



An ultrasensitive and stable potentiometric immunosensor

D. Purvis^{a,*}, O. Leonardova^b, D. Farmakovskiy^b, V. Cherkasov^b

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

^b Sensor Tech Ltd, Harston Mill, Harston, Cambridge CB2 5NH, UK

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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 has no significant effect on the final measurement. New assays can be transferred and optimised readily.

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1. 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, Diametrics 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 immunosensing systems. A notable example is Light-Addressable Potentiometric Sensor (LAPS) used in the Molecular Diagnostics Cytosensor and Threshold System (Hafner, 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

* Corresponding author. Tel.: +44-1223-875-200; fax: +44-1223-875-201. <http://www.sensortech-uts.com>.

E-mail address: dpurvis@scigen.co.uk (D. Purvis).

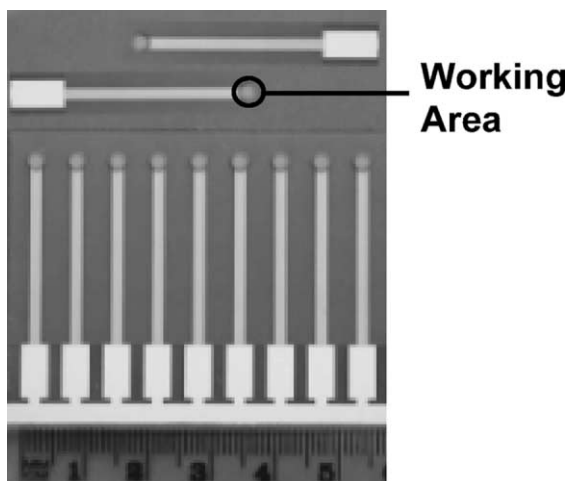


Fig. 1. The Current UTS™ Chip, working area is 1 mm².

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 on an electroconductive polypyrrole layer attached to an electrode. The enzyme conjugate becomes electrochemically active during substrate turnover, in this case we have used a horse radish 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).

2. Materials and method

2.1. Equipment

The transducer is a screenprinted gold electrode on a 175 μm 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 (μAutolab, 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.

2.2. Chemicals

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

2.2.1. 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).

2.2.2. 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-α standard from a HyCult hTNF-α 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).

2.2.3. 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.

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147 The solution for coating the polypyrrole-coated gold
 148 electrodes with antibodies was 0.05 M potassium
 149 phosphate buffer, pH 7.8, 10% sucrose. This buffer
 150 can also be used to dilute the test sample. The wash
 151 solution was 0.1 mg/ml OPD in 0.05 M sodium citrate
 152 buffer, pH 5.0. The active substrate solution comprised
 153 the wash solution with 0.014% hydrogen peroxide.

154 2.3. Methods

155 2.3.1. Potentiodynamic electro-polymerisation

156 A strip of up to 50 sensors, all connected via a
 157 conductive bus is placed in a holder. The holder was
 158 placed in a bespoke electrochemical cell with the work-
 159 ing electrodes of the sensors immersed in the polymer-
 160 isation solution. Polymerisation was achieved by cycling
 161 the potential between -0.2 and 1.9 V (vs. Ag/AgCl) at a
 162 sweep rate of 50 mV/s for four or more cycles. The total
 163 amount of charge passed through each sensor was 0.3
 164 mC (~ 30 mC/cm²). The sensors are then electrochemi-
 165 cally preconditioned by applying a final potential of 0 V
 166 and allowing the polypyrrole to relax into that state.
 167 After polymerisation the sensors were removed from the
 168 holder, washed several times with high purity water then
 169 dried at 37 °C.

170 2.3.2. Preparation of transducers

171 Strips of polymer coated sensors were spot-coated
 172 with the bioreceptor solution containing either strepta-
 173 vidin, antibodies or antigens and then dried at 37 °C.

174 2.3.3. Procedures

175 Sandwich assays were developed for HBsAg and
 176 Troponin I and a competitive assay was developed for
 177 digoxin. Two antibody coating procedures were used to
 178 prepare the transducers: direct adsorption of specific
 179 monoclonal antibodies to the polypyrrole layer, and the
 180 binding of biotinylated antibodies to the polypyrrole
 181 layer pre-coated with streptavidin. The enzyme conju-
 182 gate becomes an electrochemically active label during
 183 substrate turnover.

184 The universally applicable protocol for the sandwich
 185 assays is as follows. A transducer coated with specific
 186 antibodies is placed into the sample and incubated for a
 187 set time (depending on the affinity of the capture
 188 antibody, 2–15 min), washed with 0.01 M PBS, pH
 189 7.4, incubated with conjugate solution (5 µg/ml con-
 190 jugate in 1% BSA 10 mM PBS, pH 7.4) for ~ 2 – 5 min
 191 and washed again. The sensor is placed in to the
 192 measuring cell and exposed to the wash solution. A
 193 potentiometric measurement is taken after 20 s or when
 194 the signal has stabilised. This is followed by the active
 195 substrate solution which catalyses enzyme turnover and
 196 a second measurement is recorded after 60 s. The change
 197 in potential is related to the concentration of the formed
 198 receptor–target complex. The total time for the assay is

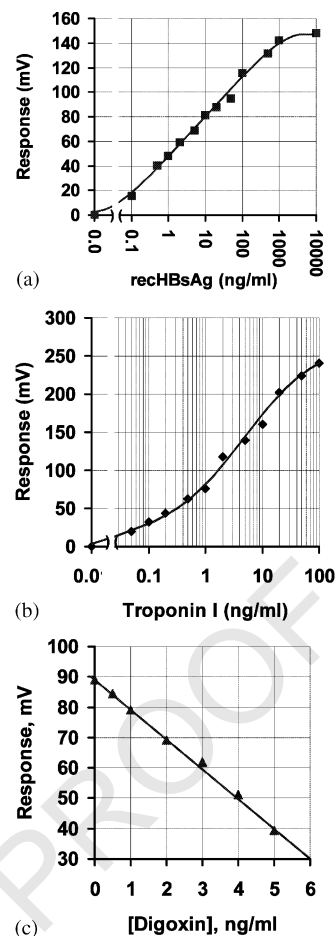


Fig. 2. Typical calibration curves for three analytes, UTS™ system. (a) HBsAg (streptavidin-coated transducers). (b) Cardiac Troponin I (antibody-coated transducers). (c) Digoxin (antibody-coated transducers).

199 in the range 5–20 min. In most cases, converting a
 200 multi-step assay to a one-step sandwich assay can reduce
 201 this time.

202 3. Results

203 3.1. HBsAg assay

204 The minimum detectable concentration of HBsAg (\sim
 205 300–1000 kDa) was 0.05 IU/ml (corresponding to ~ 50
 206 fM assuming a molecular weight of ca. 1 MDa). A
 207 measuring range of at least four orders of magnitude is
 208 demonstrated, and the coefficients of variation (CV)
 209 were between 2 and 5% at the lower concentrations (Fig.
 210 2a). The two different coating procedures used (direct
 211 and biotin–streptavidin coating) did not affect the
 212 calibration curve slopes, CV or standard deviation
 213 (S.D.). ‘Blind’ clinical trials of the technology using
 214 real samples carried out for the HBsAg assay at the
 215 North London Blood Transfusion Centre showed

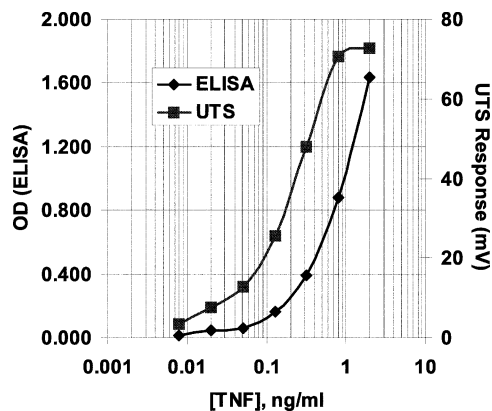


Fig. 3. TNF-alpha assay transferred from ELISA to UTS™ with a reduction in time and an increase in sensitivity. The time of the assay was reduced from the 5 h recommended for the ELISA in 0.1% BSA (PBS buffer) to 45 min for the UTS™ in 50% serum (PBS buffer). There is nearly an order of magnitude improvement in sensitivity with the UTS™. The background (0) response has been subtracted to highlight differences.

216 excellent correlation with results obtained using a
217 commercial kit in routine clinical use in that institute.

218 3.2. Troponin I assay

219 A range of concentrations of cardiac Troponin I (~
220 23 kDa) complex in Troponin I Free Serum were
221 prepared for construction of the calibration curve. The
222 functional sensitivity was 10 pg/ml (0.4 pM). A measur-
223 ing range of at least three to four orders of magnitude
224 (0.01–100 ng/ml) was shown (Fig. 2b).

225 3.3. Digoxin assay

226 A two-step competitive assay or sequential saturation
227 assay (Tijssen, 1985) was performed for this small
228 molecule model. The sensors were coated with mono-
229 clonal anti-digoxin antibody (10 µg/ml in 0.01 M PBS,
230 pH 7.4), incubated with neat serum samples for 10 min
231 followed by a 5 min incubation with digoxin–HRP
232 conjugate (7 ng/ml in 0.01 M PBS with 1% BSA, pH
233 7.4). The concentration of the active components was
234 designed to give good discrimination in the clinical
235 therapeutic window of digoxin (0.5–2.0 ng/ml; see Fig.
236 2c). The assay conditions required for digoxin are
237 different from the other two analytes, which require
238 high sensitivity and wide dynamic range. Digoxin has a
239 low molecular weight (780 Da) and a narrow therapeutic
240 range between efficacy and toxicity (0.5–5.0 ng/ml).
241 Therefore, sensitivity and dynamic range can be com-
242 promised to give good discriminatory power within the
243 narrow window of detection. As a small molecule,
244 digoxin also serves as a model for food, drug and
245 environmental monitoring assays.

3.4. Direct transfer of commercial hTNF-alpha assay onto UTS™

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A commercial ELISA (HyCult, hTNF-α DECA kit) 248
was used as a model to demonstrate the simplicity of 249
transferring an existing assay to the UTS™ format. The 250
ELISA was performed in our laboratory according to 251
the directions supplied with the kit using standards in 252
0.1% BSA with PBS buffer (0.01 M pH 7.4). The 253
calibration curve constructed (Fig. 3) matched that in 254
the enclosed batch quality control record. The compo- 255
nents from the kit were used for the UTS™ assay 256
development. The sensors were coated with polyclonal 257
anti-hTNF-α antibodies. The range of hTNF-α (~23 258
kDa) standards used in the commercial ELISA was used 259
for the UTS™ assay. The UTS™ assay was conducted 260
using the ELISA protocol, using standards in 50% 261
bovine calf serum with PBS buffer (0.01 M, pH 7.4) 262
and using shorter incubation times. This reduced the 263
total test time from 5 h to 45 min. The shape of 264
calibration curves for ELISA and UTS™ were similar 265
(see Fig. 3). Despite the use of complex sample solutions 266
and reduced incubation times the UTS™ assay showed 267
better differentiation and higher sensitivity despite the 268
higher protein content in the buffer used for the 269
standards. These results were collected after only four 270
optimisation experiments. 271

3.5. Accelerated stability studies

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Polypyrrole-coated sensors with no further treatment 273
were shown to be stable at 37 °C over a 4-month period. 274

Polypyrrole sensors coated with streptavidin and 275
treated with a simple sucrose stabilisation layer were 276
also stable at 4 and 37 °C for 4 months. These sensors 277
were dipped in 10% sucrose solution and air-dried. 278

A calibration curve for a quality control (QC) assay 279
based on the capture of biotinylated-HRP was per- 280
formed once a week for first 7 weeks, and then once 281
every 2 weeks for the next 10 weeks. The resultant curves 282
were plotted, no significant change in shape was shown. 283
Any deterioration in performance would be highlighted 284
by a change in the shape of the calibration curve over 285
time. 286

Improvements in stability can be made by adopting 287
well-known optimised stabilisation procedures currently 288
used in the immunoassay community. 289

3.6. Comparison study

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Initial clinical trials of a HBsAg assay at the North 291
London Blood Transfusion Centre in 1998 produced 292
excellent results, having perfect correlation with results 293
obtained using a state-of-the-art commercially available 294
ELISA test system (*bioelisa HBsAg colour*, Biokit, 295
Spain). These commercially available assays meet the 296

Table 1
Troponin I assay comparison: UTS™ vs. Stratus® CS

Troponin I	Assay range (ng/ml)	Analyte sensitivity (ng/ml)	CV's at ~0.5 ng/ml	CV's at 5 ng/ml
UTS™	0–100	<0.05	4%	2.0%
Stratus® CS	0–50	0.03	>5%	>3.4%

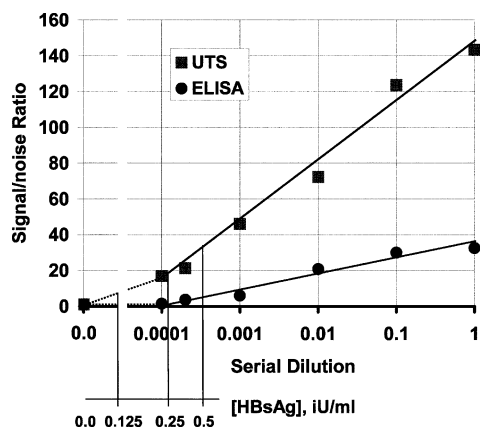


Fig. 4. Signal/noise comparison between UTS™ and a commercially available ELISA test system (*bioelisa HBsAg colour*, Biokit, Spain) used in blood bank screening laboratories.

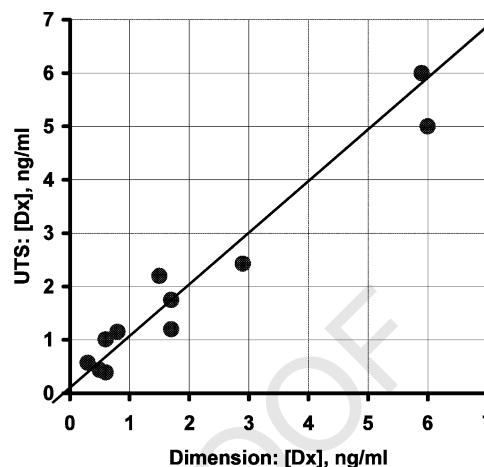


Fig. 5. Digoxin assay comparison: UTS™ vs. Dimension™ (Dade Behring).

297 strict demands regarding clinical sensitivity and speci- 323
298 ficity for blood banking assays in the UK (Barbara, 324
299 1993).

300 In order to evaluate further the performance of 325
301 UTS™ HBsAg determination, a direct comparison was 326
302 performed. Using serial dilution of a highly positive 327
303 sample it was demonstrated that the working range of 328
304 UTS™ Technology is significantly larger than that of 329
305 the reference ELISA. Using dilutions of the 2nd British 330
306 Standard (0.5 IU/ml) and the 2nd NIBSC/UKBTS 331
307 Monitor Sample (0.125 IU/ml), it was found that 332
308 discriminatory power at low concentrations was clearly 333
309 superior in the case of the UTS™ assay (Fig. 4).

310 Comparative studies were carried out for Troponin I 334
311 and digoxin, using clinical samples supplied by hospital 335
312 clinical laboratories. Again, good correlation was ob- 336
313 served between results from UTS™ assays and state-of- 337
314 the-art Dade Behring instruments. For the Troponin I 338
315 assay, the UTS™ performed very well against Stratus® 339
316 CS (Table 1), showing comparative sensitivity but better 340
317 assay range, and precision. For the digoxin assay, the 341
318 UTS™ shows excellent correlation to Dimension™ (Fig. 342
319 5).

320 4. Discussion and conclusions

321 These results are somewhat contrary to the current 343
322 understanding of what is possible using potentiometry 344

323 for detection of biological molecules and to that end 324
325 interesting.

326 The reasons for the sensitivity, reproducibility and 327
328 stability are not clear, but it is extremely dependent on 329
330 the mode of polymerisation and concentrations of 331
332 monomer and counterions used to form the polypyrrole 333

334 layer that provides commercially viable polypyrrole 335
336 based potentiometric sensors. It is also important that 337
338 the measurement is performed under controlled condi- 339
340 tions. 341
342 The conditions we have used for the electropolymer- 343
344 isation of polypyrrole onto the electrodes are unique in 344
345 that much lower concentration (up to 100 ×) of both 345
346 monomer (pyrrole) and counter-ion (SDS) are used than 346
347 is generally reported. In addition we have shifted the 347
348 potentiodynamic range for electropolymerisation 348
349 further to the right (positive), we use from ~ -0.2 to 349
350 +1.9 V over only four cycles. Most reported electro- 350
351 chemical polymerisations of polypyrrole, for whatever 351

352 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).
353 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 ultrasensitivity 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.

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