ORIGINAL ARTICLE

PROTEIN G MAGNETIC BEADS BASED IMMUNOSENSOR FOR SENSITIVE DETECTION OF ACETAMINOPHEN

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Abstract

An ultrasensitive and specific label-free immunosensor for the detection of acetaminophen is presented. Protein G-magnetic microparticles retained by the external magnetic field at the surface of carbon based screen printed electrodes were used as immobilization platform. The modified beads were then used to capture the anti-acetaminophen antibody immediately followed by the immunological reaction between the antibody and its antigen, acetaminophen. After optimizing all the steps involved in the immunosensor development, a limit of detection of 1.76 μM was obtained on differential pulse voltammetry measurements.

Rezumat

Prin utilizarea microparticulelor magnetice a fost elaborat un nou imunosenzor nemarcat cu enzimă, ultrasensibil şi specific pentru detecţia paracetamolului. Microparticulele magnetice modificate cu proteina G au fost utilizate ca platformă de imobilizare a biocomponentului, iar imobilizarea acestora la suprafaţa electrozilor planari imprimaţi pe bază de carbon s-a realizat prin aplicarea unui câmp magnetic extern. Microparticulele magnetice au fost utilizate pentru capturarea anticorpului anti-acetaminofen, urmată de reacţia imunologică dintre anticorp şi antigenul corespunzător, acetaminofenul. După optimizarea tuturor parametrilor implicaţi în dezvoltarea imunosenzorului, s-a obţinut o limită de detecţie de 1,76 μM, utilizînd voltametria puls diferenţială.

Keywords: magnetic beads, immunosensor, acetaminophen, screen printed electrodes

Introduction

Development of affinity biosensor with applications in biomedical and environmental fields increased in the last years.

Immunosensors are miniaturized devices, which selectively detect their targets by means of antibodies and provide concentration-dependent signals, the binding of the antigen leading to variations in electric charge, mass, heat or optical properties, which can be detected by a variety of transducers [11, 23]. The development of electrochemical sensors for pharmaceutical analysis has been the object of intense studies over the past years, due to some advantages such as, less time- and reagent-consuming, ease of use and automation. Antibodies have been widely applied as recognition elements in immunosensor's design, because their analytical performances depend on the affinity and specificity of the antibody to its antigen, as well as the immobilization method providing an optimum density and a better orientation for the antigen binding [6]. The type of antibody, the quantity of immobilized molecules, the reactivity upon immobilization, and the proper orientation on the sensing interface have a major influence on the immunosensors` sensitivity, repetability and stability [18]. The choice of the immobilization platform is a critical step in the immunosensors development, several immobilization techniques, such as: self-assembled monolayers [5, 22], protein A or G-based magnetic beads (MBs) [12, 17], gold nanoparticles [10] etc. being reported in the literature. MBs are an ideal platform candidate, allowing the formation of the immunocomplex, thereby eliminating most of the pretreatment steps, as well as the matrix effect. The large surface area of MBs, capable to immobilize an important quantity of biomolecules, can also provide a low detection limit. Additionally, MBs allows a simple manipulation, the immunocomplex being first formed on the beads surface and then immobilized on the surface of the electrode [1], effectively reducing the complexity and time required for sensing application [14].

Acetaminophen (APAP), chemically named *N*-acetyl-*p*-aminophenol, is a widely used analgesic and antipyretic. Being an over the counter drug, over dosage, especially in the case of children is often reported. Hepatic toxicity and renal failure at

overdoses after long term use were also frequently reported [7].

A MBs-based label-free immunoassay was achieved by using monoclonal anti-acetaminophen antibody (APAP Ab) immobilized at the surface of protein G-coated MBs, followed by the addition of APAP. The modified MBs retained at the surface of carbon based screen printed electrodes (SPEs) allowed the APAP detection by using differential pulse voltammetry (DPV). The APAP immunosensor development, step by step, is presented in Figure 1.

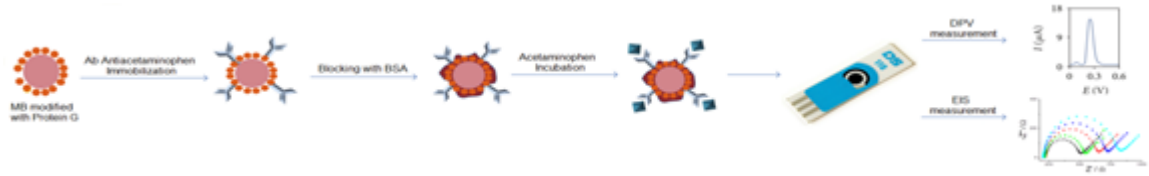


Figure 1.
APAP immunosensor development

SPEs are widely used as electrochemical sensing due to their simple, rapid and inexpensive manufacturing process, the possibility of mass production and the ability to print the entire electrode system on one solid support to obtain disposable devices and eliminating the contamination issues. They present also other advantages, such as relatively low price per item, low background currents and broad potential windows [3, 16]. Several disposable immunosensors have been developed on SPEs in the field of pharmaceutical, clinical or food analysis [1, 19, 20]. The developed immunosensor was successfully tested on real samples.

Materials and Methods

Dynabeads protein G-coated magnetic beads (MBs) (supermagnetic 2.8 µm particles modified with 17 kDa G-protein covalently bonded to the surface), suspension of 30 mg/mL in PBS with 0.01% Tween 20 and 0.09% NaN_3 were purchased from Invitrogen Dynal AS. APAP Ab was from Novus Biotechnology, Bovine serum albumin (BSA), NaCl and Tween 20 were purchased from Sigma-Aldrich. Na_2HPO_4, KH_2PO_4, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ and APAP were purchased from Merck.

The supporting electrolyte was 0.02 M phosphate buffer saline (PBS) prepared with Na_2HPO_4, KH_2PO_4 and NaCl at pH 7.4 and PBS with 0.005% w/w Tween 20 (PBS-T) was used as washing buffer. All the solutions were prepared using Milli-Q grade water.

Prior to use, protein G MBs suspension was homogenized and 50 µL were placed into a 1.5 mL Eppendorf vial, washed three times with 500 µL PBS-T buffer, suspended in 500 µL PBS, and stored at 4°C for further use. A volume of MBs suspension was incubated with APAP Ab solution (1:1 with PBS) for 30 min in a HulaMixer® (Invitrogen), was washed and the remaining free active sites were blocked by incubation with 5% BSA solution (PBS pH 7.4) for 15 min, in order to minimize the nonspecific binding during the detection step. At last, the incubation with APAP solution was done. The optimized immunosensors were tested by using electrochemical impedance spectroscopy (EIS) and DPV with an Autolab PGSTAT100 electrochemical analyser (Metrohm/Eco Chemie Netherlands) controlled by Nova 1.10.4 software. Graphite based SPEs (type DRP 110) were purchased from Dropsens (Spain).

Standard solutions of APAP and of commercially available pharmaceuticals were prepared and kept in refrigerator for further use. All the tested APAP solutions were prepared by diluting the stock solutions to the desired concentration. For the preparation of real samples solutions, twenty tablets of Panadol® and Panadol Extra® (GlaxoSmithKline containing 500 mg APAP/tablet) were fine by grounded, then an accurately quantity of powder was dissolved in distilled water and was sonicated for 30 min. After centrifugation, the removed supernatant was diluted to the desired concentration.

The UV-Vis spectra were performed in order to validate the data obtained by using the electrochemical immunosensor. A Specord 250 PLUS spectrophotometer (Analytik Jena, Germany) equipped with WinAspect software was used, and APAP was determined at 243 nm, in accordance with the 7th edition of the European Pharmacopoeia. A quantity of 0.1 g APAP was dissolved in 100 ml MeOH. 1 mL of this solution was transferred in a 100 mL volumetric flask with 0.5 mL of 10.3 g/L HCl and MeOH. The specific absorbance between 860 and 980 was recorded for APAP solution at 243 nm [8].

Results and Discussion

A sandwich type bioassay for the detection of APAP based on MBs functionalized with G-protein was developed. All the steps and the experimental parameters involved in the elaboration process and

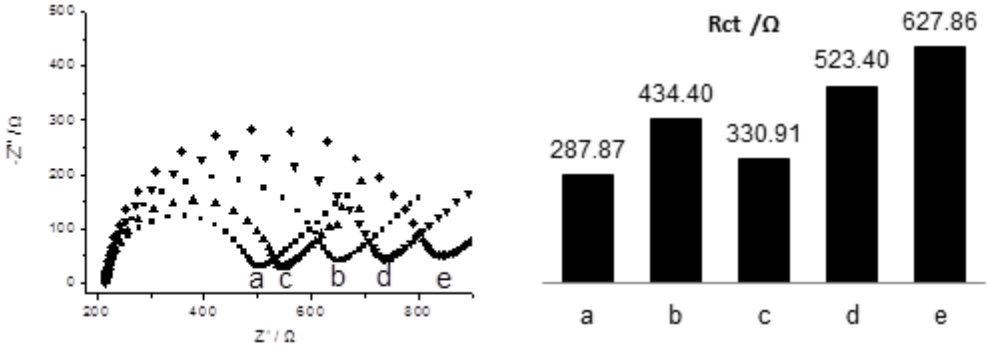
testing were optimized in order to find the best conditions for the assay. The main steps are as follows: the APAP Ab immobilization at MBs, the free remaining active sites blocking with BSA, followed by the affinity reaction with APAP (Figure 1). The elaborated immunosensor was then tested using DPV in PBS (0.02 M; pH 7.4).

Experimental parameters such as: the antibody concentration, the incubation time with APAP Ab and BSA, as well as the type and the concentration of the blocking agents, were optimized. The measurements were performed for 0.1 mM APAP and compared with the blank.

Optimization of the assay

EIS measurements were carried out with the redox probe $[Fe(CN)_6]^{3-/4-}$ in order to characterize the electron transfer properties of the modified electrodes. The Nyquist plot of EIS includes a semicircle and a linear zone, which correspond to the electron transfer limited process and the diffusion limited process, respectively. It can be observed that the obtained Nyquist diagrams show variations in charge transfer resistance (R_{ct}) after each incubation step, proving the MBs surface modification (Figure 2 A and Figure 2 B).

The proposed equivalent circuit is $R_{sol}(C_{dl}[R_{ct}W])$, both for unmodified SPE and after successive incubation steps. This is a Randles type circuit that consists of an active electrolyte resistance R_{sol} in series with the parallel combination of the double-layer capacitance C_{dl} and an impedance of a faradic reaction W. Values of R_{ct} and Warburg coefficient depend on the physico-chemical parameters of a system under investigation [13]. The reaction seems to occur in a single step and a combination of kinetic and diffusion processes with infinite thickness describe the whole process. The R_{ct} values increase in the presence of protein-G modified MBs from 287.87 Ω (SPE) to 434.40 Ω due to the electrode surface coverage with non-conductive particles which obstruct the electron transfer (Figure 2 A). After the incubation of MBs with APAP Ab, the R_{ct} value decreased at 330.91 Ω, while successive increases were registered for the R_{ct} to 523.40 Ω, after the active sites blocking of with BSA and at 627.86 Ω, after the incubation with APAP solution. The experimental results obtained using EIS confirmed that the analyte was captured at the modified MBs surface because the electron transfer rate of $[Fe(CN)_6]^{3-/4-}$ electroactive species decreased (Figure 2).



A. **B.**

Figure 2.

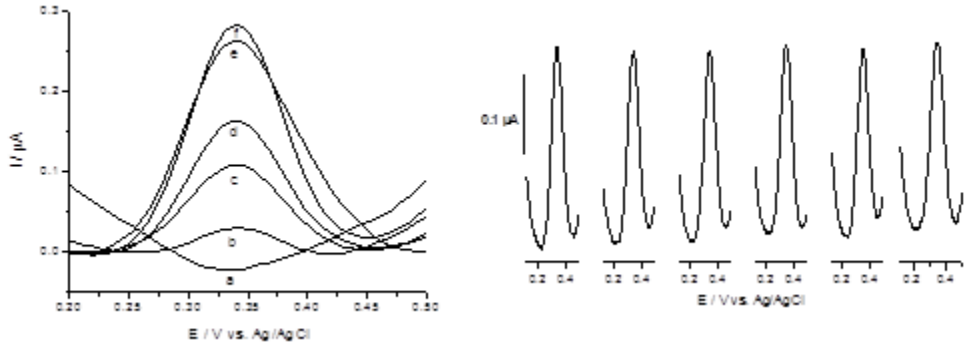
(A) EIS spectra of 10 mM $[Fe(CN)_6]^{3-/4-}$ in PBS (0.02 M; pH 7.4) and **(B)** R_{ct} values registered for: (a) bare SPE and with (b) MBs modified with protein G; (c) incubated with APAP Ab; (d) after blocking with 5% BSA solution and (e) after incubation with 10^{-4} M APAP solution for 10 min. (Inset: the equivalent circuit proposed for the electrode surface process fitting and simulation)

A decrease of R_{ct} value was observed during the APAP Ab immobilization on MBs. An incubation time of 30 min was chosen for further experiments, because the descendent trend with the increase of the incubation time does not influence the R_{ct} beyond this value. BSA and casein from milk powder were tested as blocking agents and the first one was chosen for an optimum concentration of 5%. The evolution of the affinity reaction between the APAP Ab immobilized by covalent binding at the MBs surface and the APAP is improving with the increasing of the incubation time. An incubation time of 10 min was chosen for further experiments because further increasing times do not determine a higher immunosensor sensitivity (in terms of R_{ct} value; data not shown).

DPV determinations were also performed during the optimization process and the results were consistent with those obtained using EIS. As example, in Figure 3 A are presented the DPVs obtained in the case of the APAP incubation time optimization.

The reproducibility of the immunosensor was repeatedly tested by using DPV and the optimized

protocol employed for MBs functionalization. The MBs were immobilized onto several SPEs using a magnetic stand, incubated with 10^{-4} M APAP solution in PBS (0.02 M; pH 7.4) and the obtained values for the peak current intensity were compared after DPV measurements.



A. **B.**

Figure 3.

(A) DPVs registered in PBS (0.02 M; pH 7.4) at SPEs with MBs modified with APAP Ab and 5% BSA solution after the incubation with 10^{-4} M APAP solution for: (a) 0 min, (b) 1 min, (c) 3 min, (d) 5 min, (e) 10 min, (f) and 15 min. **(B)** The reproducibility tests: DPVs performed after 5 min incubation with 10^{-4} M APAP solution on different immunosensors

It can be observed that the optimized immunoassay shows a good reproducibility for the peak current intensity (Figure 3 B), with 2.46 % relative standard deviation. These results are a proof that the immobilization of the functionalized MBs at the graphite SPE surface can be easily used for repetitive and reproducible measurements.

Calibration data for acetaminophen immunosensor

DPV was used to determine the relationship between the analyte concentration (APAP) and the peak current intensity in the oxidation range of potentials, by using the optimized immunoassay. None anodic peak can be noticed in the voltammograms performed for APAP free solutions (Figure 4).

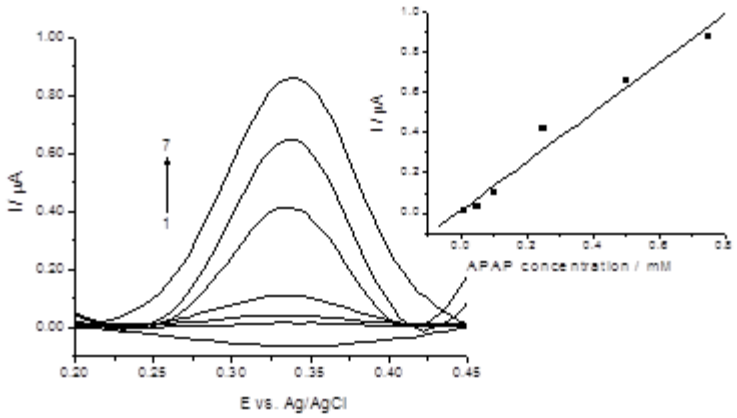


Figure 4.

DPVs registered at MBs modified with Protein-G, APAP Ab, and BSA immobilized on several SPEs after 10 min incubation for: (1) 0; (2) 0.01; (3) 0.05; (4) 0.10; (5) 0.25; (6) 0.50; (7) 0.75 mM APAP solutions in PBS (0.02 M; pH 7.4) (Inset: the calibration curve for APAP)

An anodic peak appears at + 0.34 V *vs.* Ag/AgCl after 10 min of accumulation in open-circuit for APAP solution and the peak current increased with the analyte concentration. The DPV tests were performed in PBS solution (0.02 M; pH 7.4) after APAP solution removal from the MBs surface. The LOD values were calculated on the basis of a signal-to-noise ratio of 3, while the LOQ values were estimated on the basis of a signal-to-noise ratio of 10 [15].

A linear response was observed between 0 and 0.75 mM, and the relationship between APAP concentration and the peak current intensity is expressed by the equation:

$I(\mu A) = 1.21356*Conc.(mM) + 0.01356$; $R^2=0.975$.

The immunosensor's LOD and LOQ are 1.76 μM and 5.28 μM, respectively.

Real sample analysis

The APAP concentration was measured using the optimized immunoassay for two pharmaceutical products: Panadol® and Panadol Extra®. The APAP Ab functionalized protein-G modified MBs were incubated in the presence of APAP solution, obtained from these products, and the results, in term of peak current intensity, were compared with those obtained using standard APAP solution. The DPVs were performed in PBS (0.02 M; pH 7.4) at 25 °C. The results are consistent with those reported by the manufacturer: 500 mg APAP/tablet (Table I). For all the investigated pharmaceuticals the oxidation potential of APAP was found at about the same value (E = + 0.34 V *vs.* Ag/AgCl) as in the case of standard substance.

Table I

APAP recoveries using the immunoassay and UV-Vis spectroscopy

Analyte	Immunoassay			UV-Vis spectroscopy 243 nm	
	Recovery % APAP conc./mM		RSD %		
	0.1	0.5		Recovery %	RSD %
APAP standard	101.02	100.42	0.53	100.00	0.16
APAP Panadol®	102.58	96.60	0.01	98.84	0.30
APAP Panadol Extra®	103.97	100.81	1.71	104.21	0.21

From the obtained data on bicomponent solutions it can be observed that the oxidation peak potentials and currents have almost the same values as in case of the standard samples (data not shown). The recovery rates of the main active substance, APAP in both pharmaceuticals are between 96.6 % and 103.7 %, whether alone (Panadol®) or with caffeine (Panadol Extra®). Taking this in consideration it can be assumed that all the excipients that are present in tablets do not interfere with APAP determination in terms of electrochemical activity or that their electrochemical activity is negligible in the applied domain of the potential. The recovery and interference data obtained using this immunoassay are better than those previously obtained using electrochemically activated glassy carbon electrodes [2] or different types of glassy carbon electrodes modified with carbon nanotubes and horseradish peroxidase immobilized in polymeric films [4, 21]. In order to compare the electroanalytical results, acetaminophen solutions (standard and real samples) were analyzed by UV-Vis spectroscopy at 243 nm, the official analytical method recommended by the 7th European Pharmacopoeia [8]. The achieved results are in good correlation with those obtained by electrochemical method using the new immunosensors, for both mono and bicomponent pharmaceutical products. Regarding the declared content of the active substance in tablets, the Romanian Pharmacopoeia 10th edition requests a deviation of ± 5% [9].

Conclusions

An electrochemical immunosensor based on MBs modified with protein G, and APAP Ab was developed and optimized for the specific and sensitive detection of APAP, using EIS and DPV. A LOD of 1.76 μM was calculated based on the calibration data and the performances of the immunoassay were tested by using two pharmaceutical products containing APAP with excellent recoveries. The immunoassay could be used for the detection of APAP in drug formulations without prior separation. A similar configuration of this assay could be imagined for detection of other compounds by simply changing the sensing bioelement.

Acknowledgements

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