Development of a New Immunosensor for the Detection of Dopamine

Dina Fouad

Chemistry Department, Faculty of Science, Assiut University, Assiut 71516, Egypt.
Fax: 0020882342708. E-mail: dinafouad93@hotmail.com

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Graphite immunoelectrodes as immunosensors using indirect immobilization of a hapten were investigated for their applicability to detect dopamine hydrochloride at low levels. Conditions were optimized to achieve the highest sensitivity using the indirect immobilization of dopamine hydrochloride through a polymerized glutaraldehyde network on microtiter plates using ELISA technique. The conditions were later transferred to the graphite rods (ø 0.8 m × 20 mm) and a comparison between the two different sensitivities (IC_{50} midpoint of test) was carried out. Graphite electrodes showed higher sensitivity towards dopamine than ELISA, since they were able to detect dopamine with a midpoint of test of 0.2 mmol/l while using ELISA they were able to detect dopamine hydrochloride at 2 mmol/l.

Key words: Indirect Format ELISA, Graphite, Dopamine

Introduction

Electrochemical immunosensors (Janata, 1975; Bakker, 2004), which combine specific immunoreactions with electrochemical transduction, have attracted growing attention in recent years due to their quick and sensitive immunological response (Ghindilis *et al*., 1998; Bäumner and Schmid, 1998; Yulaev *et al*., 2001; Wilson and Rauh, 2004; Darain *et al*., 2007).

Immunosensors can be used in environmental monitoring since they can provide continuous, *in situ*, and rapid measurement of contaminants. The working principle of an immunosensor is based on the formation of an immunochemical interaction step that produces a physicochemical change in the system which can be converted into a readable signal. In recent years, different types of immunoelectrodes have been developed which are capable to detect potential changes owing to the antigen–antibody complex formation which leads to a change in the surface properties and in turn results in a potential change. The measured potential is a physical response to changes in the charge distribution and density on the electrode surface, due to the solvated polar groups of the antibodies and the complexing behaviour with the coating hapten (Cooper and Hall, 1988; Lowe *et al*., 1990; Yeo-Heung *et al.*; 2006; Dianping *et al*., 2007).

Dopamine is an important neurotransmitter since it is involved in motor and cognitive functions. Deficits in brain dopamine cause Parkinson's disease in humans. According to this importance several chemical sensors have been developed and investigated for their ability of monitoring the dopamine concentration in order to produce a sensor with high sensitivity and selectivity (Tu and Chen, 2002).

The goal of this study is derived from a previous series of experiments (Pfeifer, 1989; Engel and Baumann, 1993, 1994; Fouad, 2003). This is to follow the reaction between the antibody and a related small hapten through a change of the electrode potential when the immunoreaction takes place on the electrode surface. In order to design an analytical procedure to detect an immunoreaction on an electrode surface, great care must be taken to optimize all parameters which determine the equilibria involved in the immunoreactions, particularly if competitive immunoassays are considered. Hence this work must start from the development and optimization of an ELISA on standard microtiter plates, then transfer of these experiences to similar assays on an electrode surface, which here was chosen as a graphite pencil mine surface, and finally modification and optimization of the procedure with respect to direct potentiometric detection of the immunoreactions.

Materials and Methods

Antibodies

Dopamine monoclonal antibodies 2B11 (Acris Antibodies, Herford, Germany) have been used. The concentration of the clone was 7.2 mg/ml in PBS, pH 7, containing 0.1% of sodium azide, and

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aliquots of the antibody were prepared and stored at + 4 °C. Dopamine antibodies are highly specific for the determination of dopamine.

Apparatus

Adjustable pipettes (Finpette 40–200 μl and finpette 200–1000 μl; Labsystems, Helsinki, Finland), Eppendorf pipettes (0.5–10 μl), and Eppendorf multi pipettes (Eppendorf, Hamburg, Germany) were used to dispense the liquids. ELISAs were performed in 96-well flat bottom polystyrene microtiter plates (MTPs, Maxisorb No. 442404, Nunc, Wiesbaden, Germany). A Dynatech MR 5000 (Dynatech Laboratories, Chantilly, Virginia, USA) ELISA reader was used. The software Biolinx 2.21 (Dynatech Laboratories) was used to control the reader. Data evaluation has been performed by Kaleidgraph 3.5 (Synergy Software, Reading, PA, USA). Pencil rods (graphite rods) were a gift from Staedler (Nuremberg, Germany). As pH-meter the Inolab 2 (WTW, Weilheim, Germany) was used. As a voltmeter a home-made differential instrumentation pre-amplifier (2× operational amplifier type LMC 6001, using their non-inverting inputs) had been connected to a digital voltmeter ME-31 (Metex Corp., Seoul, Korea) which by its RS-232C interface could be directly connected to any PC; the software ScopeView™ (Metex Corp.) has been used for first visualization.

Chemicals

Dopamine hydrochloride [dopamine (DOP), 4-(2-aminoethyl) benzene-1,2-diol] from Sigma has been used as a representative ELISA hapten and an analyte. Goat anti-mouse IgG-peroxidase conjugate and bovine serum albumin (BSA) were obtained from Sigma. Glutaraldehyde (GA) (25% in water) was purchased from Baker (Griesheim, Germany). All other chemicals were of analytical grade.

Experimental

The ELISA was carried out in the competitive indirect mode: Dopamine hydrochloride was immobilized (im Do) on a carrier surface (microtiter plate or ø 0.8 mm × 20 mm). The immobilized dopamine and the dopamine present freely (free Do) compete in a solution to which a mouse antibody against dopamine was added. After a washing step the bound amount of mouse antibody was determined by a peroxidase (POD)-labelled goat anti-mouse antibody (GaM-POD). The anti-antibody concentration was determined by the peroxidase-induced reaction and photometric detection at 450 nm in the usual way in an ELISA reader and was inversely correlated to the dopamine concentration. All these steps have been thoroughly optimized, with all details given in Fouad (2003).

A polymerized GA network has been used to immobilize macromolecules on polystyrene (Weigand *et al*., 1981; Suter, 1982).

Assay procedure

Dopamine hydrochloride was immobilized on MTP using a GA network. The assay on MTPs was carried out as described previously (Holthues *et al*., 2001, 2005). MTPs (96 wells) were coated by GA (6.25% v/v in 0.05 M Na-carbonate buffer). A conjugate between dopamine, GA and BSA was also investigated as an ELISA hapten on MTP and prepared according to Changnaud *et al*. (1987).

Incubation was for 2 h at room temperature. After washing with (PBS+SMB) (sodium metabisulfite) Tween phosphate-buffered saline containing 10^{-3} M SMB (Changnaud *et al*., 1987), coating with dopamine hydrochloride was performed through imino coupling of dopamine to the free aldehyde groups of the GA polymer network using 200 μl coating dopamine hydrochloride solution prepared in PBS buffer at pH 8. Reaction was over night at room temperature. A second coating with 250 μl (PBS+BSA), 6.25%, pH 7.5, has been applied in order to block unspecific antibody or anti-antibody binding sites yet available on the GA polymer network or polystyrene (Ahluwalia *et al*., 1995; Sadana and Chen, 1996). All presented conditions are the result of careful optimization procedures (Fouad, 2003). The assay was continued following the assay procedure mentioned by Fouad *et al*. (2006).

Preparation of the electrodes

Potentiometric measurements on graphite rods required the preparation of two types of electrodes:

Sample electrode: Graphite rods have been incubated for 4 h in 400 μl reaction vessels, each with 200 ml GA solution as described with MTPs. After washing with distilled water, coating with 200 ml dopamine hydrochloride in (PBS+BSA) buffer, pH 8, was performed over night at room temperature.

A second coating with 250 ml of BSA, 0.05 M PBS (phosphate-buffered saline buffer) at pH 7.5 has been applied in order to block the unspecific sites on graphite. Incubation was carried out for 2 h.

Reference electrodes: The electrodes were coated with GA in similar manner like the sample electrodes. After washing with distilled water, coating with 200 ml (PBS+BSA) buffer, pH 8, was performed over night at room temperature. The second step was carried out as for the sample electrodes.

Two types of solutions have been used in the following measurements: Test solutions are the solutions in which the electrodes were incubated, such as buffer solutions which contained both the antibody and the analyte. Measuring solutions were the solutions used for measuring the potential change, such as distilled water.

The general experiment was carried out with graphite immunoelectrodes: Measure U_0, the potential of the electrode pair in a reference solution (usually distilled water), using a high input resistance differential voltmeter. Incubate the electrode pair in the analyte (and antibody) containing the sample solution, which had been pre-incubated with the antibody. During incubation the voltmeter was disconnected from the electrodes in order to avoid even small electrochemical destructions of the electrode coatings due to the voltmeter input bias current. Rinse the electrode pair by distilled water. Measure U_1, the potential of the electrode pair after incubation, in the same reference solution. $\Delta U = U_1 - U_0$ is related to the unknown analyte concentration and can be determined after calibration.

Data evaluation and error considerations

The ELISA reader results have been evaluated in the usual way and normalized plots of $\%B/B_0$ ($\%B/B_0$ presents the relation of the amount of bound ELISA hapten in the presence of the analyte and the amount of the bound ELISA hapten in the absence of the analyte with the neglect of any unspecific binding which may arise) are presented according to

$$\%B/B_0 = (A - A_{ex})/(A_{ex} - A_0), \quad (1)$$

where A is the absorption of the considered analyte sample, A_{ex} the absorption at excess of the analyte, and A_0 the absorption in the absence of the analyte. In case of the use of the electrodes this normalized value could be written as $\Delta U/U_0$ since it represents the relation of the potential change in the presence and in the absence of the analyte.

The so-called four-parameter curve was used to fit the sigmoidal calibration curves (Rodbard, 1974):

$$Y = (a - d)/(1 + (x/c)^b) + d. \quad (2)$$

Here Y is the dose response absorption, a is the response at zero dose, c is the midpoint of test, most often called IC_{50}, d is the response at infinite dose of the analyte, x is the dose of the unlabelled ligand, and b is the slope of the sigmoidal curve.

R^2 as a measure for the fit quality was in all cases larger than 0.992, mostly between 0.995 and 0.997.

All assay signals in this communication are means of four replicates. The errors reported here may be compared to the IUPAC guidelines (Krotzky and Zeeh, 1995), which recommend for ELISAs that the variation coefficient of the IC_{50} values and of the limit of detection should not exceed 15% and 25%, respectively. The reproducibility of the IC_{50} values at the same day and under different conditions should not exceed 12.5%, and for different days not 25%.

Effect of different immobilized dopamine

Tow different immobilized forms of dopamine were compared with respect to their sensitivity. The first was a continuation of the work carried out in our previous publication (Fouad *et al*., 2006) where a glutaraldehyde network was used for immobilizing of dopamine (im Do) on MTP and graphite. The second was the use of a dopamine conjugate (conj Do) which has been commonly used (Changnaud *et al.,* 1987) and is very much similar to the conjugate used in the immunization for the production of the antibody (immunogene).

The first measurement was carried out by coating the MTP with the immobilized dopamine which competes with the dopamine conjugate in solution to the antibody present in solution as shown in Fig. 1. The second measurement was carried out by coating the MTP with the dopamine conjugate which competes with the dopamine free in solution to the antibody present in solution as shown in Fig. 2.

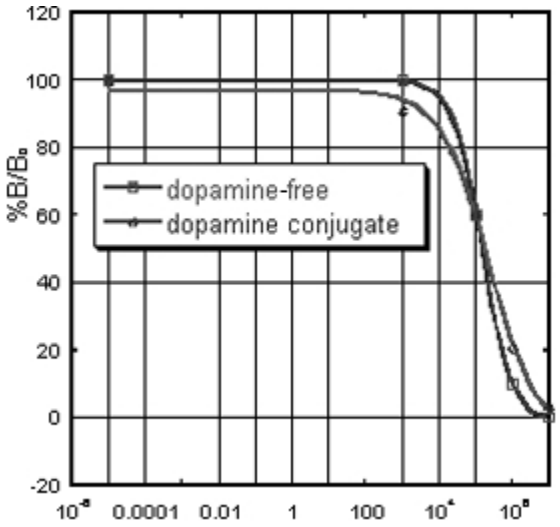

Fig. 1. Calibration curve on MTP, shown as normalized absorption response $\%B/B_0$ against dopamine conjugate; antibody dilution, 1:5000; Elisa hapten, 1 nmol/l.

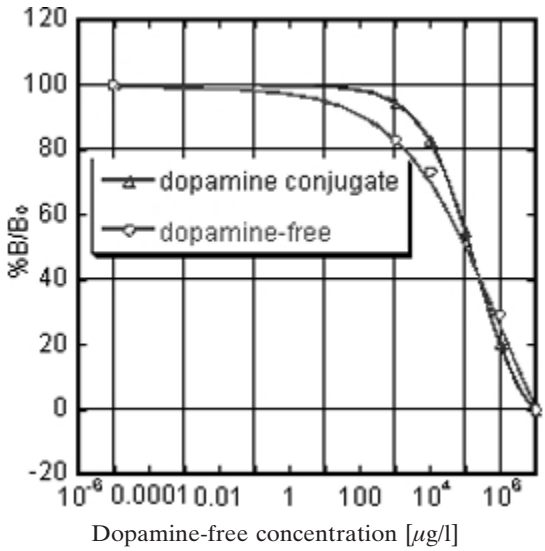

Fig. 2. Calibration curve on MTP, shown as normalized absorption response $\%B/B_0$ against dopamine; antibody dilution, 1:5000; Elisa hapten, 1 nmol/l.

Application of the immunoelectrodes

The conditions with respect to antibody concentrations and hapten concentrations used in the ELISA were directly transferred to the graphite immunoelelectrodes and by following the assay procedures. ΔU (potential change) for five differ-

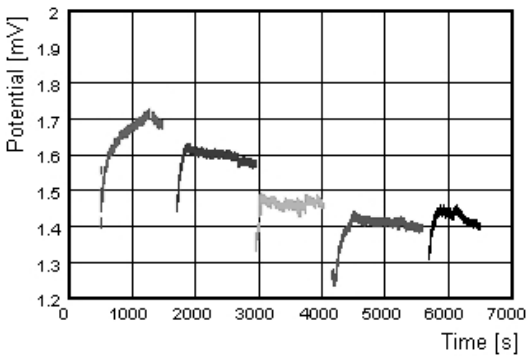

Fig. 3. ΔU (potential change) for five different dopamine concentrations.

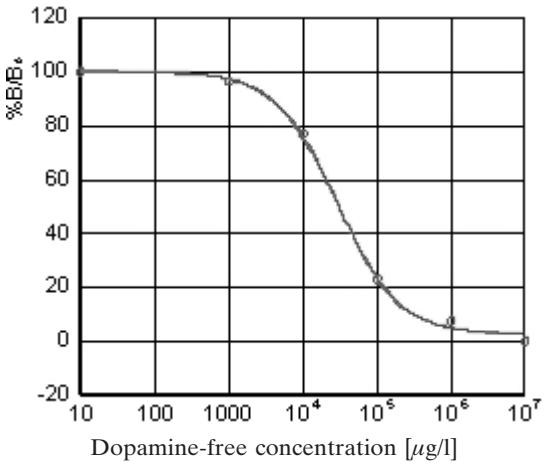

Fig. 4. Calibration curve carried out between graphite electrodes using the antibody dilution 1:5000 and the hapten concentration 1 nmol/l.

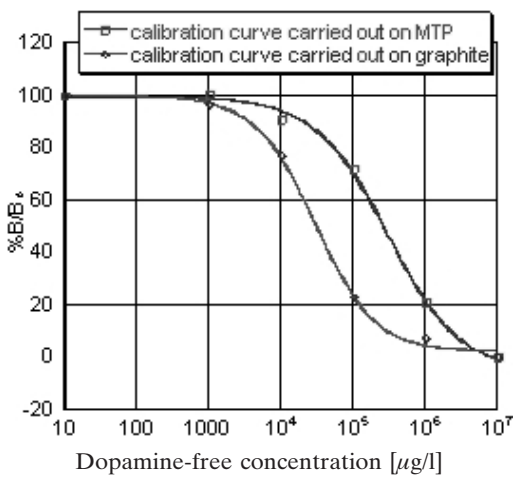

Fig. 5. Comparison between IC_{50} of ELISA and immunoelectrodes; hapten concentration 1 nmol/l; antibody dilution 1:1000.

ent dopamine hydrochloride concentrations is shown in Fig. 3. A stable plateau was reached. Fig. 4 shows the calibrations curve.

Results and Discussion

Developing a biosensor with high sensitivity and selectivity is a demanding task of all researchers in recent years (YeoHeung *et al*., 2006; Samuel and Esteve, 2007). In continuation of the experiments carried out to detect trace levels of triazins using immunoelectrodes (Pfeifer, 1989; Engel and Baumann, 1993; Fouad, 2003), a new approach is to try investigating whether this technique could be transferred to haptens of medical interest such as dopamine.

The potential change ΔU in the immunoelectrode is a response to the antibody-coating hapten complexation on the sample electrode surface, only, against the reference electrode which in turn depends on the analyte concentration since it competes with the coating hapten for the antibody's reactive sites.

Immunoassay conditions such as blocking agent, washing solutions antibody dilution, and hapten concentration were carefully optimized through an immunoassay carried out on the MTP in order to transfer them to the immunoelectrodes. Comparing the IC_{50} values of the two forms of dopamine (conj Do and im Do) resulted in the assurance that dopamine can be immobilized directly through the coupling with glutaraldehyde and used as an immobilized hapten. Results in Figs. 1 and 2 show that the sensitivities of the two forms are very much similar, which shows that the indirect competitive ELISA could be successfully applied for the detection of dopamine. It was possible to detect dopamine with an IC_{50} value of 2 mmol/l.

Even more sensitivity could be achieved if one uses a series of antibodies all generated against dopamine but from different immunogens, since the immunongene and several aspects play a great role in modulating the characteristics, selectivity and sensitivity of the antibody produced (Steward and Lew, 1985; Wie and Hammock, 1984; Chan and Ho, 2002; Singh *et al*., 2004).

On transferring all the immunoassay conditions carried out on ELISA to the immunoelectrodes, the immunoelectrodes were able to detect dopamine with a sensitivity of 0.2 mmol/l (Fig. 5). By comparing this sensitivity with that obtained on MTP it could be shown that immunoelectrodes are more sensitive than ELISA in the detection of dopamine. These results are in agreement with the concept that using immunoelectrodes is a sensitive technique.

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