An Ultrasensitive and Stable Potentiometric Immunosensor
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An Ultrasensitive and Stable Potentiometric Immunosensor

D. Purvis \(^a\)

O. Leonardova \(^b\)

D. Farmakovsky \(^b\)

V. Cherkasov \(^b\)

\(^a\) Scientific Generics Ltd, Harston Mill, Harston, Cambridge CB2 5NH, UK

\(^b\) vantix® DIAGNOSTICS

Abbey Barns Unit 4

Duxford Road

Ickleton, Cambridge CB10 1SX

UK +44 (0) 1799 533160

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Abstract

We describe a novel quantitative polypyrrole based potentiometric biosensor that provides broad-spectrum assay capability. The biosensor allows for capture of analytes of interest from complex real samples such as serum and whole blood, and subsequent measurement in a controlled matrix environment.

The technology is rapid (≤15 min), ultrasensitive (≤50 fM) and reproducible (CV ≤5% at 0.1 ng/ml). In addition the system has shown a wide dynamic range (four to five orders of magnitude), and good stability, 37 °C for at least 4 months.

This potentiometric biosensor detects enzyme labelled immuno-complexes formed at the surface of a polypyrrole coated, screenprinted gold electrode. Detection is mediated by a secondary reaction that produces charged products (a ‘charge-step’ procedure). A shift in potential is measured at the sensor surface, caused by local changes in redox state, pH and/or ionic strength. The magnitude of the difference in potential is related to the concentration of the formed receptor-target complex.

The potentiometric sensing technology has been demonstrated in assays for hepatitis B surface antigen (HBsAg) (Mw > 300 kDa), Troponin I (Mw ~23 kDa), Digoxin (Mw 780 Da) and tumour necrosis factor (hTNF-α) (Mw ~23 kDa). These model targets were chosen to represent analytes of a range of molecular weights, and because of their requirement for assays of high analytical sensitivity and precision.

All these assays were performed using complex fluid samples and the presence of any non-specific binding had no significant effect on the final measurement. New assays can be transferred and optimised readily.
Introduction

Conducting polymer based potentiometric devices derive their responses from the change in redox conditions in the electroconductive polymer. Changes in the steady state potential of the potentiometric sensor can be induced by changes in ionic, pH or redox state at the surface. These changes can be caused by electrochemical, chemical or biological interactions. (Bobacka et al., 1994; Lemuel et al., 1987; Lewenstam et al., 1994; Ghindilis et al., 1998; Michalska et al., 1997). Examples of potentiometric sensors are the solid state ion selective field effect transistors (ISFETs) and pH electrode-based glass ion selective electrodes (ISE’s). These are used for pH, ion, chemical or gas sensing and can be found in blood gas analysers such as those marketed by iStat Corp, Diame- trics and others. Recently there has been several reports of sensitive immunosensors based on ISFET’s, e.g. for detection of bungarotoxin (Selvanayagam et al., 2002) and simazine (Starodub et al., 2000). There are few examples of potentiometric biosensors that are generally applicable to enzyme or immuno-sensing systems. A notable example is Light-Addressable Potentiometric Sensor (LAPS) used in the Molecular Diagnostics Cytosensor and Threshold System (Hafner, 51 2000; Lee et al., 2000).

The reason for the relative unpopularity of potentiometric biosensors is partially related to problems in the late 1980s and early 1990s with sensitivity, accuracy, precision and stability. It was felt that potentiometric sensors would not work because interference from the sample matrix would occlude any signal derived from the specific binding of the analyte. In addition, the success of amperometric sensors, such as those used for glucose sensing and the introduction of optical systems such as BIAcore™ and IAsys™ attracted much interest at the expense of the development of potentiometric systems.

The technology presented here overcomes many of the problems associated with potentiometric biosensors. There are two processes that lead to these results. The first is that time and environment separate the capture and measurement of the analyte. In effect, an immunoaffinity separation is performed and then measured under controlled conditions. The second is that the polypyrrole layer is grown under conditions different to all other published protocols (Bobacka et al., 1994; Lewenstam et al., 1994, Cosnier, 1999). The new polymerisation regime imparts an unexpected robustness and sensitivity to the polypyrrole layer.

Using established ELISA techniques the analyte is captured from the sample and subsequently complexed with a secondary enzyme labelled antibody and measured in a controlled environment. The sensor detects a change in potential due to the activation of receptor target complexes (e.g. enzyme linked immunocomplexes) formed at the surface of an electroconductive polypyrrole layer attached to an electrode. The enzyme conjugate becomes electrochemically active during substrate turnover, in this case we have used a horseradish peroxidase (HRP), o-phenylenediamine dihydrochloride (OPD) substrate system. Consecutive exposure of the electrode to two electrolyte solutions (a wash solution and an enzyme substrate solution) causes a change in the potential of the polypyrrole film, which is measured with respect to a Ag/AgCl reference electrode. The change in potential is related to the concentration of the formed receptor-target complex, and therefore, the concentration of target in the sample. The transducer comprises the electrode with a polypyrrole layer, coated with streptavidin or specific bioreceptors. This technology has been called UTS™ (universal transducer system).
Materials

Equipment
The transducer is a screenprinted gold electrode on a 175 mm thick polyethylene terephthalate (PET) substrate (Fig. 1), coated with polypyrrole and specific bioreagents.

Polymerisation of the polypyrrole layer was carried out using a computer controlled potentiostat (mAutolab, type II, EcoChemie), an auxiliary electrode (platinum wire, Aldrich), an Ag/AgCl reference electrode (BioAnalytical Systems), a bespoke cell for electrochemical polymerisation, and transducer(s) (working electrode(s)).

The data is collected and results presented using a PC with an analogue to digital converter (ADC) card and bespoke software.

Chemicals
All materials were purchased from Sigma Ltd, unless otherwise stated.

Assays and measurement
PBS tablets, OPD tablets; mono and dibasic potassium phosphates, hydrogen peroxide, sodium citrate, citric acid, BSA, bovine calf serum, biotinylated-HRP, streptavidin (Sorbent, Russia).

Biochemicals
Monoclonal anti-HBsAg antibody, enzyme conjugate and recombinant HBsAg (Sorbent, Russia); British Standards HBsAg preparations (National Institute for Biological Standards and Control, UK), Monoclonal anti-Troponin I antibodies, antibody conjugate and cardiac Troponin complex (HyTest, Finland), anti-digoxin antibodies and antibody conjugates (Biogenesis, UK), Digoxin standards (OEM Concepts, USA), anti-TNF antibodies, conjugate and hTNF-a standard from a HyCult hTNF-a DECA kit (Abcam, UK). HBsAg positive and negative samples (North London Blood Transfusion Centre, UK), Troponin I standards (Department of Clinical Biochemistry, South Glasgow University Hospitals, UK), Troponin T positive and negative serum samples (St George’s Hospital Medical School, London, UK), Digoxin serum samples (Addenbrookes Hospital, Cambridge, UK).

Solutions
Polymerisation solution varies depending on application and electrode morphology but the components were in the range 0.001 - 0.02 M pyrrole (Merck) and 0.0003 - 0.001 M sodium dodecyl sulphate (SDS) in de-ionised water. The solution for coating the polypyrrole-coated gold electrodes with antibodies was 0.05 M potassium phosphate buffer, pH 7.8, 10% sucrose. This buffer can also be used to dilute the test sample. The wash solution was 0.1 mg/ml OPD in 0.05 M sodium citrate buffer, pH 5.0. The active substrate solution comprised the wash solution with 0.014% hydrogen peroxide.
Methods

Potentiodynamic electro-polymerisation
A strip of up to 50 sensors, all connected via a conductive bus is placed in a holder. The holder was placed in a bespoke electrochemical cell with the working electrodes of the sensors immersed in the polymerisation solution. Polymerisation was achieved by cycling the potential between -0.2 and 1.9 V (vs. Ag/AgCl) at a sweep rate of 50 mV/s for four or more cycles. The total amount of charge passed through each sensor was 0.3 mC (~30 mC/cm2). The sensors are then electrochemically preconditioned by applying a final potential of 0 V and allowing the polypyrrole to relax into that state. After polymerisation the sensors were removed from the holder, washed several times with high purity water then dried at 37 °C.

Preparation of transducers
Strips of polymer coated sensors were spot-coated with the bioreceptor solution containing either streptavidin, antibodies or antigens and then dried at 37 °C.

Procedures
Sandwich assays were developed for HBsAg and Troponin I and a competitive assay was developed for digoxin. Two antibody coating procedures were used to prepare the transducers: direct adsorption of specific monoclonal antibodies to the polypyrrole layer, and the binding of biotinylated antibodies to the polypyrrole layer pre-coated with streptavidin. The enzyme conjugate becomes an electrochemically active label during substrate turnover.

The universally applicable protocol for the sandwich assays is as follows. A transducer coated with specific antibodies is placed into the sample and incubated for a set time (depending on the affinity of the capture antibody, 2-15 min), washed with 0.01 M PBS, pH 7.4, incubated with conjugate solution (5 mg/ml conjugate in 1% BSA 10 mM PBS, pH 7.4) for ~2-5 min and washed again. The sensor is placed in to the measuring cell and exposed to the wash solution. A potentiometric measurement is taken after 20 s or when the signal has stabilised. This is followed by the active substrate solution which catalyses enzyme turnover and a second measurement is recorded after 60 s. The change in potential is related to the concentration of the formed receptor-target complex. The total time for the assay is in the range 5-20 min. In most cases, converting a multi-step assay to a one-step sandwich assay can reduce this time.

Charge Steps

![Charge Steps Diagram]
Results

HBsAg assay
The minimum detectable concentration of HBsAg ("300 -1000 kDa) was 0.05 IU/ml (corresponding to "50 fM assuming a molecular weight of ca. 1 MDa). A measuring range of at least four orders of magnitude is demonstrated, and the coefficients of variation (CV) were between 2 and 5% at the lower concentrations (Fig. 2a). The two different coating procedures used (direct and biotin-streptavidin coating) did not affect the calibration curve slopes, CV or standard deviation (S.D.). 'Blind' clinical trials of the technology using real samples carried out for the HBsAg assay at the North London Blood Transfusion Centre showed excellent correlation with results obtained using a commercial kit in routine clinical use in that institute.

Troponin I assay
A range of concentrations of cardiac Troponin I ("23 kDa) complex in Troponin I Free Serum were prepared for construction of the calibration curve. The functional sensitivity was 10 pg/ml (0.4 pM). A measuring range of at least three to four orders of magnitude (0.01-100 ng/ml) was shown (Fig. 2b).

Digoxin assay
A two-step competitive assay or sequential saturation assay (Tijssen, 1985) was performed for this small molecule model. The sensors were coated with monoclonal anti-digoxin antibody (10 mg/ml in 0.01 M PBS, pH 7.4), incubated with neat serum samples for 10 min followed by a 5 min incubation with digoxin-HRP conjugate (7 ng/ml in 0.01 M PBS with 1% BSA, pH 7.4). The concentration of the active components was designed to give good discrimination in the clinical therapeutic window of digoxin (0.5-2.0 ng/ml; see Fig. 2c). The assay conditions required for digoxin are different from the other two analytes, which require high sensitivity and wide dynamic range. Digoxin has a low molecular weight (780 Da) and a narrow therapeutic range between efficacy and toxicity (0.5 -5.0 ng/ml). Therefore, sensitivity and dynamic range can be compromised to give good discriminatory power within the narrow window of detection. As a small molecule, digoxin also serves as a model for food, drug and environmental monitoring assays.

![Typical calibration curves for three analytes, UTS™ system.](image)

(a) HBsAg (streptavidin-coated transducers)
(b) Cardiac Troponin I (antibody-coated transducers)
(c) Digoxin (antibody-coated transducers)
Results (cont.)

Direct transfer of commercial hTNF-alpha assay onto UTS™

A commercial ELISA (HyCult, hTNF-a DECA kit) was used as a model to demonstrate the simplicity of transferring an existing assay to the UTS™ format. The ELISA was performed in our laboratory according to the directions supplied with the kit using standards in 0.1% BSA with PBS buffer (0.01 M pH 7.4). The calibration curve constructed (Fig. 3) matched that in the enclosed batch quality control record. The components from the kit were used for the UTS™ assay development. The sensors were coated with polyclonal anti-hTNF-a antibodies. The range of hTNF-a (~23 kDa) standards used in the commercial ELISA was used for the UTS™ assay. The UTS™ assay was conducted using the ELISA protocol, using standards in 50% bovine calf serum with PBS buffer (0.01 M, pH 7.4) and using shorter incubation times. This reduced the total test time from 5 h to 45 min. The shape of calibration curves for ELISA and UTS™ were similar (see Fig. 3). Despite the use of complex sample solutions and reduced incubation times the UTS™ assay showed better differentiation and higher sensitivity despite the higher protein content in the buffer used for the standards. These results were collected after only four optimisation experiments.

Accelerated stability studies

Polypyrrole-coated sensors with no further treatment were shown to be stable at 37°C over a 4-month period. Polypyrrole sensors coated with streptavidin and treated with a simple sucrose stabilisation layer were also stable at 4 and 37°C for 4 months. These sensors were dipped in 10% sucrose solution and air-dried.

A calibration curve for a quality control (QC) assay based on the capture of biotinylated-HRP was performed once a week for first 7 weeks, and then once every 2 weeks for the next 10 weeks. The resultant curves were plotted, no significant change in shape was shown. Any deterioration in performance would be highlighted by a change in the shape of the calibration curve over time.

Improvements in stability can be made by adopting well-known optimised stabilisation procedures currently used in the immunoassay community.
Results (cont.)

Comparison study

Initial clinical trials of a HBsAg assay at the North London Blood Transfusion Centre in 1998 produced excellent results, having perfect correlation with results obtained using a state-of-the-art commercially available ELISA test system (bioelisa HBsAg colour, Biokit, Spain). These commercially available assays meet the strict demands regarding clinical sensitivity and specificity for blood banking assays in the UK (Barbara, 1993).

In order to evaluate further the performance of UTSTM HBsAg determination, a direct comparison was performed. Using serial dilution of a highly positive sample it was demonstrated that the working range of UTSTM Technology is significantly larger than that of the reference ELISA. Using dilutions of the 2nd British Standard (0.5 IU/ml) and the 2nd NIBSC/UKBTS Monitor Sample (0.125 IU/ml), it was found that discriminatory power at low concentrations was clearly superior in the case of the UTSTM assay (Fig. 4).

Comparative studies were carried out for Troponin I and digoxin, using clinical samples supplied by hospital clinical laboratories. Again, good correlation was observed between results from UTSTM assays and state-of-the-art Dade Behring instruments. For the Troponin I assay, the UTSTM performed very well against Stratus† CS (Table 1), showing comparative sensitivity but better assay range, and precision. For the digoxin assay, the UTSTM shows excellent correlation to DimensionTM (Fig. 5).

![Fig. 4 Comparison between UTSTM and commercial ELISA — HBsAg](image)

Signal/noise comparison between UTSTM and a commercially available ELISA test system (bioelisa HBsAg colour, Biokit, Spain) used in blood bank screening laboratories.

![Fig. 5 Comparison between UTSTM and commercial ELISA — Digoxin](image)

Digoxin assay comparison: UTSTM vs. DimensionTM (Dade Behring).

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<tr>
<td>Troponin I</td>
<td>Assay range (ng/ml)</td>
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<tr>
<td>UTSTM</td>
<td>0-100</td>
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<tr>
<td>Stratus†</td>
<td>0-50</td>
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Discussion and Conclusions

These results are somewhat contrary to the current understanding of what is possible using potentiometry for detection of biological molecules and to that end interesting.

The reasons for the sensitivity, reproducibility and stability are extremely dependent on the mode of polymerisation and concentrations of monomer and counterions used to form the polypyrrole layer that provides commercially viable polypyrrole based potentiometric sensors. It is also important that the measurement is performed under controlled conditions.

The conditions we have used for the electropolymerisation of polypyrrole onto the electrodes are unique in that much lower concentration (up to 100x) of both monomer (pyrrole) and counter-ion (SDS) are used than is generally reported. In addition we have shifted the potentiodynamic range for electropolymerisation further to the right (positive), we use from \"-0.2 to +1.9\ V over only four cycles. Most reported electro-chemical polymerisations of polypyrrole, for whatever electrochemical sensor format, amperometric, impedance or potentiometric, use typical concentrations of \"0.1 M monomer and \"0.1 M counter-ion carried out over up to 50 cycles between \-1.0 and \+0.75 V (Smela, 1999; Lillie et al., 2001).

The mechanism for this technology is an electro-physiochemical phenomenon we refer to as a charge-step procedure. This may be described as an induced change in potential due in part to electron depletion of the polypyrrole layer. Changes in pH or ionic strength of the solution immediately adjacent to the surface could also explain the change in potential. The process is passively induced by electrochemical activity at the polypyrrole surface provided by the enzyme HRP converting OPD into 2,3-diaminophenazine (DAP) in the presence of H2O2. It is likely that it is the combination of the redox, pH and ionic events which change the physical (porosity, density, thickness) and electrochemical properties (conductivity, charge) of the polypyrrole layer leading to the observed shift in potential of the sensor.

The results show the development of a stable, redox sensitive polypyrrole layer that is the basis of a potentiometric immunosensor exhibiting ultra-sensitivity with good precision. It has been shown that the technology is applicable to high, medium and low molecular weight analytes, and therefore, has the ability to perform a wide range of immunoassays currently required in routine and special clinical laboratories.

In essence, this is an enzyme sensor being used as an immunosensor.
Acknowledgements

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References

Barbara, J., 1993. Questions of quality: how much HBsAg is there in this sample and is our assay sensitive enough to detect it. Vox Sang. 65, 249-250.


