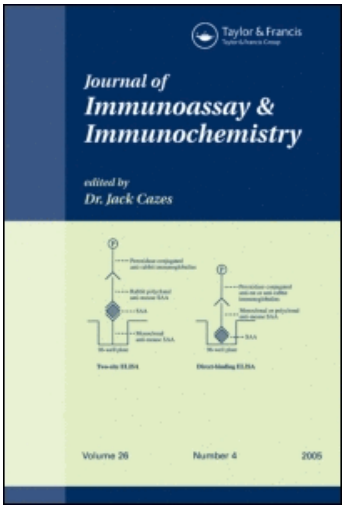


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# A Novel, Generic, Electroanalytical Immunoassay Format Utilising Silver Nano-Particles as a Bio-Label

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**Abstract:** The aim of this study was to evaluate a novel, generic, sensitive electroanalytical platform method for binding reactions, such as immuno or DNA assays. It was thought that silver nano-particles of 40 nM when attached to the analyte of interest would give an electroanalytical amplification of approx. $10^6$ resulting from silver ions produced by dissolution of the nano-particles by a mild chemical oxidant such as ferricyanide. Ferricyanide has been widely used in biochemical measurement and has been shown to relatively stable in a biosensor device. Here we have demonstrated the use of silver nano-particles as a bio-conjugate in a 96 well sandwich assay format for measuring human chorionic gonadotropin (hCG) to a concentration of 0.2 mIU.

**Keywords:** Assay, Bioassay, Electroanalytical, Electrochemical, Immunoassay, Silver nano-particles

## INTRODUCTION

Immunoassays have been an important diagnostic tool since their discovery in the 1950s, first described by Berson and Yalow,[1] which resulted in a Nobel prize for medicine in 1977. Over the years immunoassays have

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been used extensively in hospitals, clinical laboratories, medical and research facilities, driving to improve health and well being of humans, animals and plants. Information gained from clinical immunoassay testing has had the impact of shorting hospital waiting times and in-patient stay. There have also been significant reductions in damage to patient long-term health by mitigating the severity of presenting symptoms. Recently, there has been a drive to bring immunoassay measurement to the patient's bedside, in order to further improve speed and effectiveness of treatment. To do do this, the method selected needs to be sensitive, speedy and specific with the ability to work with micro litre volumes within turbid biomatrices, with little user preparation and handling.

One route to delivering the required high sensitivity assays is to use metal nano-particles as antibody labelled conjugates. The general term used for describing immuno assays using metal nano-particles as detection conjugates is referred to as a metalloimmunoassay.[2] Metalloimmunoassays were first developed in the 1970's as a potential improvement to assays using radioisotopes and fluorescent or enzyme labels. Gold nano-particles are the most common metal nano-particle used in metalloimmunoassay and have been widely employed within lateral flow assays,[4] as well as in other measurement formats, such as the bio specific aggregation of gold nano-particles conjugates, followed by standard spectrophotometry[5] to give a calibrated analytical response, or using an indirect mass spectrum measurement.[6] Photothermal deflection spectroscopy,[7,8] acoustic plate mode sensor,[9] surface plasmon resonance,[10] scanning force microscopy,[11] infrared[12] or Raman[13] spectroscopy, time-resolved fluorescence,[14] and electrochemical techniques such as polarography[15] or voltammetry[16] have all been used to measure gold nano-particles as a measure of a biochemical interaction.



The work on electrochemical measurement of gold nano-particles has shown promise[17,18] with the potential ability to work in turbid and small-volume samples,[19] with the prime advantage of miniaturising electronics to create a cost-effective hand-held instrument. With these benefits, the electrochemical approach utilising metal nano-particles offers a huge potential in medical devices and in portable point of care tests environments. Many electrochemical formats using gold nano-particles have been published, e.g., Limoges et al. to detect IgG, streptavidin[19] or by Authier et al. in detecting human cytomegalovirus DNA.[20] These methods involve the oxidation of gold to form soluble gold ions by $HBr/Br_2$ solution. The gold(III) ions (more precisely gold(III) complexes: $AuBr_4^-$) are then directly determined by anodic stripping voltammetry (ASV) or cyclic voltammetry (CV).[21] A drawback of this method is the use of harsh oxidants such as $HBr/Br_2$ to convert the gold nano-particles to gold ions, which makes this type of assay impractical in a one-step immunoassay format and, hence, for a point of care device. Here, we report an assay format

**Table 1.** The estimation for $10^6$ silver ions per 40 nm silver nano-particle

| Abbreviation code | | | | Equation |
|---|---|---|---|---|
| N | Avogadro number | 6.02E + 23 | $mol^{-1}$ | |
| S | silver atomic mass | 107.9 | g/mol | |
| P | particle size/nm | 40 | nm | |
| D | density of silver | 10.5 | $g/cm^3$ | |
| V | particle volume | 3.35093E−17 | $cm^3$ | $V = 4/3 \times \pi\ (P/2)^3$ |
| M | mass in 1 particle | 3.51848E−16 | g | $m = D \times V$ |
| M | moles in 1 particle | 3.26087E−18 | mol | $M = m/S$ |
| A | atoms in 1 particle | 1.96E+06 | | $A = M \times N$ |

using silver enhancement[22] and metalloimmunoassay based on silver nano-particles with the mild oxidant, ferricyanide.

The reasons for employing nano silver particles in this way are: they can be made roughly mono-disperse (as gold are nano-particles), they have been shown to form good biological conjugates for use in the area of 'Dark Field Microscopy' immunoassay applications,[23] and on dissolution produce approx. $10^6$ silver ions per nano-particle. Ferrocyanide is routinely used in glucose assays with minimal ill effect on the biology of the assay. So, using ferricyanide as a mild oxidant, instead of the harsh oxidants used in gold nano-particles assays,[21] is one aspect of the chemistry being simplified for a one step assay. Within the format, the use of ammonium thiocyanate adds additional value, as reported by Dilleen et al.[24] since ammonium thiocyanate forms a stable electrochemical chelate with silver. The ammonium thiocyanate also forms stable chelates with other metal ions, which may appear in biological samples and removes their electrochemistry away from that of silver ions, leaving a suitable silver peak for stripping voltammetry analysis. Silver ions in a biological solution will also form silver chloride which will reduce the efficacy of the analysis; ammonium thiocyanate dissolves silver chloride to form the electroactive soluble form of the silver ion.[24] Table 1 also shows that a single 40 nm particle can give a large amplification in signal of $10^6$ silver ions. These data match well with estimates from electrochemical analysis.

## EXPERIMENTAL

### Material

Silver nano particles (40 nM) (British Biocell International) were size validated on a Malvern Zetasizer Nano. Antibodies were specific

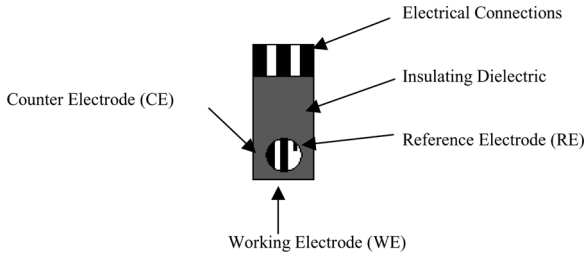



**Figure 1.** The electrode construct used in the assay (working electrode 1 mm by 5 mm).

in-house antibodies used by Unipath in their clearblue pregnancy test range. Electrodes were screen-printed carbon electrodes (D2 from GEM) on ceramic using a standard blue dielectric ink (GEM). Figure 1 shows the electrode construct. Silver nitrate (analytical grade), ammonium thiocyanate, potassium ferrocyanide and potassium ferricyanide were purchased from Aldrich.



## Electrochemical Measurement Procedure

The volume of sample applied in each case to the electrode surfaces was 10 uL. Carbon/ammonium thiocyanate/ferri/ferrocyanide was used as a pseudo reference element. This has a potential difference of approximately −450 mV vs Ag/AgCl/3.5 M KCl half cell. The electroanalytical technique used was fast square wave anodic stripping voltammetry (FQWASV). Other standard electroanalytical techniques could, however, be employed.

Measurements were made using an Autolab PGSTAT12. The parameters for electrochemical analysis by anodic stripping voltammetry of silver were:

1. 10 seconds at 0.35 V
2. 5 seconds at −1.6 V
3. 55 seconds at −1.2 V
4. Scan from −1.2 V to 0.1 V at a scan rate of $1\,V\,s^{-1}$ peak width 5ms; half cycle amplitude 25 mV; frequency 100 Hz.

The area defined by the peaks was used to provide an indication of the charge resulting from the scans and was taken as indicative of silver ion concentration in the sample.

## Silver Nano-Particles Conjugate

A PD-10 size exclusion column (Pharmacia) was equlibrated with 25 mL of pH 6.0 phosphate buffer; 2.5 mL of anti-αhCG monoclonal antibodies

(Mabs) stock was loaded onto the column and eluted with 3.5 mL of pH 6.0 phosphate buffer. To 900 μL of phosphate buffer was added 100 μL of the eluted MAb solution and the absorbance was measured at 280 nm. The concentration was calculated (Abs = εcl), ε = 1.4. The total mass of protein in solution was calculated (3.4 mL remaining). The amount of substrate of MAb was calculated (MW of MAb = 150,000).

S-acetylmercaptosuccinic acid (SAMSA) (Sigma) was dissolved in a dry DMF solution to a concentration of 50 mg/mL and the appropriate quantity was reacted with the anti-αhCG monoclonal antibodies (Mabs) solution while mixing. The resulting solution was incubated overnight, with stirring, at room temperature. The amount of substance of SAMSA needed for 35 equivalent excess was calculated (MW of SAMSA = 174.2).

Approximately 2 mg silver nano-particles solution (OD1) was incubated with a non-ionic surfactant (Pluronic F108-PMPI), 5 mg, in deionised water into an Eppendorf tube and incubated for about 1 hour at room temperature on a rotary mixer. The solution was then centrifuged at 17,000 rpm for 15 min at 5°C. The supernatant was removed and the pellet resuspended in 1 mL of 50 mM HEPES pH 7.5.



The SAMSA-MAb was de-protected to give monoclonal antibody thiols (MAb-SH) for more effective binding to the silver colloid. To a 1 mL aliquot of SAMSA-MAb solution, 40 μL of 0.1 M tris hydroxymethylaminoethane (Tris) solution was added and mixed for 5 min. 20 μL of 0.1 M ethylenediaminetetraacetic acid (EDTA) solution was added and mixed for 5 min. 40 μL of 1 M hydroxylamine solution was added and mixed for 5 min. NAP-10 column packing (Pharmacia) was equilibrated with 20 mL of pH 7.5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mL of deprotected MAb solution was loaded and eluted with 1.5 mL of HEPES.

The silver colloid was then reacted with the deprotected SAMSA-Mab. The samples were incubated overnight and centrifuged at 17,000 rpm for 30 min at 5°C; the supernatant was removed and the sample was resuspended in 500 μL of borate buffer (pH 8.6) (centrifuge repeated twice). The resulting silver particulate-labelled antibodies were stored in borate buffer (pH 8.6) at 4°C.

## Electrochemical Assay Performed in 96-Well Plate

Monoclonal anti-βhCG antibody reagent was immobilised to an ELISA plate (Greiner high binding plates) as follows: 100 μL of anti-βhCG in 50 mM borate buffer, pH 8.6 was added to each well. The plate was covered and incubated with shaking at 37°C for 1 hr, followed by washing. DBS/BSA buffer (Dulbecco's Phosphate Buffered Saline–1 tablet per 100 mL R.O. water, 1% BSA) was added to the wells and left overnight.

The buffer was then discarded. 200 μL of the desired concentration of hCG in DBS/BSA buffer (0, 0.2, 1, 5, 10, 20 and 50 mIU, respectively) was added, in duplicate, to the wells and incubated at 37°C for 1 hr, followed by washing. 50 μL of excess silver colloid conjugated to SAMSA-MAb, in borate buffer (pH 8.6) was added to each well and incubated at 37°C for 1 hr, followed by washing.

Oxidation of the immobilised silver-antibody complex was carried out by addition of 100 μL of 5 M ammonium thiocyanate, 0.05 M citrate buffer pH 4, 1 M ferricyanide, 0.5 M ferrocyanide solution to each well, followed by incubation at 37°C for 30 seconds. 10 μL of the solution was removed by pipette, placed onto the 3-electrode structure described in Figure 1, and the amount of silver ions was then measured electrochemically.

## RESULTS AND DISCUSSION



The direction of this paper was to ascertain the best approach to measure silver as a basis for a novel metalloimmunoassay. Utilising a simple three electrode construct (Figure 1), a number of electrode materials were tested, such as platinum, platinum carbon mix, gold, gold carbon mix, and various types of carbon inks. Only three electrode materials tested provided an effective surface at which to measure the silver ammonium thiocyanate complex. These were D2 and D14 carbon inks from Gem and platinum carbon mix, the latter being the best surface. D2 carbon was selected, however, as the platinum carbon mix was viewed as an expensive ink for single use sensors and D2 gave similar results to the platinum carbon mix ink. Figure 2 shows the effectiveness of the measurement of silver ions on this surface using silver nitrate (analytical grade from Aldrich) as the ion source. The addition of the oxidant ferricyanide (Figure 3) had a small effect on the electroanalytical measurement of silver ions by reducing the total peak area. The ferricyanide did form a weak complex with silver ions and, therefore, there is an equilibrium of the two silver complexes, the ammonium thiocyanate being the stronger. Ammonium thiocyanate was selected for three reasons, firstly, its ability to form a stable electrochemical complex, secondly, the removal of other potential interfering metallic electrochemistry, therefore, giving a clean silver peak, and thirdly, the ability of ammonium thiocyanate to dissolve any silver chloride formed in the sample. The optimum pH was evaluated and this was shown to be around pH 4 (Figure 4) probably due to favouring the ammonium thiocyanate complex over the ferricyanide, as well as favourable conditions for the accumulation stripping experiments. It has, therefore, been shown that silver ions can be measured on a specific carbon electrode in the presence of

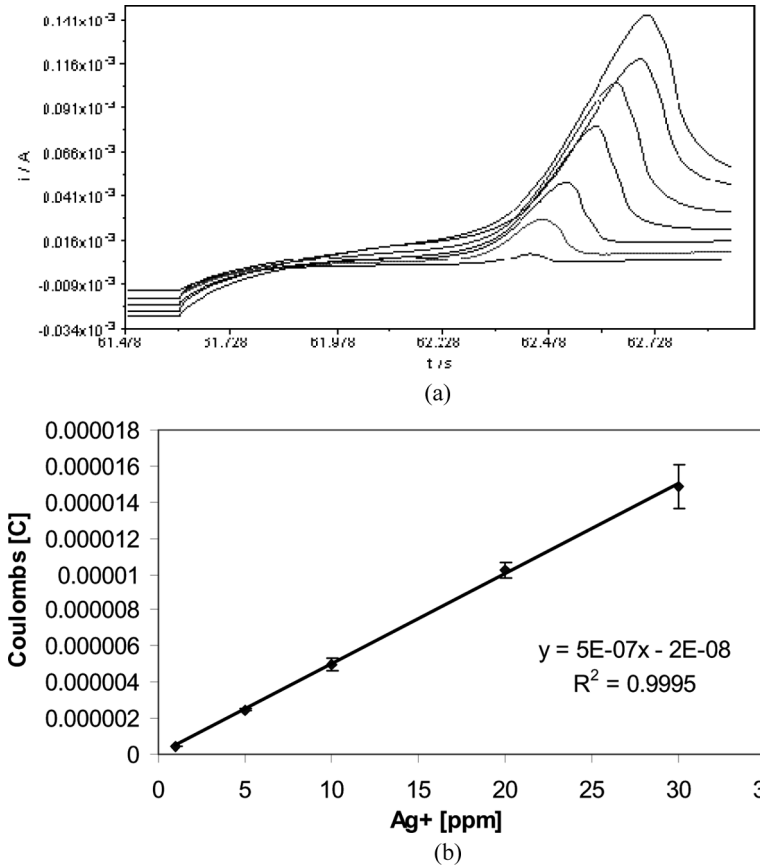
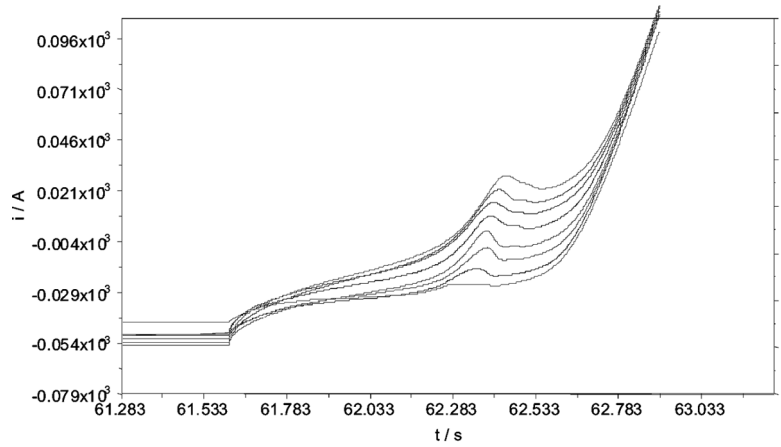


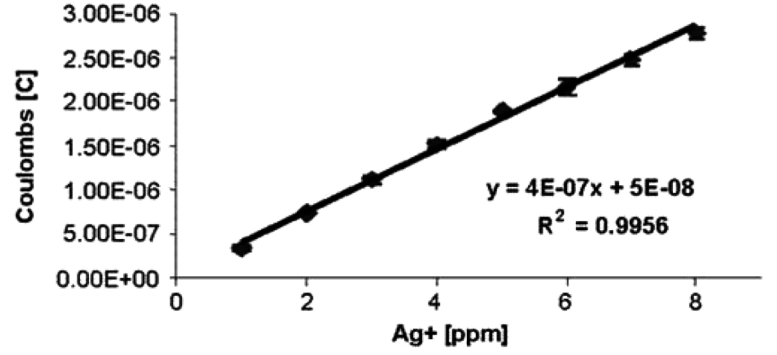

**Figure 2.** Electroanalytical measurement of the silver ammonium thiocyanate complex verses a carbon ammonium thiocyanate reference electrode (a) the recorded scans (b) the graph of the integration under the peaks against concentration of silver ions in ppm.

ammonium thiocyanate as an electroactive chelate and ferricyanide as a potential oxidant.

The next phase was to evaluate the effectiveness of the ferricyanide in converting silver nano-particles to silver ions. The first physical method to evaluate the effect of ferricyanide on silver nano-particles was to analyse changes in the OD (optical density) value. This experiment showed that the silver nano-particles were completely dissolved within a few seconds of adding the chemical oxidant, ferricyanide (Figure 5). Figure 6 is a representation of the data that shows that silver nano-particles in the presence of ferricyanide as a chemical oxidant and ammonium thiocyanate




(a)




(b)

**Figure 3.** A calibration curve and spectra for the electrochemical measurement of silver ions in the presence of ferricyanide and thiocyanate vs a carbon ammonium thiocyanate ferri/ferro cyanide reference element (a) the recorded scans and (b) the graph of the integration under the peaks against concentration of silver ions in ppm.

as an electrochemical chelate, can measure different concentrations of silver nano-particles. Here, we have laid out the framework to show that, utilising this principle, silver nano-particles can be used as an electroactive label. It is shown below that attaching the biology to this system does not interfere with the electrochemical measurement.

The approach that has been employed for this paper utilised a sandwich assay format and has been carried out using antibody labelled silver nano-particle conjugate and an antibody capture solid phase area of a 96-well plate. The immobilised silver nano-particles were converted into

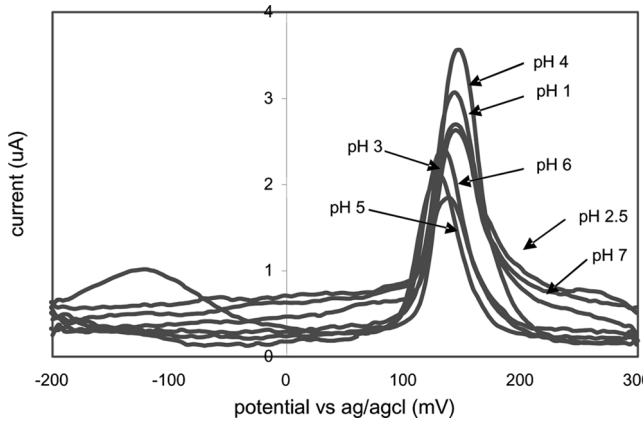



**Figure 4.** Effect of pH on the stripping current vs a silver/silver chloride reference electrode.



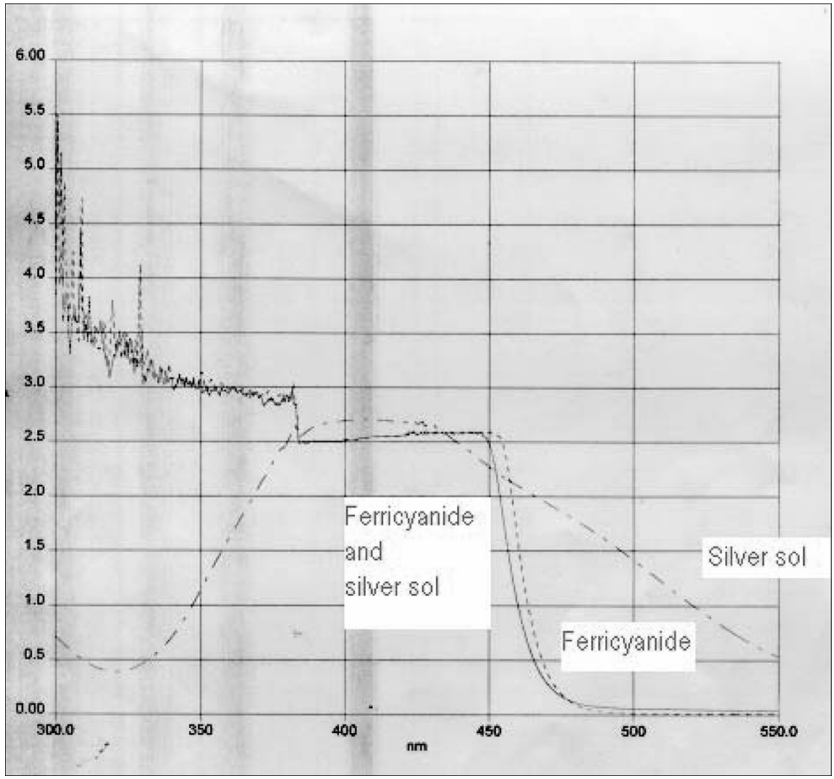



**Figure 5.** UV spectrometric data showing the that silver sol is dissolved using ferricyanide in water.

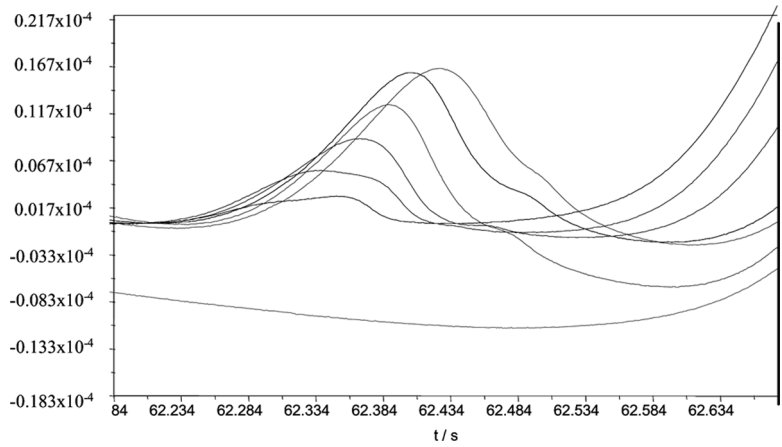



**Figure 6.** Measurement of silver ions from dissolved 40 nm silver nano-particles using ferricyanide as oxidant and ammonium thiocyanate as a chelate. The red line is the blank and the subsequent curves are sequential increase in silver nano-particle concentrations taken from dilutions of the stock solution obtained by BBI.



silver ions by chemical oxidation with ferricyanide and pipetted onto an electrode (Figure 7). Before the silver ions could diffuse away from the working electrode, the silver ions (or a substantial fraction) were electroplated onto that electrode by a suitable reduction potential for a

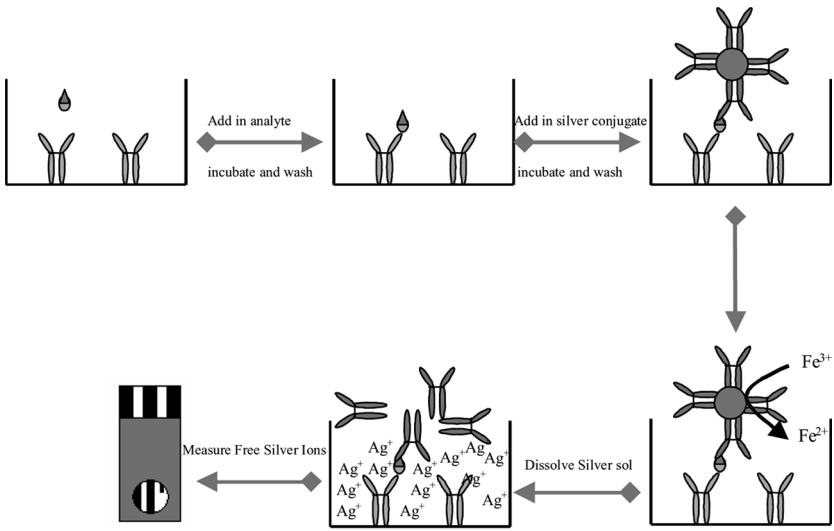



**Figure 7.** Schematic showing the steps used in the 96 well plate format.

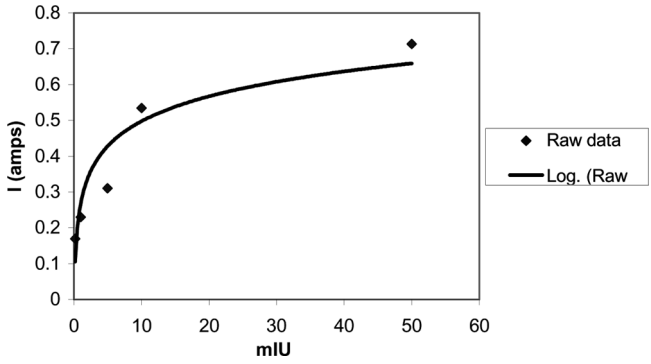



**Figure 8.** Electroanalytical immunoassay utilising antibody labelled silver nano-particles to measure the pregnancy hormone HCG down to 0.2 mIU, in the 96 well plate format (zero levels of HCG gave no silver peak).



known time. An anodic potential was then used to strip the silver from the electrode surface at a characteristic potential. Signal processing resulted in a stripping peak, the area of which was indicative of amount of silver ions, hence silver nano-particles, bound analyte. Figure 8 shows an assay for human chorionic gonadotropin (hCG) showing sensitivities down to 0.2 mIU utilising this method, indicating that the biology has little effect on the measurement of the silver nano-particles.

## CONCLUSION

We have shown that one 40 nM silver nano-particle results in approximately $10^6$ silver ions on dissolution, through estimating the mass of a 40 nM silver nano-particle and electroanalytical measurement. This gives a large amplification factor for the assay, thus improving the electroanalytical sensitivity. We have reduced the influence of the biological samples, which contain relatively high levels of chloride, together with amounts of bromide and sulphur containing materials. These form precipitates or complexes with silver ions and can interfere seriously with the stripping analysis. An excess of ammonium thiocyanate in the reagent layer, together with the preferred oxidant (e.g., potassium ferricyanide or ferric acetate/EDTA) at pH 4 reduces this difficulty by preferentially complexing silver ions, whilst retaining electroactivity for the stripping analysis. Finally, we have shown that we can effectively use this method within an immunoassay format directed to hCG.

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