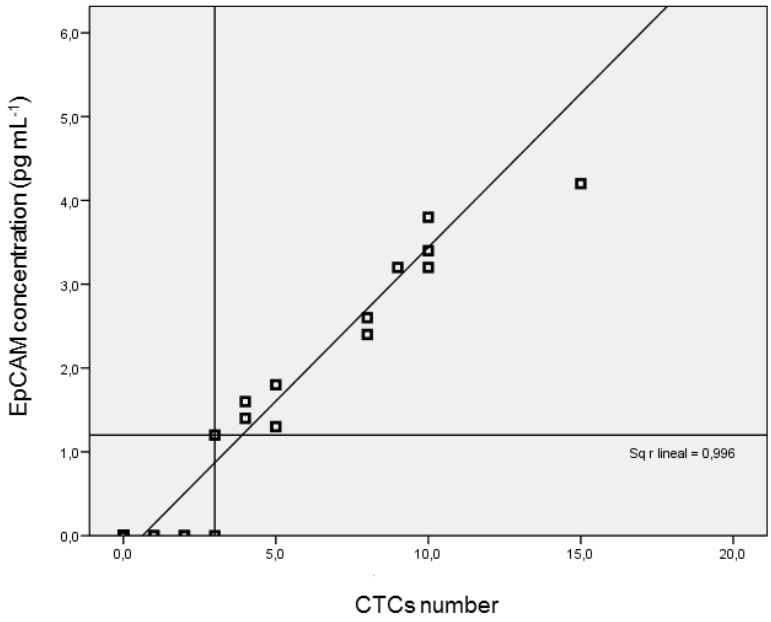

Fig. 4

Highlights

►A microfluidic immunosensor for epithelial cancer biomarker EpCAM determination was developed.

►The ZnONPs-PVA were used as nano-platform of biomolecules immobilization.

►The LIF method offers a truthful and useful analytical tool to be easily applied in diagnosis and prognosis of epithelial cancer.