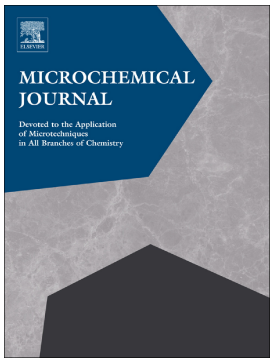

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Development of a nanostructured electrochemical immunosensor applied to the early detection of invasive aspergillosis

Sofía V. Piguillem [1], *Francisco G. Ortega* [2], *Julio Raba* [1], *Germán A. Messina* [1], Martín *A. Fernández-Baldo* [1*]

[1] INQUISAL, Departamento de Química. Universidad Nacional de San Luis, CONICET. Chacabuco 917. D5700BWS. San Luis, Argentina.

[2] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel.

Authors to whom correspondence should be addressed: (e-mail) mbaldo@unsl.edu.ar. (Tel.) +54-266-4425385; (Fax) +54-266-443-0224.

INQUISAL, Departamento de Química. Universidad Nacional de San Luis, CONICET. Chacabuco 917. D5700BWS. San Luis, Argentina.

Abstract

The use of a biological marker as an adjunct for screening for invasive aspergillosis (IA) in high-risk patients is attractive, because it is noninvasive and may detect evidence of IA prior to the appearance of clinical signs and symptoms. Galactomannan (GMN) is a polysaccharide cell-wall component that is released by *Aspergillus* species. In the present work, an electrochemical microfluidic immunosensor for GMN detection was developed. Moreover, copper nanoparticles covered with polyvinylpyrrolidone (CuNPs-PVP) were synthesized, characterized and used in a microfluidic immunosensor based on the use of anti-GMN antibodies as a trapping agent. The concentration of trapped GMN is then electrochemically quantified by HRP-conjugated anti-GMN-antibody. HRP reacted with its enzymatic substrate in a redox process which resulted in the appearance of a current whose magnitude is directly proportional to the concentration of GMN. The immunosensor was validated by analyzing patient samples, and a good correlation with a commercial ELISA was obtained. This microfluidic immunosensor can be applied to the early diagnosis of IA.

Keywords: Galactomannan; invasive aspergillosis; clinical diagnosis; nano-platform; microfluidic immunosensor; electrochemical.

1. Introduction

The concept of aspergillosis involves a wide variety of infections, ranging from the aspergillosis bronchopulmonary allergic to the aspergillosis disseminated. Invasive aspergillosis (IA) is one of these infections that in immunocompetent people can cause allergic reactions, but in people, who have an immunocompromised state of health, the disease is invasive, serious and if it is not treated it can be fatal [1]. In recent decades, there has been a significant increase in the number of patients who have HIV, cancer patients, transplant patients or those who receive immunomodulatory drugs, that is why the rate of people suffering from IA has also increased [2].

IA is caused by inhalation of spores of a type of mold called *Aspergillus fumigatus* [3]. This fungus can often be found in dead leaves, organic fertilizer and decomposing plant matter. Mold spores can be transported on shoes and clothes and can grow on the carpet of homes. Air conditioners are suitable places for mold growth if the filters are not kept clean and the water does not drain as it should. The walls of old houses may be contaminated with their spores [3]. The symptoms of IA can be nonspecific [4], causing cough, fever, hemoptysis, pleuritic pain, etc. Usually present one or more nodules with peribronchial infiltrate or pleural effusion. When this infection spreads to the brain it causes seizures, cerebral infarcts and meningitis [4]. Moreover, diagnosis of IA is challenging, because clinical and radiologic signs are very insensitive or nonspecific [4]. Tissue biopsy as a means of making the diagnosis is invasive and is not always possible, especially among patients with thrombocytopenia [3, 4]. Early diagnosis leading to prompt institution of appropriate therapy may result in improved patient outcomes. IA has many antigenic components, but the most useful is galactomannan (GMN) and its determination for the

early detection of this serious infection is relevant in the clinical diagnosis [4]. GMN is a heteropolysaccharide present in the cell wall of most *Aspergillus* species [4]. In the last years, much attention has been focused on developing a noninvasive test for diagnosing IA, particularly methods to detect GMN [3, 4]. Due to the risk of contamination and colonization of bacteria, especially sputum, detection of GMN in serum is better [4]. Currently, there is a commercial method for the detection of GMN called ELISA Platelia™ *Aspergillus* kit (Bio-Rad, France) which is a "sandwich" immunoenzymatic (EIA) assay [5]. This method that incorporates the β 1-5 galactofuranose-specific EBA_2 monoclonal antibody as both the detector and acceptor for GMN showed a high sensitivity and has been approved by the US Food and Drug Administration for use with serum samples [5]. However, these methods consume a lot of time and need sophisticated instrumentation.

In recent years, electrochemical microfluidic immunosensors have been used in the clinical field, especially for the diagnosis of infectious diseases [6]. These devices are considered to be valuable and promising ones due to their robustness, simplicity, sensitivity, ease of handling, cost-effectiveness, rapid analysis and miniaturization ability [7-10]. Furthermore, when these microfluidic immunosensors are modified with nanoparticles acquire a greater selectivity than those naked sensors and also have the capacity to increase the sensitivity of the method due to the increase of the surface area that said nanoparticles provide [7]. The specific use of some metallic nanoparticles as such as copper nanoparticles (CuNPs) can be very interesting for the modification of the central channel of a microfluidic immunosensor and as platform for biomolecules immobilization (antigens, antibodies, enzymes or DNA) [7-15].

In this work, we report a sensitive electrochemical microfluidic immunosensor based on copper nanoparticles covered with polyvinylpyrrolidone (CuNPs-PVP) as nano-platform for anti-GMN monoclonal antibodies immobilization applied to specifically determination of the GMN present in serum samples. To the best of our knowledge, an electrochemical microfluidic immunosensor based on CuNPs-PVP as nano-platform and specific monoclonal antibodies for GMN quantification has not reported to date.

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). AZ4330 photoresist (PR) and AZ 400 K were obtained from Dow Corning (Midland, MI, USA) and Clariant Corporation (Sommerville, NJ, USA), respectively. Glutaraldehyde (25% aqueous solution) and hydrogen peroxide 30% were purchased from Merck (Darmstadt, Germany). Polyvinylpyrrolidone (PVP k30, Mw= 82,500), ethylenediamminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$), copper sulphate ($CuSO_4$), 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 Platelia™ *Aspergillus* kit (enzyme immunoassay) for the quantitative determination of GMN was purchased from Bio-Rad (France), and it was used according to the manufacturer's instructions. Mouse monoclonal specific antibody to GMN (1 mg mL^{-1}) and HRP-conjugated anti-GMN-antibody (1 mg mL^{-1}) were purchased from Agrisera (Sweden) and Abcam® (USA), respectively. All buffer solutions were prepared with Milli-Q water.

2.2. Apparatus

Amperometric measurements were performed using the BAS LC 4 C (Bioanalytical Systems, West Lafayette, IN, USA). The BAS 100 B electrochemical analyzer Bioanalytical Systems) was used for cyclic voltammetry 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 CuNPs-PVP 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) and transmission electron microscopy (TEM) (Carl Zeiss CEM902, USA). Infrared (FTIR) spectroscopic measurements were obtained in a Spectrum 65 FIIR spectrometer Perkin Elmer, in a region from 4000 to 400 cm^{-1}. Textural characterization was carried out by N_2 adsorption-desorption isotherms at 77 K using a manometric adsorption equipment (ASAP 2000, Micromeritics), where the samples were previously degassed at 250°C for 12 h, up to a residual pressure smaller than 3 Pa.

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. Synthesis of nano-platform: CuNPs-PVP

CuNPs-PVP were synthesized by a chemical reduction method using $NaBH_4$ with some own modifications [16]. $CuSO_4$ (1 mM) and EDTA were dissolved in 25 mL of ultrapure water (MilliQ-Plus®) in which 10 mL of PVP (1 g L^{-1}) was added. Later, $NaBH_4$ (0.1 M) was added to the above solution as reducing agent, and then the solution was heated to 30°C under constant stirring for 30 min. After, the NPs solution was spilled out and filtered. Subsequently, the product was washed 3 times with ethanol and acetone separately and finally was sonicated for 30 min before use. Finally, the CuNPs-PVP were re-suspended in 1 mL of ultrapure water.

2.4. Microfluidic immunosensor fabrication

The construction of microfluidic immunosensor was carried out according to the procedure proposed by Moraes et al. (2012) with own modifications [17]. The microfluidic immunosensor 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 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. 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^{-1}.

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^{-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-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 [18]. After that, glutaraldehyde solution (0.21 M) in 0.1 M 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 CuNPs-PVP (0.5 mg L^{-1}) was performed at 25°C for 12 h according to the similar procedure proposed by Yu et al. (2007) [19].

Later, CC was washed with 0.1 M PBS (pH 7.2) at 25°C for 1 h. Once CuNPs-PVP were covalently attached to CC, anti-GMN monoclonal 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). In this case this cross-linker allowed the binding of the antibodies amino groups with those residual amino moieties present on the surface of CuNPs-PVP (Figure 1). Finally, the CC was rinsed with 0.1 M PBS (pH 7.2) to remove the unbound anti-GMN monoclonal antibodies and stored in the same buffer at 4°C. The overall time required for the immobilization procedure was 16 h. The immobilized monoclonal antibody preparation was stable for at least 1 month.

Figure 1

2.6. Analytical procedure for GMN determination

The electrochemical determination of GMN 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 (0.1M 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 0.01M 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 immunosensor was washed with 0.01 M PBS, pH 7.2 for 4 min. Once the GMN was recognized and captured by anti-GMN monoclonal antibody on CuNPs-PVP, the HRP-conjugated anti-GMN-antibody (dilution of 1/1500 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 x 10^{-3} M H_2O_2 and 1 x 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 (Figure 1).

In order to start with the next determination of GMN, 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 and then washed with PBS, pH 7.2. The purpose of this treatment is to have immobilized capture monoclonal 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 4ºC.

3. Results and discussion

3.1. Characterization of synthesized CuNPs-PVP

CuNPs-PVP obtained by a chemical reduction method have been characterized by UV-visible spectroscopy, SEM, EDS, TEM and FTIR. UV-visible absorption spectroscopy is widely being used technique to examine the optical properties of synthesized nanoparticles. Surface plasmon resonance (SPR) band at 550-650 nm in the UV-visible spectra indicates the formation of CuNPs-PVP [20]. Figure 2 (a) shows the characteristic SPR band at 630 nm indicating the formation of CuNPs-PVA [20]. This band presents a considerable width to speak of a polydispersity but in its characteristic range, these are spherical nanoparticles.

Figure 2 (b) represents the SEM picture of CuNPs-PVP. 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 [12-14, 16, 20, 21]. The elemental composition was disclosed by EDS analysis in which strong signals of Cu were observed at 8 keV, while signals from C and O were also recorded confirming the presence of CuNPs-PVP (Figure 2 (c)) [20, 21]. Peaks of C and O reflecting the presence of elements constituting PVP. TEM image of CuNPs-PVP is showed in Figure 2 (d), 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 10–25 nm. Small nanoparticles can be observed in Figure 2 (d), indicating good stabilization by the PVP. Moreover, the FTIR characterization was done by comparing two spectra, one of the PVP polymer and other of the PVP-stabilized nanoparticles, in order to obtain information of links involved in interactions (Figure 2 (e)). It is observed that the bands decreased in intensity when the nanomaterial is present with the PVP, producing interactions between

both. The carbonyl band in the polymer alone appears at 1645 cm^{-1} while stabilizing the nanoparticles at slight shift occurs at 1643 cm^{-1} [22]. It is presumed that this link is responsible for interacting with the metal nanoparticles [23] leaving the carbon with a positive charge density, which is then stabilized by the free electron pair of the adjacent nitrogen in the ring, and then there is increase said signal of N-C from 1292 cm^{-1} for the PVP alone, to 1340 cm^{-1} when the CuNPs are linked.

Figure 2

3.2. Optimization of experimental variables

Several studies of experimental variables that affect the performance of microfluidic immunosensor for GMN determination in biological samples were done. For this purpose a GMN control of 1.5 ng 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 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

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 GMN in the developed microfluidic immunosensor through electrochemical signal. This first

immunological stage was followed by adding HRP-conjugated anti-GMN-antibody. After washing, 4-TBC was incorporated. This enzymatic substrate suffered an oxidation process whose signal was measured as a function of reaction time. Figure 3 shows the obtained signals for 0.5, 1.5 and 2.5 ng mL^{-1} GMN control concentrations. The signal grew with increasing of the GMN 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 GMN control concentration, which corresponds to the saturation of the antigen–antibody reaction in the microfluidic immunosensor. As a result, the reaction time used in all sample measurements was 10 min.

Figure 3

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^{-1} (Supplementary Material).

Finally, the rate of enzymatic response using a GMN control of 1.5 ng mL^{-1} 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 (Figure S2, Supplementary Material).

Figure S2

3.3. Quantitative determination of GMN in the microfluidic immunosensor

The determination of GMN was performed in 15 serum samples with the proposed method and under the optimized conditions. The calibration curve of the proposed immunosensor and commercial ELISA were constructed using different GMN control samples. The linear regression equation was $i\ (nA) = 3.76 + 92.08 \times C_{GMN}$, with the linear

regression coefficient r = 0.998. A linear relation was observed between the concentration range 0-2.5 ng mL^{-1}. The coefficient of variation (CV) for the determination of 2.5 ng mL^{-1} GMN was 3.76 % (five replicates). An ELISA procedure was also carried out plotting absorbance changes against the corresponding GMN concentration then, a calibration curve was constructed. The linear regression equation was A = 0.03 + 0.78 x C_{GMN}, with the linear regression coefficient r = 0.996, and the CV for the determination of 2.5 ng mL^{-1} GMN was 6.54 % (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 0.23 ng mL^{-1} and 1 ng 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 GMN controls of 0.5, 1.5 and 2.5 ng 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 showed good precision; the CV within-assay values were below 3.95 % and the between assay values below 6.33 %.

Table 1

The accuracy was tested with a dilution test which was performed with a GMN control of 1.5 ng mL^{-1} which was serially diluted in 0.01 M PBS, pH 7.2. The linear regression equation was i (nA) = 0.01 + 144.72 x C_{GMN}, with the linear regression coefficient r = 0.998 (Figure 4).

Figure 4

Moreover, the proposed method was compared with a commercial ELISA procedure for the quantification of GMN in serum samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Figure 5). For this comparison, 5 high level and 10 low level GMN serum 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 GMN in all of them. The low levels GMN serum samples and blanks were also confirmed by our proposed microfluidic immunosensor. The results obtained in Figure 5 correspond only to a modified CC of the proposed method.

Figure 5

Microfluidic immunosensors are widely studied because of their relevant advantages. Some advantages of these methods include: compactness, low sample, low cost production, better general control of the process, in real time analysis and a quick response [24, 25]. These characteristics open the possibility of performing in situ, real-time measurements. Also, microfluidic immunosensors operate in such a manner that sample pre-treatment as well as chemical assay can be performed therein [26, 27]. Their ergonomic and user-friendly design allows being easily adapted to perform the desired analysis just by simply modifying the surface of the channels with nanomaterials as platform for the biomolecules immobilization [28]. Metal nanoparticles as such as CuNPs are beneficial for the development of biosensing techniques due to high surface areas, strong stability, limited aggregation, and high electrocatalytic activity [29]. Moreover, the electrochemical deposition of metal NPs into the microchannel can accurately control the position of nanostructures, maximize the stability of the electrode, and ensure that miniaturized

electrochemical sensors with NPs are more specific and highly sensitive for clinical diagnostics [30].

Furthermore, PVP is known to be a good stabilizer of small metal particles [30-33] and has been frequently used as particle stabilizers in chemical synthesis of different metal nanoparticles. Besides, this polymer combined with CuNPs can be easily integrated into microfluidic devices as nano-platform for biomolecules immobilization.

4. Conclusions

In the present work, we present an electrochemical microfluidic immunosensor based on CuNPs-PVP as nano-platform for immobilization of specific monoclonal antibodies applied to sensitive quantification of GMN in serum samples. The overall assay time employed (34 min) was shorter than the time reported for commercial ELISA test kit frequently used in clinical diagnosis (120 min), with no reduction on the sensibility and selectivity.

Finally, our microfluidic immunosensor with CuNPs-PVP as immobilizing platform of specific monoclonal antibodies for GMN determination can be applied to the early diagnosis of IA.

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Figure captions

Figure 1. Representation of the glass microfluidic surface modification and the immunological reaction. Anti-GMN monoclonal antibodies were covalently bounded onto CuNPs-PVP, which were covalently attached over 3-APTES modified glass microfluidic surface. GMN present in the sample reacted immunologically with anti-GMN monoclonal antibody immobilized on CuNPs-PVP-3-APTES-modified glass microfluidic immunosensor. The bound GMN was quantified by HRP-conjugated anti-GMN antibody using 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 GMN.

Figure 2. Characterization of CuNPs-PVP nano-platform. (a) UV-visible spectra. (b) SEM image. This image confirmed the formation of spherical nanoparticles with a size <20±5 nm. (c) EDS spectra for CuNPs-PVP. Peak of Cu were observed at 8 keV, while signals from C and O were also recorded confirming the presence of CuNPs-PVP. Peaks of C and O reflecting the presence of elements constituting PVP. (d) TEM image. (e) FTIR spectra.

Figure 3. Study of reaction time effect using GMN controls of 0.5, 1.5, and 2.5 ng mL^{-1}; 0.01 M phosphate–citrate buffer, pH 5, containing 1 x 10^{-3} M H_2O_2 and 1 x 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. Each value of current is based on five determinations.

Figure 4. Dilution test results for GMN control of 1.5 ng mL^{-1} in 0.01 M PBS, pH 7.2. Each value of current is based on five determinations.

Figure 5. Correlation between proposed method and commercial ELISA.

Tables

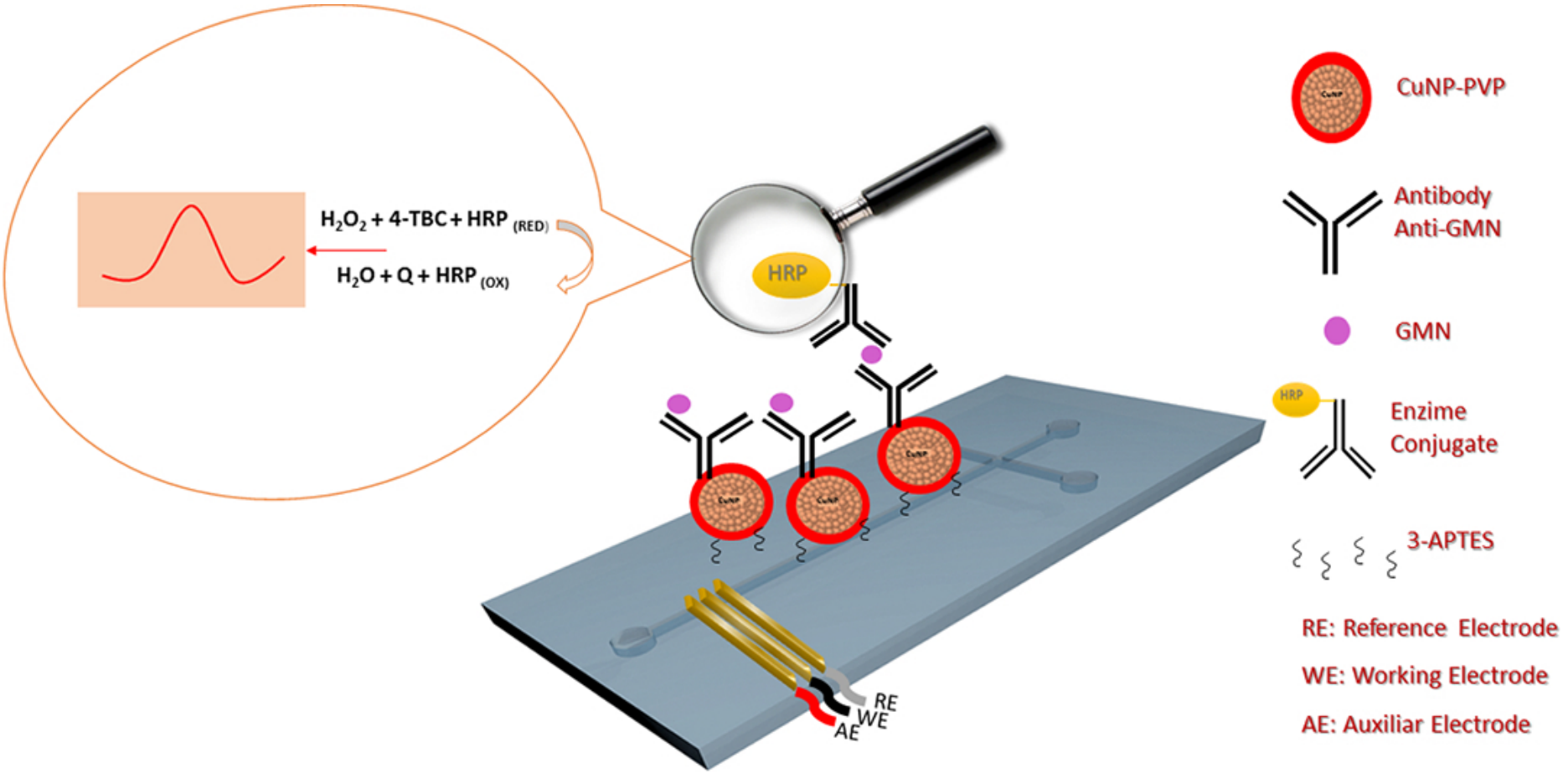
Table 1. Within-assay precision (five measurements in the same run for each control sample) and between-assay precision (five measurements for each control sample, repeated for three consecutive days).

[a] Control sample	Within-assay		Between-assay	
	Mean	**CV %**	**Mean**	**CV %**
0.5	0.48	2.83	0.52	4,85
1.5	1.48	3.95	1.53	5.63
2.5	2.49	3.76	2.51	6.33

[a] ng mL^{-1} GMN

Highlights

- A novel microfluidic immunosensor for GMN quantification was developed.
- This is based on the use of CuNPs-PVP as platform.
- The electrochemical method presented a good analytical performance.
- This device could be used for diagnosis of invasive aspergillosis.



Graphics Abstract

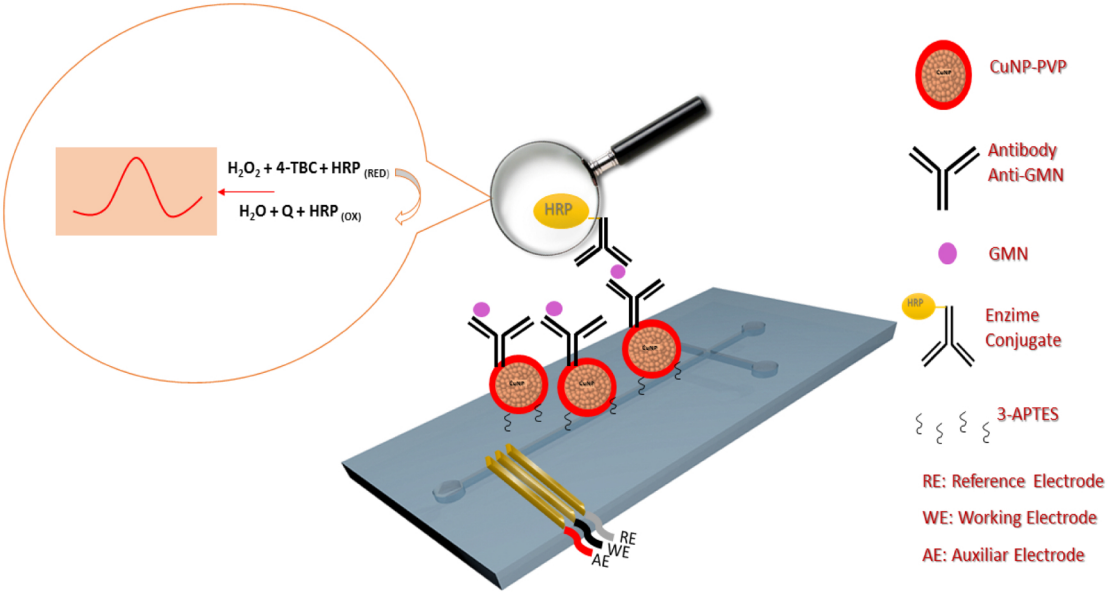
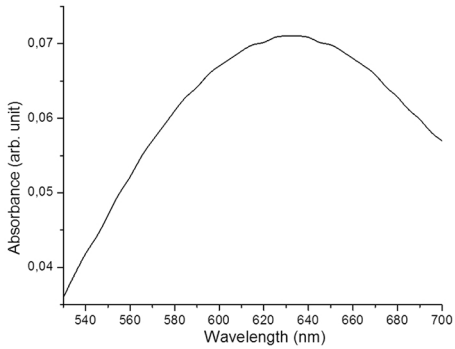

H_2O_2 + 4-TBC + HRP (RED)
H_2O + Q + HRP (OX)
HRP
CuNP-PVP
Antibody Anti-GMN
GMN
HRP
Enzime Conjugate
3-APTES
RE: Reference Electrode
WE: Working Electrode
AE: Auxiliar Electrode
RE
WE
AE

Figure 1



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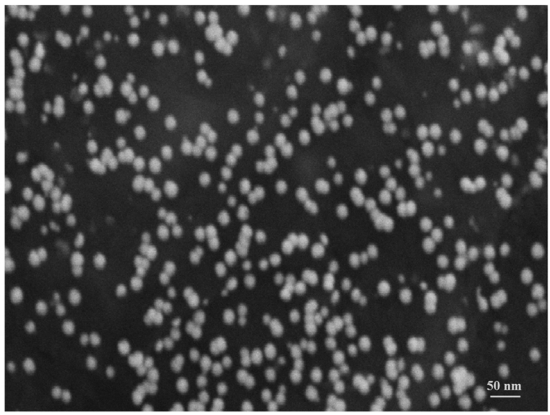

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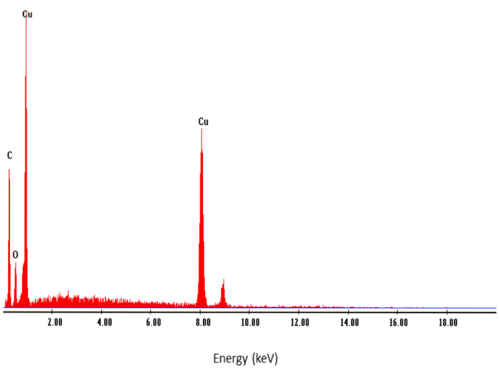

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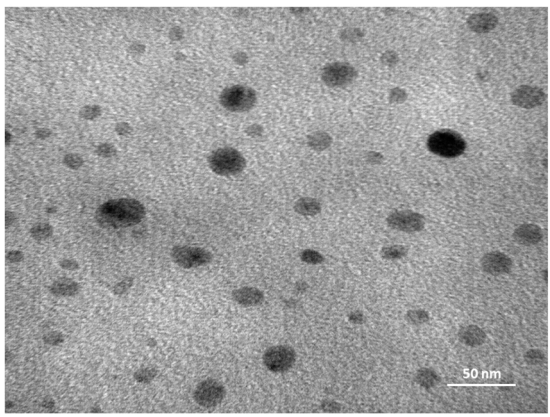

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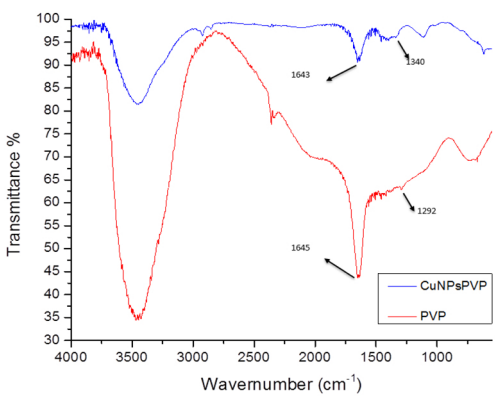

(e)

Figure 2

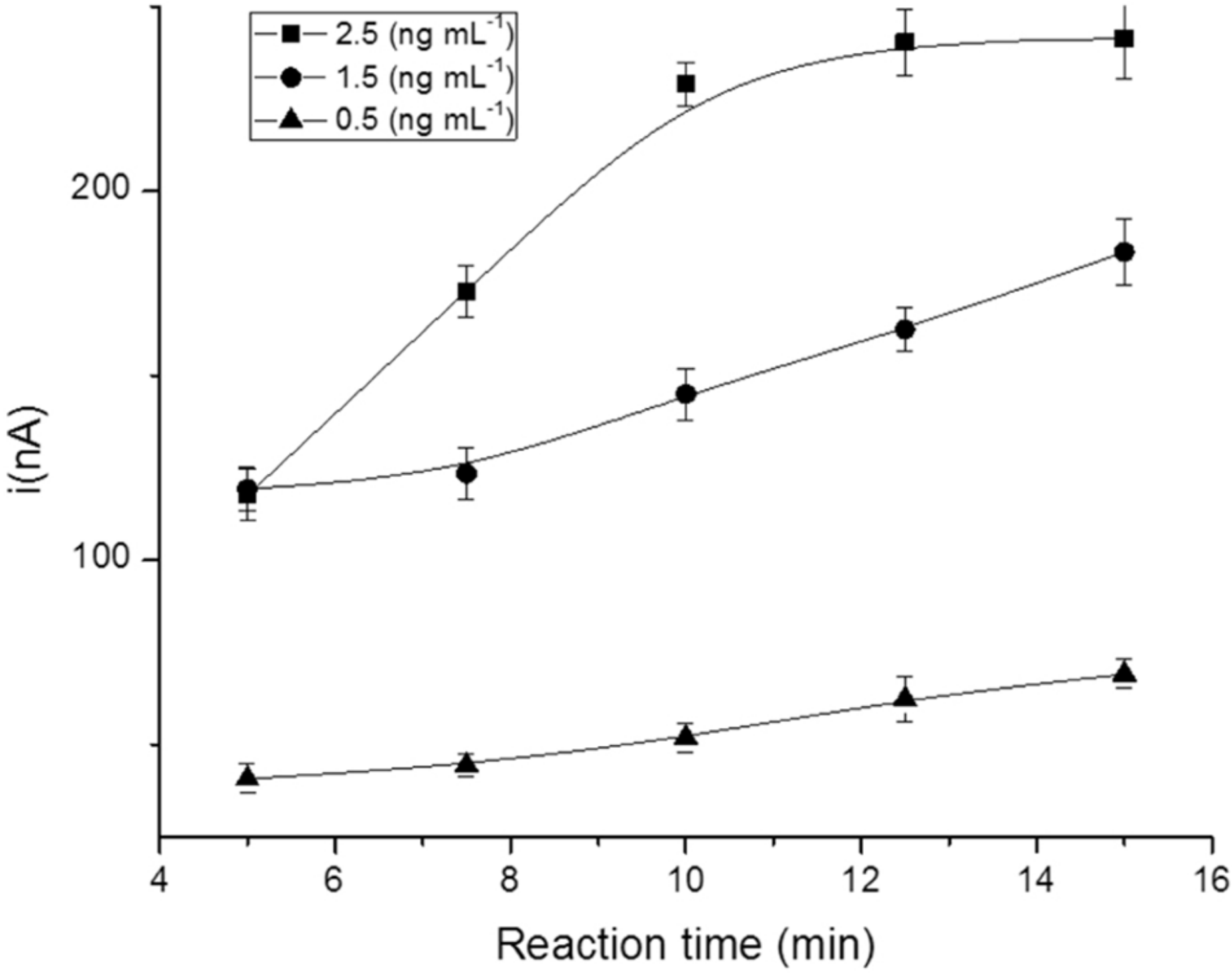
2.5 (ng mL-1)
1.5 (ng mL-1)
0.5 (ng mL-1)
200
100
i(nA)
4
6
8
10
12
14
16
Reaction time (min)

Figure 3

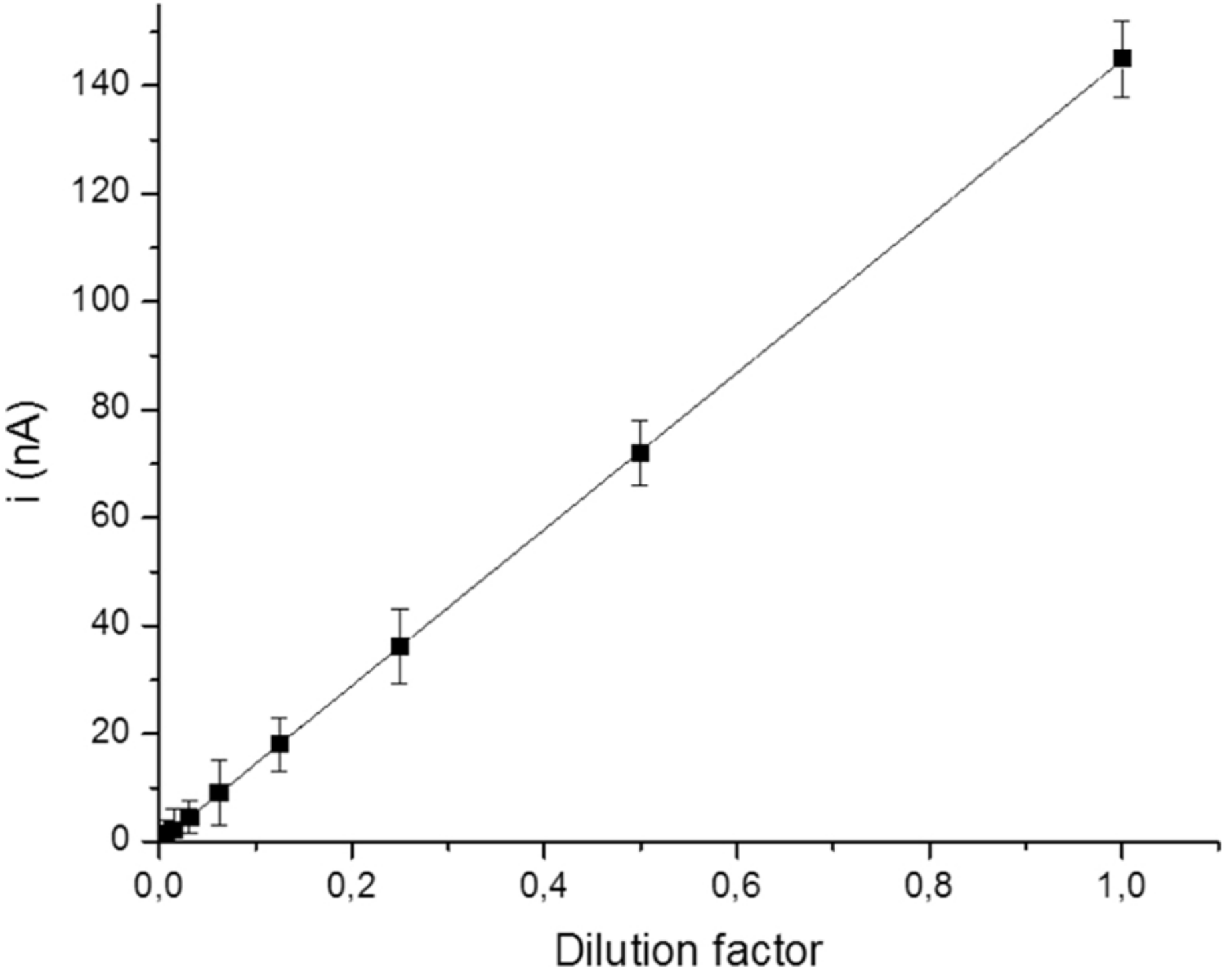

Figure 4

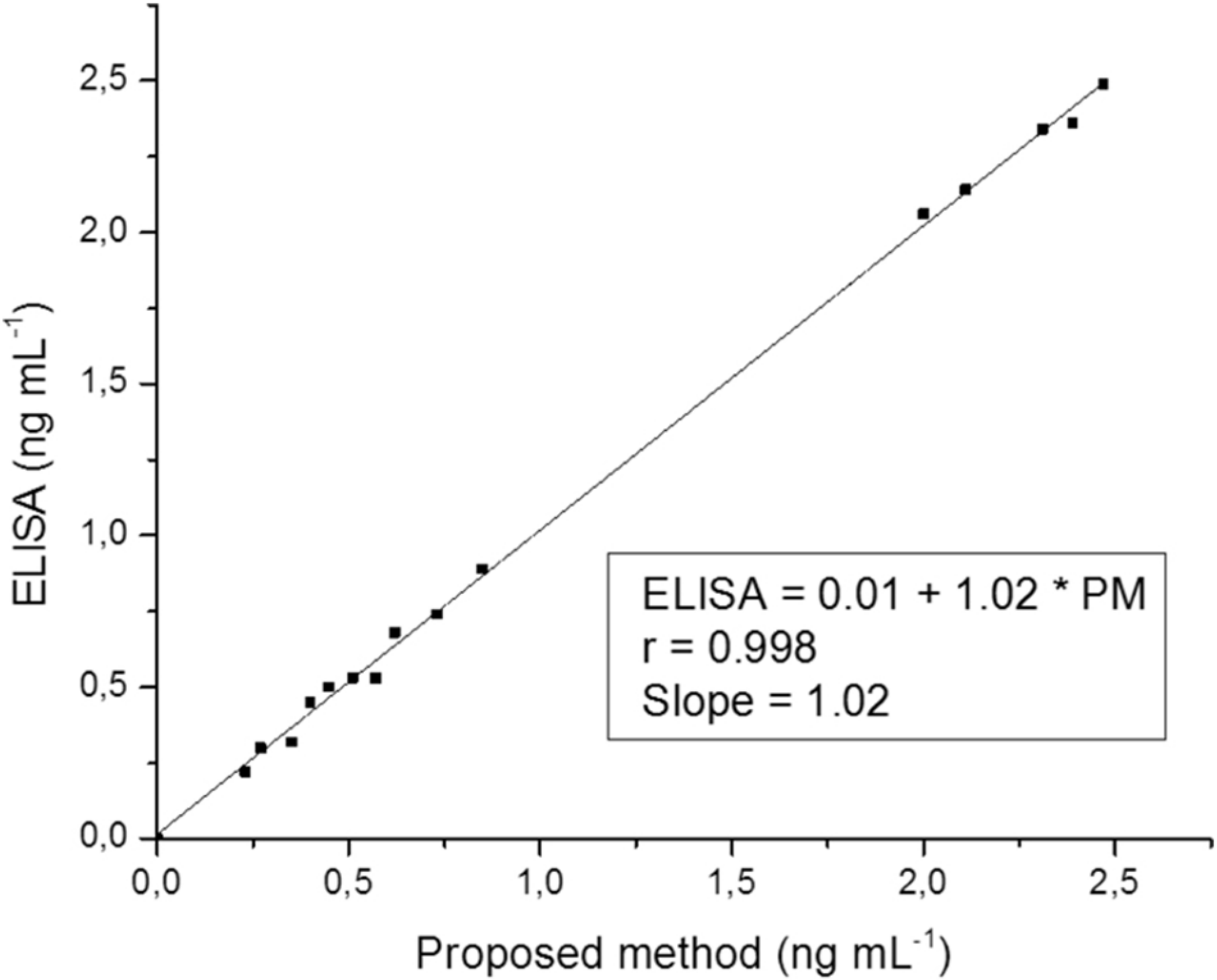
ELISA (ng mL-1)
Proposed method (ng mL-1)
0,0
0,5
1,0
1,5
2,0
2,5
ELISA = 0.01 + 1.02 * PM
r = 0.998
Slope = 1.02

Figure 5