ELECTRONIC SUPPORTING INFORMATION

Recognition of anti-mycolic acid antibody at self-assembled mycolic acid antigens on gold electrode: A potential impedimetric immunosensing platform for active tuberculosis†

Nsovo S. Mathebula,[a] Jeseelan Pillay[a], Gianna Toschi[a], Jan A. Verschoor[b] and Kenneth I. Ozoemena*[a,c]

Experimental section

Cysteamine (~95% pure) and stearic acid were obtained from Sigma and Hopkin and Williams Ltd (Great Britain). Saponin was obtained from Sigma. N, N-Dimethylformamide (DMF) was purchased from Applied Biosystems(a division of Perkin-Elmer, Great Britain), was dried and distilled before use. Phosphatidylcholine (PC) used for preparing liposomes was obtained from Sigma. Mycobacterial mycolic acids (MA) were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as previously described.[1] Human sera (one tuberculosis-positive (TB^+) and one tuberculosis-negative (TB^-) as a control), which are part of a collection for another study[2] from the general medical wards of the Helen Joseph Hospital, Johannesburg (South Africa) were used. The TB^+ serum was from a patient with newly-diagnosed smear-positive active pulmonary tuberculosis who was not on anti-TB chemotherapy at the time of serum collection. The TB^- patient had a medical condition other than TB.

Phosphate-buffered saline containing sodium azide (0.025%, m/v) and 1 mM EDTA (PBS/AE, pH 7.4) was prepared with appropriate amounts of Na_2HPO_4 (7.5 mM), KH_2PO_4 (1.5 mM), NaCl (0.14 M) and KCl (2.5 mM). Dicyclohexylcarbodiimide (DCC 99% pure) used as condensing agent was obtained from Aldrich. Ultrapure water of resistivity 18.2 MΩcm was obtained from a Milli-Q Water System (Millipore Corp., Bedford, MA, USA) and was used throughout for the preparation of solutions. All electrochemical experiments were performed with nitrogen-saturated PBS/AE. All other reagents were of analytical grade and were used as received from the suppliers.

All electrochemical experiments were carried out using an Autolab Potentiostat PGSTAT 302 (Eco-Chemie, Utrecht, Netherlands) driven by the GPES and FRA softwares version 4.9). Electrochemical impedance spectroscopy (EIS) measurements were performed between 1.0 Hz and 10 kHz using a 5 mV rms sinusoidal modulation in PBS/AE solution of 1 mM $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl, and at the $E_{1/2}$ of the $[Fe(CN)_6]^{3-/4-}$ (0.27 V vs. Ag|AgCl, sat'd KCl). Polycrystalline gold electrode (BAS, r = 0.8 mm) was used as the working electrode. Ag|AgCl sat'd KCl and platinum rod were used as reference and counter electrodes, respectively. All solutions were de-aerated by bubbling pure nitrogen (Afrox) prior to each electrochemical experiment. All experiments were performed at 25±1°C.

AFM images were obtained at SAM-modified SPR gold disks (Eco-Chemie) using an AFM 5100 System (Agilent Technologies, AC mode AFM scanner interfaced with a PicoScan controller, scan range 1.25 μm in x–y and 2.322 μm in z, silicon type PPP-NCH-20 (Nanosensors®) of thickness 4.0±1.0 μm, length 125±10 μm, width 30±7.5 μm, spring constants 10 – 130 N m^{-1}, resonant frequencies of 204 – 497 kHz and tip height of 10-15 μm). All images (256 samples/line × 256 lines) were taken in air at room temperature and at scan rates 0.9–1.0 lines s^{-1}. The X-ray photoelectron spectra (XPS) were obtained at SAM-modified SPR gold disks using a Physical Electronics model 5400 spectrometer system with monochromatic Mg K α radiation at 1253.6 eV at take-off angles of 15° and 45°.

The cleaning of the gold electrode and the subsequent formation of the cysteamine monolayer followed the previous reports.[3,4,5] The stearic acid was attached onto the cysteamine monolayer to form the Au-MEODA SAM by covalent attachment of the stearic acid using the well established carbodiimide coupling chemistry in 8.4 mM M Stearic acid DMF/DCC solution to form an amide bond.[6] After the formation of the Au-MEODA SAM, the modified electrode was thoroughly rinsed in copious amount of distilled deionised water and ethanol to remove physically adsorbed stearic acid species. The MA antigens were integrated into the MEODA SAM (to form the Au-MEODA-MA) by incubating the electrode in dry DMF solution of MA (0.5 mg mL^{-1}) for about 48 h at room temperature. After the immobilisation of the MA antigens, the electrode was rinsed several times with PBS/AE solution (pH 7.4) to remove the excess of physically bound MA species and immersed in 1.5 mg mL^{-1} PBS/AE solution of Saponin for 10 min to block nonspecific binding sites.

First, liposomes with and without mycolic acids (MA) were prepared from a phosphatidylcholine (PC)/$CHCl_3$ stock solution (100 mg/ml) as previously reported.[7,8] Briefly, MA-containing liposomes were prepared by mixing 90 μL of PC/$CHCl_3$ stock solution (100 mg/ml) with 1 mg of dried mycolic acids. Empty liposomes (i.e., without MA) were prepared consisting of only PC/$CHCl_3$ solution. The PC/$CHCl_3$ contents in an amber glass vial were initially vortexed to ensure thorough mixing, dried at 85 °C (using a heat block) under a stream of pure nitrogen gas for

about 10 min. Liposome formation was induced by addition of 2 ml of saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated using Branson sonifier (Model B-30, Branson Sonifier Co., USA) for 2 min at 30% duty cycle at an output of 3%. Subsequently, the liposomes were divided into 200 µl aliquots, freeze-dried and stored at –70 ^{o}C ready for use. Before use, the liposomes were reconstituted with 2 ml of PBS/AE (pH 7.4), heated at 80 ^{o}C for 20 min and then sonicated as before. The final liposome concentration was 500 µg/ml.

Human sera (HIV^{+}/TB^{+} and HIV^{-}/TB^{-}) were appropriately diluted in empty liposome PBS/AE (pH 7.4) (1:2000; 1:1000 and 1:500 v/v, i.e., 0.05, 0.1 and 0.2% serum, respectively). For control experiments, a similar procedure was followed using liposomes containing MA. The modified electrode (working electrode) was incubated in the required serum solution for 10 min, rinsed in a copious amount of PBS/AE (pH 7.4) to remove any physically adsorbed species before performing the EIS experiment in a test solution of 1 mM $[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$ (PBS/AE, pH 7.4) at a bias potential of 0.27 V (vs Ag|AgCl, sat'd KCl).

Fitting the impedance spectra

The impedance spectra of the Au-MEODA-MA/SAP before and after interaction with the HIV^{+}/TB^{+} patient or the HIV^{-}/TB^{-} patient sera were fitted to extract the actual charge transfer resistance (Rct) arising from the respective semi-circular arc. The semi-circular arcs exhibited non-ideal behaviour (i.e., departure from a single arc centered on the real axis of Z'), thus every attempt to fit their spectra with the Randles circuit or a one-reaction RC time-constant circuit was unsuccessful as they led to very large fitting error values. The data suggest that the impedance spectra are influenced by distributed-time-constant phenomena.[9] The spectra of the Au-MEODA-MA/SAP were satisfactorily fitted with equivalent electrical circuit models comprising Randles circuit and Voigt circuit of two RC elements in series, involving the electrolyte resistance (R_s), electron-transfer resistance (R_{ct}), constant phase element (CPE) due to the inherent roughness of the electrode, double layer capacitance (C_{dl}) and Warburg-type impedance (Z_w) which is associated with the diffusion of the redox probe ions. The spectra for the HIV^{-}/TB^{-} patient serum (Figure SI 2) and its control experiments were fitted the same circuit as for the Au-MEODA-MA/SAP. Interestingly, spectra resulting from the interaction of the Au-MEODA-MAP/SAP with HIV^{+}/TB^{+} patient serum dilutions were best fitted using Voigt circuit model comprising four RC elements in series with a solution resistance (R_s). The absence of the diffusion parameter (Z_w) in the Au-MEODA-MAP/SAP-HIV^{+}/TB^{+} patient sera, clearly suggesting complete blockage of the redox probe from reaching the underlying Au electrode. In this communication, the most relevant parameter is the R_{ct} values, the total of which is the sum of individual R_{ct} values in series. Detailed analysis of the implications of the impedimetric data is necessary and will be the subject of next investigation.

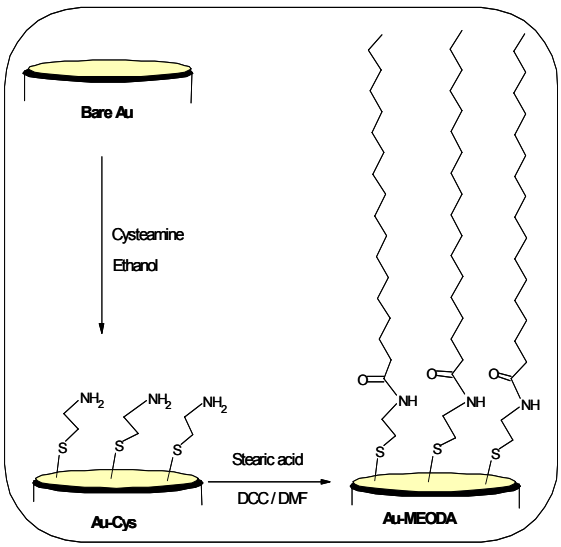
Notes and References:

The liposomes resemble the way that mycolic acids are transported in the blood circulation of the patient. By presenting the mycolic acid antigen in liposomes and performing an inhibition of binding of antibodies, the accuracy of detection could be increase by around 30% (see ref 7) compared to direct binding of antibodies from serum to surface-confined mycolic acids.

***Safety Note**: All glassware in contact with human sera (both TB positive and negative) must be sterilized by autoclaving for 20 min at 115 ^{o}C after use. Waste solutions must be carefully collected and sterilized before disposal. Intending workers must ensure they are vaccinated against hepatitis B.*

[a] *Department of Chemistry, University of Pretoria, South Africa.*
[b] *Department of Biochemistry, University of Pretoria, South Africa.*
[c] *Energy & Processes Unit, Materials Sciences & Manufacturing, Council for Scientific and Industrial Research (CSIR), Pretoria, Pretoria 0001, South Africa. Tel: +27-12-8413664; Fax: +27-12-8412135; E-mail: kozoemena@csir.co.za*

1. M.A. Goodrum, D.G.R. Siko, T. Niehues, D. Eichelbauer and J.A. Verschoor, *Microbios* 2001, **106**, 55.
2. G.K. Schleicher, C. Feldman, Y. Vermaak and J.A. Verschoor, *Clin. Chem. Lab. Med.* 2002, **40**, 882.
3. B.O. Agboola, K.I. Ozoemena, Phys.Chem.Chem.Phys. 2008, **10**, 2399.
4. Duduzile Nkosi, Kenneth I. Ozoemena, *J. Electroanal. Chem.* 2008, **621**, 304.
5. Duduzile Nkosi, Kenneth I. Ozoemena, *Electrochim. Acta* 2008, **53**, 2782.
6. J.J. Gooding, R. Wibowo, J. Liu, W. Yang, D. Losic, S. Orbons, F.J. Meams, J.G. Shapter and D.B. Hibbert, *J. Am. Chem. Soc.* 2003, **125**, 9006.
7. S.T. Thanyani, V.Roberts, D.G.R. Siko, P. Vrey, J.A. Verschoor, *J. Immunol. Methods* 2008, **332**, 61.
8. Y. Benadie, M. Deysel, D. G. R. Siko, V. V. Roberts, S. Van Wyngaardt, S.T. Thanyani, G. Sekanka, A.M.C. Ten Bokum, L.A. Collett, J. Grooten, M.S. Baird, J.A. Verschoor, *Chem. Phys. Lipids* 2008, **152**, 95.
9. M.E. Orazem, B. Tribollet, Electrochemical Impedance Spectroscopy, John Wiley & Sons Inc, Hoboken, NJ., 2008, Chapter 13.



Scheme SI: Schematic representation of the self-assembly process for the formation of the Au-MEODA

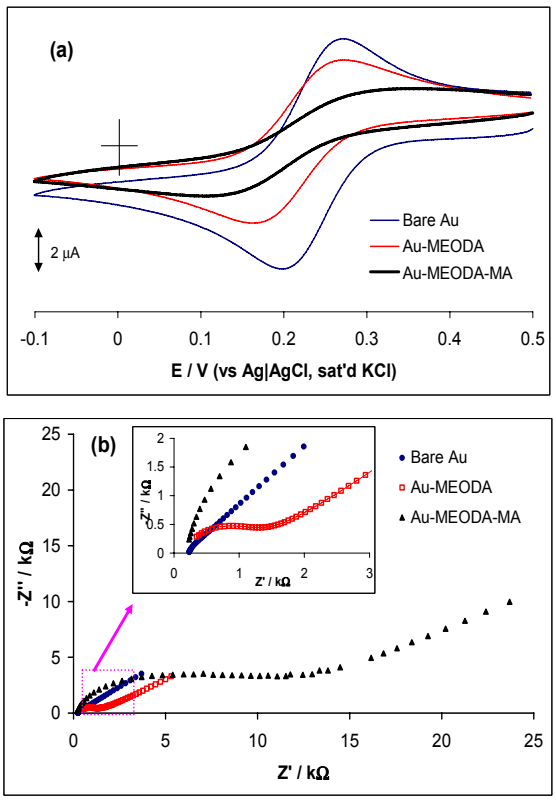


Figure SI 1: Typical comparative CV and EIS evolutions of the bare Au, Au-MEODA and Au-MEODA-MA in 1 mM $[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$ (PBS/AE, pH 7.4). Potential bias = 0.27 V (vs Ag|AgCl, sat'd KCl). Scan rate = 50 mVs^{-1}.

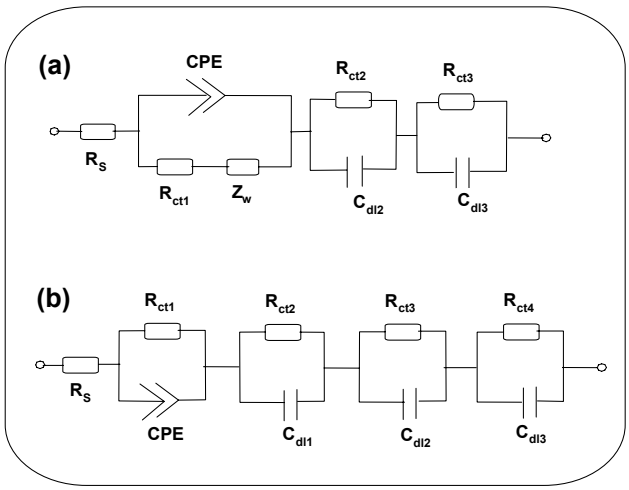


Figure SI 2: Equivalent circuit model used to fit the impedance spectra of the Au-MEODA-MA-SAP electrode before (a) and after interaction with different dilutions of the HIV^+/TB^+ patient serum. Circuit (a) was also used to fit different dilutions of the HIV^-/TB^- patient serum.

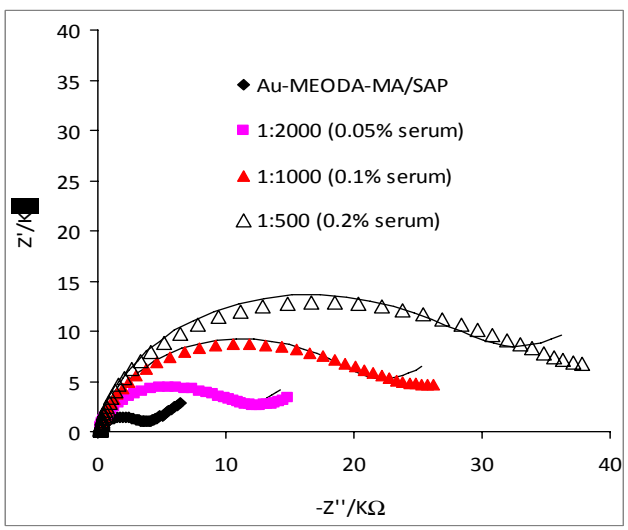


Figure SI 3: Typical serum concentration-dependent Nyquist plots of Au-MEODA-MA-SAP electrode incubated in human TB negative serum. The serum was diluted at 1:500 (0.20% serum); 1:1000 (0.10% serum) and 1:2000 (0.05% serum). The symbols represent the experimental data, while solid lines are fitted curves using equivalent circuit Figure SI 2a. Test solution: 1 mM $[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$ (PBS/AE, pH 7.4); Potential bias = 0.27 V (vs Ag|AgCl, sat'd KCl).

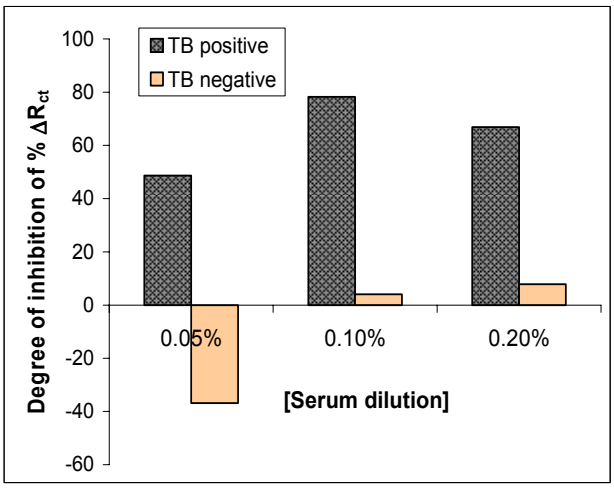


Figure SI 4: Comparative bar chart representation of the degree of inhibition of the impedimetric detection signal recorded at the Au-MEODA-MA-SAP electrode for human TB positive and negative sera.

Binding of self-assembled mycolic acid antigens with anti-mycolic acid antibodies in tuberculosis patient's blood serum, co-infected with HIV, can be conveniently monitored by impedance spectroscopy, a crucial step towards developing an electrochemical binding assay for active tuberculosis in HIV-infected patients.