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# ELECTROCHEMICAL IMMUNOASSAY FOR CARDIAC MARKERS WITH MAGNETIC PARTICLES AS A SOLID PHASE AND SILVER NANOPARTICLES AS AN ELECTROACTIVE BIO-LABEL

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**Abstract** – This paper introduces a novel electrochemical detection method for measuring the formation of an immunological complex. The described electrochemical immunoassay consists of several consecutive steps. First the sample is mixed with silver colloid and magnetic particles both previously conjugated to appropriate antibodies. The analyte from the sample binds to both silver and magnetic beads forming a sandwich immunocomplex: magnetic particle – analyte – silver nanoparticle (Fig. 1). That complex is separated from the sample with the use of magnetic field and then washed to remove any unbound silver nanoparticle. The bound silver nanoparticles are dissolved electrochemically into silver ions. This process is extensively investigated and the detailed mechanism of nanoparticles dissolution is presented. After the silver dissolution the released silver ions $Ag^+$ are detected with anodic stripping voltammetry (ASV) and the ASV signal of silver oxidation is proportional to the analyte concentration. This approach allows for fast detection of cardiac markers like myoglobin and troponin in low volume, turbid samples with the detection limit of 200 and 150 pg/mL respectively.

**Keywords**: silver nanoparticle, cardiac marker, electrochemical immunoassay

## 1. INTRODUCTION

Heart diseases are one of the main causes of deaths worldwide. When damage to the heart occurs, protein like myoglobin and troponin are released into bloodstream. We call them cardiac markers and their increased level in the blood is indicative of the extent of damage to the heart. These proteins can be detected in clinical laboratories with techniques such as ELISA [1-2]. There is though a need for fast point of care detection of myoglobin and troponin to facilitate early diagnosis and treatment.

The electrochemical immunoassay described in this paper can be a point of care alternative to ELSIA and other clinical methods. To develop the assay antibodies were immobilised on magnetic particles and we also optimised the immobilisation of antibodies on silver colloid. Both conjugates were then incubated with the sample. The analyte present in the sample binds to both particles forming a sandwich immunocomplex (Fig. 1). This binding can occur within microfluidics channels and by applying magnetic field any unbound silver is washed away. The silver that does bind to magnetic particles is thereafter dissolved electrochemically to produce silver ions, which are next measured with ASV on carbon electrode. The oxidation signal of silver ions is directly proportional to myoglobin/troponin concentration.

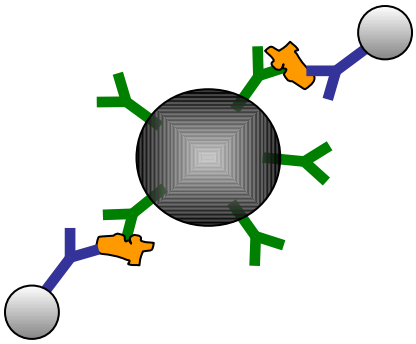

Fig. 1. The sandwich immunocomplex (magnetic bead-analyte-silver nanoparticle) that is formed during the electrochemical immunoassay.

## 2. MATERIALS AND METHODS

### *2.1 Antibody immobilisation on the silver colloid:*

1. Spin down 2 mL of silver colloid (British Biocell International, 40 nm) at 16110 x g for 10 minutes at 4°C.
2. Remove the supernatant very carefully as the pellet is fragile.
3. Resuspend the pellet in 2 mL of 0.2 M borate buffer pH 6.5 with 30 µg/mL antibody concentration (Anti-myoglobin clone 7C3 from HyTest).
4. Incubate at room temperature for 2 hours gently mixing.
5. Spin down again at 16110 x g for 10 minutes at 4°C.
6. Resuspend the pellet in 2 mL of 0.2 M borate buffer pH 6.5 + 0.1 % bovine serum albumin (BSA) (Sigma).
7. Spin down again (as above).

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8. Resuspend the pellet in 0.5 mL of 0.2 M borate buffer pH 6.5 + 0.1 % BSA and store at 4°C.

(For anti-troponin antibodies, clone 19C7 0.1 M borate buffer pH 7.5 was used).

### *2.2 Antibody immobilisation on the magnetic beads:*

PBS = Phosphate Buffered Saline
CB = Coating Buffer (0.1 M $H_3BO_3$ pH 9.5)
BB = Blocking Buffer (0.01 M PBS pH 7.4; 0.5% BSA; 0.05% Tween20)
WB = Washing and Storage Buffer (0.01 M PBS; 0.1% BSA; 0.05% Tween20)

1. Resuspend magnetic particles (Dynabeads® MyOne™ Tosylactivated) by vortexing thoroughly.
2. Transfer 25 mg of beads /250 µL/ to 1 mL tube.
3. Pull down the particles using a magnet bar and pipette off the supernatant.
4. Resuspend the beads in 0.5 mL of CB
5. Repeat steps 3-4.
6. Pull down particles and resuspend the beads in 207.5 µL of CB.
7. Add 1 mg /210 µl/ of antibody: clone 4E2 (antibody solution was previously buffer exchanged with CB to remove $NaN_3$).
8. Add 207.5 µL of 3 M $(NH_4)_2 SO_4$
9. Incubate 24h at 37°C with slow tilt rotation.
10. Pull dawn the beads, remove supernatant and add 625 µl of BB.
11. Incubate at 37°C overnight with gentle mixing.
12. Wash 3x with 1 mL of WB.
13. After last washing resuspend in 1 mL of WB and store at 4°C.

### *2.3 Immunoassay in 96-well plate format:*

The wells of the plate (Costar 9018) were coated overnight at 4°C with 50 µL of anti-myoglobin clone 4E2 antibody (HyTest) solution (10 µg/ml in 0.01 M PBS pH 7.4). After removing the solution, the wells were rinsed three times with 200 µL of wash buffer (0.05 M Tris; 0.15 M NaCl; 0.05 % Tween20; pH 7.5) and blocked for 40 minutes with 150 µL of block buffer (Pierce #37536). The wells were rinsed again three times with wash buffer and then incubated for 60 mins with 50 µL of various concentration of myoglobin solution in wash buffer. Next, the wells were washed three times yet again and 50 µL of silver conjugate probe was added and incubated for 90 minutes. After another washing the wells were filled with 50 µL of 1 M $NH_4SCN$, incubated for 1h and the solution from the wells was transferred onto screen-printed electrode, where it was subjected to anodic stripping voltammetry (ASV).

### *2.4 Immunoassay in a tube with magnetic beads.*

1. Place 4 µL of magnetic particle-antibody conjugate suspension in a series of 0.5-mL tubes.
2. Add 70 µL of standard solutions of myoglobin/troponin diluted in wash buffer (0.01 M PBS pH 7.4, 0.1 % BSA, 0.05% Tween20).
3. Incubate for 10 minutes.
4. Add 26 µL of silver nanoparticle conjugate.
5. Incubate for 30 minutes gently shaking (particles need to be in suspension all the time).
6. Pull down the magnetic particles with a magnet and wash the pellet twice with 200 µL of wash buffer.
7. Resuspend the pellet in 50 µL of 1 M $NH_4SCN$
8. Incubate for 0.5 h.
9. Transfer the suspension onto the electrode and run ASV.

### *2.5 Immunoassay in real samples.*

1. Place 5 µL of magnetic particle-antibody conjugate suspension is a series of 0.5-mL tubes.
2. Prepare standard solutions of myoglobin in wash buffer and in human, myoglobin-free serum (HyTest).
3. Add 5 µL of myoglobin standards prepared in buffer or serum.
4. Incubate 10 minutes.
5. Add 40 µL of silver nanoparticle conjugate.
6. Incubate for 30 minutes gently shaking (partricles need to be in suspension all the time).
7. Pull down the magnetic particles with a magnet and wash the pellet twice with 200 µL of wash buffer.
8. Resuspend the pellet in 50 µL of 1 M $NH_4SCN$.
9. Incubate for 0.5 h.
10. Transfer the suspension onto the electrode and run ASV (with 120 second plating time).

### *2.6 Electrochemical detection of silver nanoparticles.*

50 µL of the solution from the plate wells or from tube with magnetic beads (silver and/or magnetic conjugate in 1 M $NH_4SCN$) was transferred onto sensor surface. The ASV was run using an electrochemical workstation (AutoLab PGSTAT 12, Windsor Scientific) with the following steps and parameters:

a. Pre-treatment: 0.6 V for 15 s
b. Nucleation step: -1.6V for 5 s
c. Deposition step: -1.2V for 55 s
d. Stripping step: staircase sweep from -1.2 to 0.1V at scan rate 1V/s and potential step 0.01V.

The results were recorded and saved with a PC software control – General Purpose Electrochemical System (GPES 4.9). The analytical signal, which is the number of coulombs generated, was calculated by measuring the area under the silver oxidation peak obtained in the stripping step.

The mentioned sensor used in voltammetric analysis was a single-use, screen-printed carbon three-electrode cell. The sensors were printed in Luton Institute of

Research in the Applied Natural Science with the D2 carbon paste manufactured by Gwent Electronic Materials.

Before measurements each sensor was cleaned by running ASV with a 50-µL-drop of 1M $NH_4SCN$. After cleaning the remaining solution on the electrode was wiped dry with a soft tissue.

## 2. RESULTS AND DISCUSSION

In order to obtain a sensitive assay we needed first to optimise the immobilisation process for both antibodies. There was no problem with magnetic beads as there are used in many applications [3]. However, the preparation of silver conjugates required much more attention. We found out that even the slight difference in the immobilisation buffer greatly affected the activity of obtained conjugates. Fig. 2 shows the influence of some parameters of immobilisation on the final activity. From our experiments we concluded that the best buffer for immobilisation of anti-myoglobin antibody, clone 7C3 was 0.2 M borate buffer pH 6.5. The best buffer for anti-troponin antibody, clone 19C7 was 0.1 M borate buffer pH 7.5.

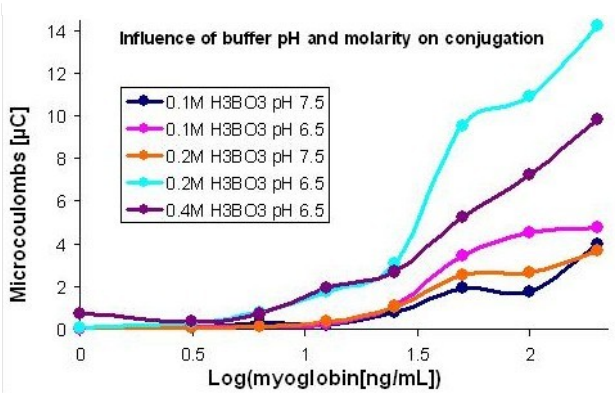


Fig. 2. The performance of 5 different conjugates prepared in different buffers. 7C3 antibody concentration = 30 µg/mL; plating 5s at -1.6V and 55s at 1,2V; pre-treatment 15s at 0.6V; stripping from -1.2 to 0.1V at scan rate 1V/s (n=2, no error bars for clarity).

The differences are due to the interaction between protein and the surface of silver colloid. The electrostatic and hydrophobic interactions have a very important role in that binding and as a result the pH and ionic strength of the buffer affect the immobilisation process [4].

The exceptional performance of electrochemical immunoassay is related to the release of huge number of metal ions from one single nanoparticle (40-nm particle contains $10^6$ Ag atoms) and their subsequent ASV determination. However, to obtain these ions the metal nanoparticle needs to be dissolved first. It involves an additional analytical step that often needs the use of toxic or concentrated chemical reagents. For instance the oxidative dissolution of gold nanoparticles requires $HBr/Br_2$ solution [5-7]. In case of copper and silver tags, the dissolution involves less severe condition since both silver and copper can be easily oxidised to soluble ions with nitric acid [8-13]. Even so, $HNO_3$ is neither practical nor desirable in hand-held point of care devices.

For the assay described in this presentation we developed a novel strategy that does not require any extra oxidants to dissolve silver metal and in that way offering advantages for applications in terms of fast response, reduced cost of analysis and procedural simplicity.

This dissolution process engages silver nanoparticles being dragged towards the surface of positively charged carbon electrode (Fig. 3). Once the metal is in direct contact with electrode surface, the positive electrode potential transforms silver nanoparticles into silver ions $Ag^+$, whereas the $SCN^-$ anion assists the silver dissolution in coordinating the generated $Ag^+$ ion with formation of a strong complex $Ag(SCN)_n^{1-n}$.

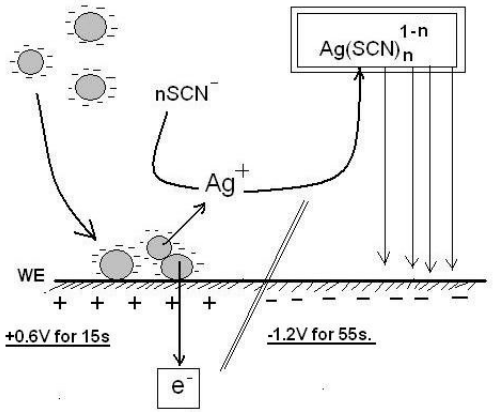


Fig. 3. The proposed mechanism of electrochemical behaviour of silver nanoparticle on the surface of carbon working electrode (WE) during the first step of ASV (pre-treatment).

The influence of the applied potential value and its duration as well as $SCN^-$ molarity was investigated and optimised. As an example figure 4 shows the potential value influence on the dissolution of silver nanoparticles in 1 M $NH_4SCN$ solution. We found out that the dissolution process is the most efficient at +0.6 V in 1 M $NH_4SCN$ and that 15 seconds in enough to dissolve all nanoparticles at that conditions.

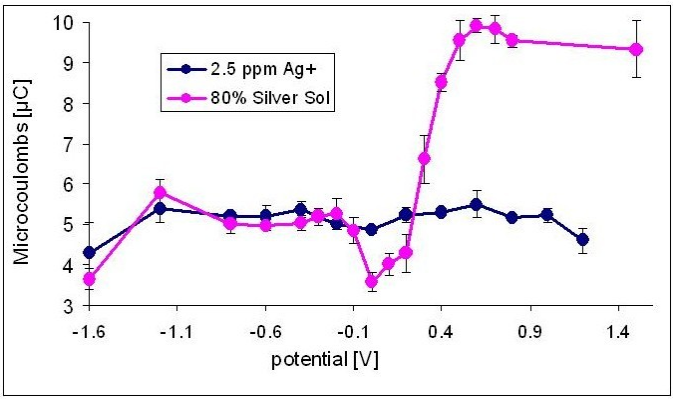


Fig. 4. The influence of pre-treatment potential (t=10s) on current signal. The signal from 2.5 ppm $AgNO_3$ is potential-independent; it affects only the silver sol, indicating that the pre-treatment potential is an important factor of silver nanoparticle dissolution.

The electrochemical dissolution and detection of silver nanoparticles, combined with the successful immobilisation of antibodies on their surface allowed us to perform an immunoassay, initially in 96-well plate format (similarly to ELISA). The calibration plot of myoglobin concentration versus electrochemical signal is presented in Fig. 5.

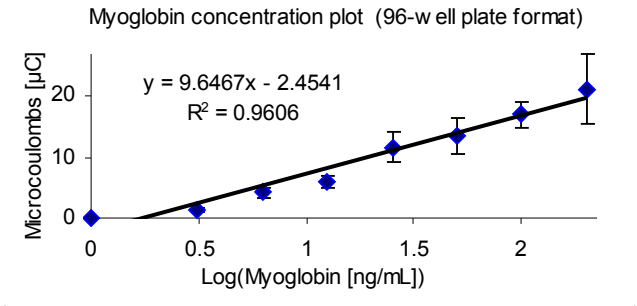


Fig. 5. The results of electrochemical immunoassay for myoglobin conducted in 96-well plate.

It can be clearly seen that the assay works well with the lowest detection limit of myoglobin - 3 ng/mL. We are not, however, really interested in doing the assay in 96-well plate format. This was only done to check the activity of the produced silver conjugates and if that conjugate can be detected electrochemically after the immunoreaction had taken place. Since it was successful we could go further, to do the assay with magnetic beads as a solid/capture phase and in the future within the microfluidics device.

The electrochemical immunoassay with magnetic beads as a solid phase is simply done by mixing magnetic particles with the sample with various analyte concentrations and then after short incubation the silver nanoparticle probe is added. (Alternatively both magnetic and silver conjugates might be mixed together first and then the sample added. However, this approach may induce the hook effect). The immunocomplex is formed (Fig. 1) and with the use of a magnet it can be separated from the sample and any unbound silver conjugates. The bound silver nanoparticles are then detected with ASV. The result of the assay is presented in Fig 6.

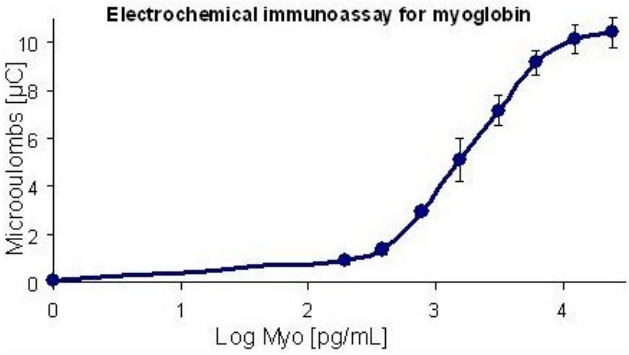


Fig. 6. The calibration plot of myoglobin concentration versus electrochemical signal. Magnetic particles used as a solid phase.

The examples of signals we obtain are shown in Fig. 7. We calculate the area under the oxidation peak of silver that is directly proportional to the analyte concentration.

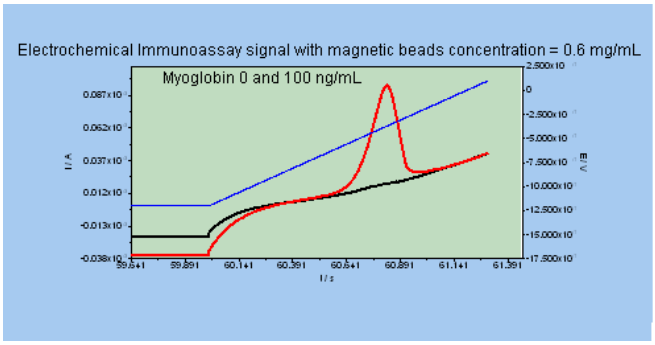


Fig. 7. Anodic stripping voltammogram of silver in the absence (black) and presence (red) of 100 ng/mL myoglobin. Magnetic particles used as a solid phase.

We can see that the assay works very well, with the lowest detection of 0.2 ng/mL. This is much lower than the same assay conducted in 96-well plate (see fig. 2). The lower detection limit is mainly due to the increased surface area of the solid phase and the amount of capture antibody immobilised on it – 0.5 µg per well and 4 µg per 0.1 mg of magnetic beads (the amount used in one sample/tube), assuming 100% immobilisation effectiveness. Moreover, the fact that magnetic particles are suspended in the whole sample volume, the contact phase is greatly increased which allowed for the shorter incubation time.

It often happens that the assay works in the buffer but when the system is moved to real samples it does not. It might be due to many reasons like non-specific binding or interferences from other molecules present in the sample. The electrochemical immunoassay for myoglobin is most likely to be used on blood samples. In order to test the behaviour/performance of the assay in real samples we

prepared a series of know dilutions of myoglobin in myoglobin-free serum. We used these samples to obtain calibration plot of myoglobin concentration in serum and we compared it with the results of the assay run just in buffer (Fig. 8).

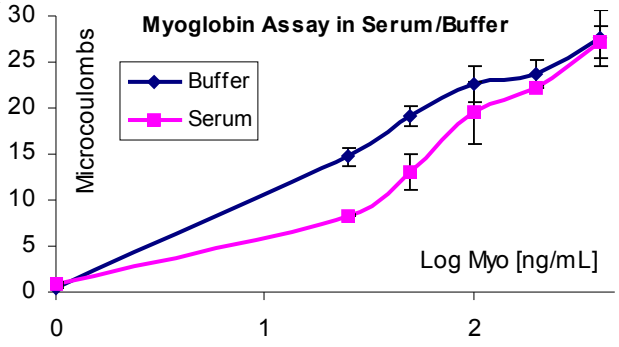


Fig. 8. Comparison of the immunoassay for myoglobin performed in buffer and serum.

The signals for myoglobin is serum samples were lower than in buffer, yet still high enough to perform the assay. It is unsure why the response is lower; nevertheless, there is a possibility that other proteins present in the serum may bind/ interact with myoglobin, blocking the epitopes for antibodies.

Normal serum myoglobin levels range from 30 to 90 ng/ mL. After 1 hour of the onset of myocardial infarction, serum myoglobin level can raise to 200 ng/mL or even higher. During the peak hour, myoglobin level can be as high as 900 ng/mL [14]. The myoglobin concentration range we covered in this experiment (Fig. 8) was from 25 to 400 ng/mL, proving that the assay is capable of working in the range of interest. Moreover, the volume of serum sample was only 5 µL. It is quite important as lower volume samples improves patients' comfort and reduces the stress of analysis.

## 4. CONCLUSIONS

The described electrochemical immunoassay is a very promising platform technology. Combined with microfluidics channels and portable electrochemical reader it can be applied to build a point of care medical device.

There are also other electrochemical immunoassays but as far as we are aware this is the first immunoassay that utilises silver nanoparticles for the protein detection. The proposed approach for silver dissolution on the surface of carbon electrode removed the need of using harsh oxidant. It is very advantageous in terms of point of care tests. We applied this technology for the determination of cardiac markers - troponin and myoglobin, but it can be applied to any protein including other diagnostic markers, to veterinary or food quality measurements or even for environmental measurements in the field.

The future work will include performing this assay in duplexing or even multiplexing format. The antibodies for different cardiac markers can be immobilised on different metal nanoparticles (gold, copper, silver). As the oxidation peak of these metals occur at different potential, the signal from each type of nanoparticle can be easily distinguished. This will allow for simultaneous detection of a number of analytes.

## ACKNOWLEDGMENTS

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