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Article

Fingerprinting Breath: Electrochemical Monitoring of Markers Indicative of Bacteria *Mycobacterium tuberculosis* Infection

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Recentemente demonstrou-se que marcadores químicos no ar inalado/exalado podem fornecer uma metodologia para a detecção de infecção de tuberculose. Esses marcadores consistem em metil fenilacetato, metil *p*-anisato, metil nicotinato e *o*-fenilanisol (2-metoxibifenil). As abordagens atuais usam cromatografia gasosa com detector de massas (GCMS) que são úteis para testes respiratórios centralizados. A Organização Mundial de Saúde (WHO) exige uma ferramenta de diagnóstico que seja portátil e não invasiva para analisar tuberculose. Para cumprir com essa exigência, demostra-se uma etapa no desenvolvimento na identificação analítica de marcadores químicos em soluções aquosas usando tecnologia baseada em eletroquímica. Demonstra-se que sensores eletroquímicos *screen-printed* podem ser usados como base da ferramenta de diagnóstico para a caracterização eletroquímica respiratória dos marcadores químicos (metil nicotinato e 2-metoxibifenil) úteis na detecção de tuberculose. Espera-se que um desenvolvimento futuro facilite a construção de uma ferramenta de diagnóstico respiratório que seja portátil e não invasivo.

Recently it has been shown that chemical markers in exhaled air/breath can provide a methodology for the detection of tuberculosis infection. These markers consist of methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and *o*-phenylanisole (2-methoxybiphenyl). Current approaches utilise gas chromatography-mass spectrometry (GCMS) which are useful for centralised testing of breath samples. The World Health Organization (WHO) require a portable, non-invasive diagnostic tool for the screening of tuberculosis infection. In order to meet this, we demonstrate proof-of-concept for the analytical sensing of the identified chemical markers in aqueous solutions using electrochemical based technology. We demonstrate that screen-printed electrochemical sensors can be used as the basis of a diagnostic tool for the electrochemical breathprinting of chemical markers (methyl nicotinate and 2-methoxybiphenyl) useful for the screening of tuberculosis infection. It is hoped that further development will facilitate the potential for a portable, hand-held, non-invasive breath diagnostic tool to be realised.

Keywords: screen-printed electrodes, electrochemistry, mycobacterium tuberculosis, electrochemical breathprints

Introduction

Tuberculosis (TB) is familiar to most as a disease which has plagued global society for time in memoriam. Although generally regarded, by the western world at least, as less of a threat than it once was, this is a misconception as global incidence rates still run into the millions of cases *per annum*. For instance, the global recorded incidence rate in 2008 was 9.3 million, roughly equivalent to the total population of Sweden; with the highest incidence of TB being in South-east Asia and Africa.[1]

Tuberculosis is a disease generally caused by the bacteria *Mycobacterium tuberculosis* (MTB). The disease has the ability to manifest in any part of the body, however, the lungs are the most common reservoir for the bacteria.[2,3] MTB is an airborne bacteria, and hence spreads rapidly from one person to another through coughing, sneezing and even simply talking. More specifically, transfer occurs through the inhalation of bacterium containing droplets in the air. A single cough generates around 3000 droplet

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nuclei, each containing around three bacilli, with droplets generated through sneezing being able to spread a distance of 10 feet. As the disease is airborne, contamination is generally via inhalation and the disease typically manifests upon droplet nuclei reaching the alveoli.[2,3]

Tuberculosis is notorious for progressing very slowly, and exhibiting only the vaguest of symptoms. It is often missed or misdiagnosed for a long period of time. One explanation for the regular delay in diagnosis is the extended period of time required for confirmatory tests of disease infection to be completed. Some test methods require a period of some of up to eight weeks to confirm diagnosis.[1] However, in view of the serious nature of this disease, it is imperative for diagnosis and treatment to be carried out as soon as practicable, with the aim of not only reducing mortality rates for those infected, but also to reduce contamination within the populous in general.

Many hours have been spent in the search for improved methods for the treatment and prevention of tuberculosis related infection and consequential disease, although despite this research a rapid, accurate method of tuberculosis detection has not been found. None of the available methods of tuberculosis detection are quick and easy to use. As noted above, rapid diagnosis of infection is key to successful treatment and the ultimate goal of survival. Rapid diagnosis also enables more efficient inhibition of the spread of infection within a given population, enabling those determined to have been infected to be isolated. Sadly, the majority of infections occur in countries with very low *per capita* income and a deficient health care. Given such circumstances, it is vital to develop a test, which is accurate, fast and inexpensive. In this way, it would be possible to medicate the patients in an early state of the infection and prevent the further spreading of the disease.[4,5]

When tuberculosis becomes active, 70-75% of cases manifest as pulmonary tuberculosis which infects the lungs and the symptoms are well known and documented. The other 25-30% of cases are termed extrapulmonary tuberculosis; these are cases where the bacteria infects other parts of the body. In the latter case, diagnosis of extrapulmonary tuberculosis can be performed by point-of-care based tests for measurement in blood or urine. However, such tests are only available for the roughly one quarter of cases which are extrapulmonary, and a point-of-care test is required for pulmonary tuberculosis.[6,7]

In the case of pulmonary tuberculosis, diagnosis is currently often undertaken using a chest X-ray combined with the microscopic analysis of sputum samples.[1] Given that the majority of incidence and mortality from tuberculosis is in Asia and Africa, often in remote locations where there is no access to equipment or appropriately trained personnel, this diagnostic approach is not favourable. In addition, X-ray methods have the distinct disadvantage, that areas which appear to be infected may appear cloudy or discoloured for other reasons. For instance, misdiagnosis could result as a result of pre-existing lung tissue scarring.[1,6]

Other diagnostic methods include acid fast smearing, T-spot, culturing of samples and biopsy.[8] However, each of these methods suffers from either the need for specialised personnel, a slow diagnosis time or a lack of precision in results.[1,6] The application of light waves or molecular amplifications to samples has also been tested, providing unsatisfactory results. Whilst light based techniques are rapid, portability is a problem; with molecular amplifications, the primary disadvantage is one of cost, although the reliability of the technique has also been questioned.[9] Evidently, methodologies for detection exist though in many cases, such as those involving the incorporation of nanoparticles combined with specific biomolecules and immunosensing[10-13] in addition to other techniques disclosed earlier, suffer from critical drawbacks when considering the most affected demographics of society; those in areas of limited healthcare and funding. These drawbacks include, but are not limited to, excessive financial implications, the requirement for skilled workforce, excessive result time and invasive procedures. Evidently, there is a real necessity to devise a method which alleviates if not all, then the majority of these factors, for example, as has been demonstrated for the monitoring of glucose,[14] the development of an electrochemical point-of-care device.

Since pulmonary tuberculosis affects the lungs, research has been carried out looking for chemical markers in exhaled breath.[3,15] In particular, volatile organic carbon compounds have been researched.[3,15] However, it is practically impossible to distinguish the volatile organics which are indicative of the presence of tuberculosis, from those which occur naturally in healthy individuals.[15] Chambers and his team have demonstrated that there are 4 chemical markers in breath that can act as useful indicators of tuberculosis (*Mycobacterium tuberculosis*) infection.[3] The chemical markers have been identified to be: methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and *o*-phenylanisole (2-methoxybiphenyl).[3] This pioneering work has been conducted using gas chromatography-mass spectroscopy (GCMS) and recently sample collection and preconcentration has been explored.[16] Such an approach will allow the testing of breath at centralized points such as clinics and/or hospitals. In order to fight tuberculosis, the World Health Organisation (WHO) require a rapid, sensitive, inexpensive, portable and non-invasive diagnostic tool.[3,9] One approach that is well proven to meet such requirements as those presented by a point-of-care clinical

device is electrochemical sensing strategies based upon disposable, one-shot screen-printed sensors[17-19] which are both effective and affordable. The ability to capitalise on such disposable devices helps to reduce the risk of infection presented by the utilisation of a single device for a large number of patients.

In this paper we explore, for the first time, the potential electrochemical determination of the proposed markers (methyl phenyl acetate, methyl *p*-anisate, methyl nicotinate and 2-methoxybiphenyl) in aqueous solutions which are indicative of *Mycobacterium tuberculosis* infection in exhaled air/breath using electrochemical based sensing technology. Due to the use of unmodified screen-printed sensors, the opportunity exists to develop an elegantly simplistic, portable and non-invasive approach for electrochemical breathprinting.

Experimental

All chemicals were of the highest grade available and were used as received (without further purification) from Sigma Aldrich (UK). All solutions were prepared using deionised water of resistivity no less than 18.2 MΩ cm and were vigorously degassed prior to electrochemical measurements with high purity, oxygen free nitrogen. Voltammetric measurements were carried out using an μ-Autolab III (ECO-Chemie, The Netherlands) potentiostat. All measurements were conducted using screen-printed three electrode configurations (see below) with a geometric working electrode area of 3 mm diameter. All measurements of the highlighted markers were carried out in solution using liquid standards. For measurements involving carbon dioxide and oxygen the solution was saturated with the required gas via vigorously bubbling the solution for 20 min prior to electrochemical measurement.

Screen-printed carbon-based electrodes (denoted as SPEs) were fabricated in-house with appropriate stencil designs using a microDEK 1760RS screen-printing machine (DEK, Weymouth, UK) as depicted in Figure 1. Note that this screen-printed electrode design has been previously reported[20-24] "as is" without electrode pre-treatment or modification in various electroanalytical endeavours. For fabrication of the SPEs, first a carbon ink formulation (Product Code: C2000802P2; Gwent Electronic Materials Ltd, UK) utilised for the efficient connection of all three electrodes and the electrode material for both the working and counter electrodes was screen-printed onto a polyester (Autostat, 250 micron thickness) flexible film. The carbon ink layer was cured in a fan oven at 60 °C for 30 min. Next, a silver/silver chloride reference electrode was included by screen-printing Ag/AgCl paste (Product Code: C2040308P2; Gwent Electronic Materials Ltd, UK) onto the polyester substrates which was subsequently cured once more in a fan oven at 60 °C for 30 min. Finally, a dielectric paste (Product Code: D2070423P5; Gwent Electronic Materials Ltd, UK) was then printed onto the polyester substrate to cover the connections and define the active electrode areas including that of the working electrode (3 mm diameter). After curing at 60 °C for 30 min, the SPEs are ready to be used. These electrodes have been characterised electrochemically in a prior paper and have heterogeneous rate constants of 1.08×10^{-3} cm s^{-1}.[25]

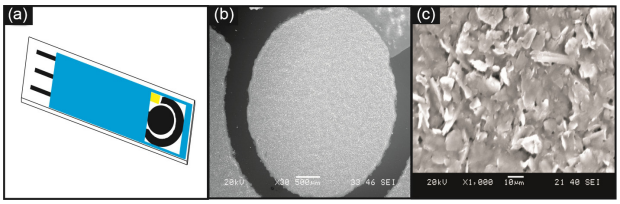

Figure 1. (a) Schematic diagram and (b) and (c) SEM images at increasing magnifications of a graphite screen-printed sensor (SPE).

The reproducibility of the fabricated batches of electrodes were explored through comparison of cyclic voltammetric responses using 1 mmol L^{-1} hexaammine-ruthenium (III) chloride/0.1 mol L^{-1} KCl. Analysis of the voltammetric data revealed the % relative standard deviation to correspond to no greater than 0.82 % ($N = 20$) for the fabricated SPEs highlighting the reproducibility of the fabricated electrodes and their use in electroanalysis.

Results and Discussion

First consideration was given to exploring the identified chemical markers[3,15] indicative of TB infection, namely: methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and 2-methoxybiphenyl. A blank solution of a pH 7 phosphate buffer solution (PBS) was used, each biomarker added and the cyclic voltammetric response determined through analysis using cyclic voltammetry using screen-printed electrodes. It was apparent upon initial studies that of the four markers proposed (methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and 2-methoxybiphenyl) only methyl nicotinate and 2-methoxybiphenyl were electrochemically active within the accessible voltammetric window. The observed voltammetric responses for both methyl nicotinate and 2-methoxybiphenyl are shown in Figures 2 and 3, respectively. For the case of methyl nicotinate (Figure 2) a single oxidative peak was noted at a potential of ca. –0.45 (*vs.* Ag/AgCl) with a reduction peak evident in the blank. The electrochemical interrogation of 2-methoxybiphenyl (Figure 3) revealed the presence an oxidative peak at a potential of ca. +0.40 V (*vs*. Ag/AgCl)

complimented with a reduction peak at a potential of ca. –0.40 V (*vs*. Ag/AgCl). Note for further electrochemical investigation the oxidation peak at a potential of ca. +0.45 V (*vs*. Ag/AgCl) was utilised for methyl nicotinate analysis while that at ca. +0.40 V (*vs*. Ag/AgCl) was monitored when studying 2-methoxybiphenyl.

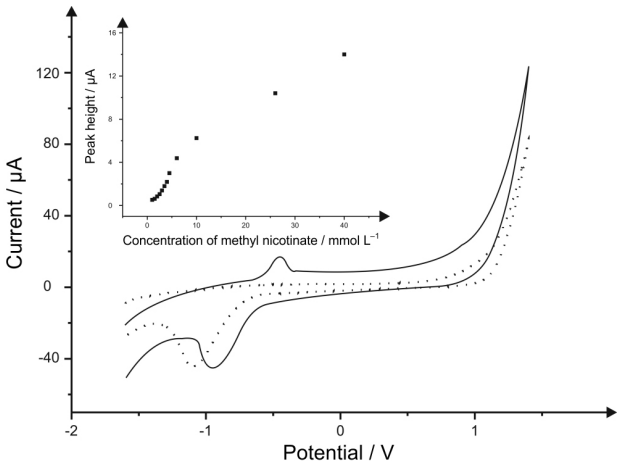

Figure 2. Typical cyclic voltammograms recorded in the absence (dotted line) and presence (solid line) of methyl nicotinate (25 mmol L^{-1}) in a pH 7 phosphate buffer solution using a screen-printed electrode. Scan rate: 100 mV s^{-1} (*vs*. Ag/AgCl). Inset: A typical corresponding calibration plot result from additions over the range 1 to 40 mmol L^{-1}.

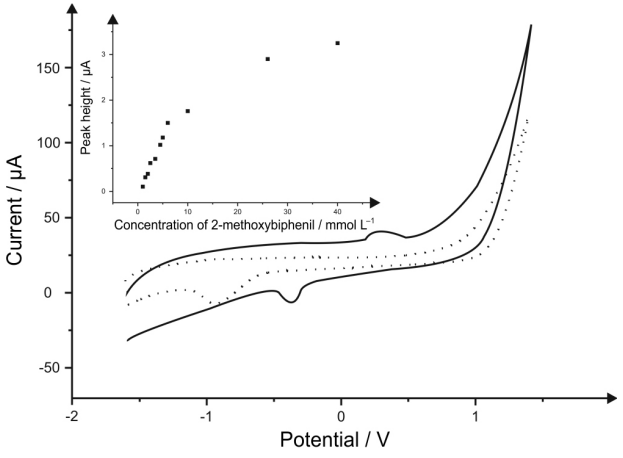

Figure 3. Typical cyclic voltammograms recorded in the absence (dotted line) and presence (solid line) of 2-methoxybiphenyl (40 mmol L^{-1}) in a pH 7 phosphate buffer solution using a screen-printed electrode. Scan rate: 100 mV s^{-1} (*vs*. Ag/AgCl). Inset: a typical corresponding calibration plot result from additions over the range 1 to 40 mmol L^{-1}.

Calibration plots for each of the compounds were constructed in order to assess the feasibility and sensitivity of detection of methyl nicotinate and 2-methoxybiphenyl (Figures 2 and 3 inset, respectively) using the screen-printed electrochemical sensors. Using cyclic voltammetry the detection of methyl nicotinate and 2-methoxybiphenyl in pH 7 PBS was found to be possible at concentrations ranging from 1 to 40 mmol L^{-1}. Analysis of the voltammetric peak height (I_p) as a function of concentration revealed a linear range over the entire concentration range explored for methyl nicotinate ($I_p/A = 3.24 \times 10^{-7}\ A$ / mmol L^{-1} + $1.46 \times 10^{-6}\ A$; $R^2 = 0.98$; $N = 15$), while 2-methoxybiphenyl was observed to exhibit linearity up to a concentration of 25 mmol L^{-1} ($I_p/A = 1.17 \times 10^{-7}\ A$ / mmol L^{-1} + $2.33 \times 10^{-7}\ A$; $R^2 = 0.98$; $N = 14$) beyond which the response begins to plateau; the exact origin of this is unknown but likely due to saturation of the electrode surface or phenomena of adsorption of the reaction product on the surface of the electrode also can be considered.

The effect of pH upon the ability of the sensor to detect biomarkers was also studied. Detection of 2-methyoxybiphenyl at pH's of 2.5, 7.3, 12 was attempted. It was noted that successful determination of the analyte was possible over the entire pH range explored. Clearly such findings, across a wide range of pH values, indicate a good tolerance to sensor storage and pH change potentially caused by other components of the breath, for example carbon dioxide which is a major component of exhaled air/breath. The electrochemical reduction of CO_2 is possible at carbon electrodes but usually employing an excessively high overpotential. Nevertheless, to ensure that the presence of CO_2 within breath will not detrimental to the observations made above, analysis of a 40 mmol L^{-1} solution of 2-methoxybiphenyl in the presence of CO_2 (not shown) was explored. The presence of CO_2 was found to not affect the observed response for 2-methoxybiphenyl in anyway. Additionally, the electrochemical reduction of O_2 is possible at carbon electrodes, occurring at potential of ca. +0.6 V and higher depending on the electrode surface. The effect of O_2 was explored on the response (in the same manner as CO_2) where it was again found that the presence of the gas had no effect upon the electrochemical system.

Further challenges faced to make this into a truly point-of-care breath device include exploring (breath) sample collection and preconcentration systems to present the breath (gas) into a conducting solution for the electrochemistry to be conducted, or a similar approach, and also in order to align the concentrations observed aqueous solution with those reported in exhaled air/breath with the electroanalytical output observed here.

Next we turn to monitoring the two markers simultaneously; a solution containing both methyl nicotinate and 2-methyloxybiphenyl was prepared with square-wave voltammetry (SWV) was utilised to attempt to improve the sensitivity of the protocol which applies a different waveform than that of cyclic voltammetry. The SWV response is depicted in Figure 4a and demonstrates the successful determination of the two markers simultaneously within a single solution at a single screen-printed electrode.

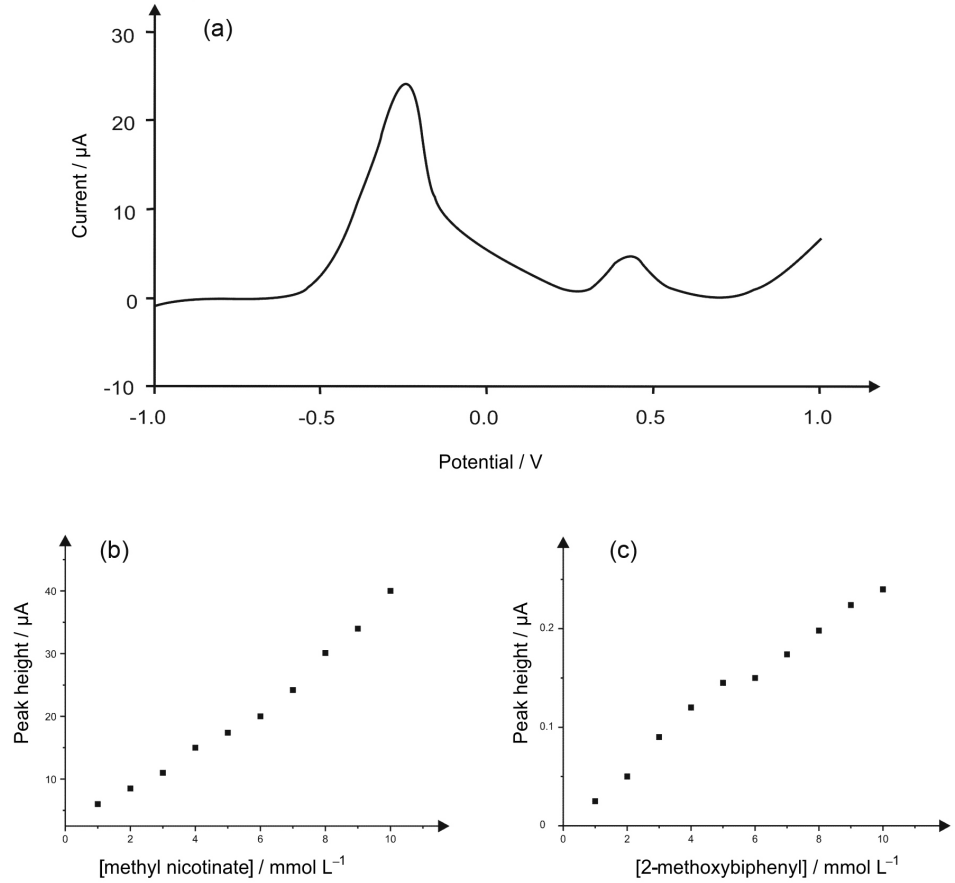

Figure 4. (a) A typical SWV recorded in a pH 7.4 phosphate buffer solution containing 4 mmol L^{-1} methyl nicotinate (ca. –0.25 V) and 4 mmol L^{-1} 2-methoxybiphenyl (ca. +0.4 V). SWV parameters: frequency 20 Hz, scan potential 10 mV and amplitude 30 mV; (b) and (c) depict calibrations plots for the simultaneous additions of methyl nicotinate and 2-methoxybiphenyl respectively over the concentration range of 1 to 10 mmol L^{-1}.

After determining that the applied system had the ability to detect the two biomarkers additions (of the solution containing both biomarkers) were made to assess the effect of increasing biomarker concentration on the observed output. The results are provided in Figure 4b and c; a clear calibration is shown for both analytes, which each exhibiting two linear responses for the concentration ranges explored. In the case of methyl nicotinate (Figure 4b) one linear range was noted between the concentrations of 1 to 6 mmol L^{-1} (I_P/A = 2.87 × 10^{-6} A / mmol L^{-1} + 2.91 × 10^{-6} A; R^2 = 0.99; N = 6) and 7 to 10 mmol L^{-1} (I_P/A = 5.13 × 10^{-6} A / mmol L^{-1} – 1.15 × 10^{-5} A; R^2 = 0.99; N = 4). Similarly, when determining the two analytes simultaneously, 2-methyloxybiphenyl (Figure 4c) exhibited one linear range between the concentrations of 1 to 5 mmol L^{-1} (I_P/A = 3.10 × 10^{-7} A / mmol L^{-1} – 7.00 × 10^{-8} A; R^2 = 0.99; N = 5) and a further linear range between 6 to 10 mmol L^{-1} (I_P/A = 2.30 × 10^{-7} A / mmol L^{-1} + 1.32 × 10^{-7} A; R^2 = 0.98; N = 5). These findings indicate that the screen-printed sensor is tolerant to a change in marker concentration. Limits of detection (3σ) of 238 and 392 µmol L^{-1} were deduced using this protocol for the simultaneous determination of methyl nicotinate and 2-methoxybiphenyl respectively.

In summary, we have shown that of the four markers proposed, only two are electrochemically active (methyl nicotinate and 2-methoxybiphenyl). These two markers have been shown to be readily determined using cost-effective, disposable and single-shot screen-printed sensors; ideal for point-of-care and clinical devices. Furthermore, the determination of the markers has been expanded to include their monitoring simultaneously within the same solution which deduced that both can be monitored down to mid-level micromolar concentrations with relative ease.

Conclusions

We have shown proof-of-concept for the simultaneous electroanalytical detection of methyl nicotinate and 2-methoxybiphenyl using screen-printed electrochemical sensors which has the potential to allow for the development of a point-of-care non-invasive breath test for the screening of tuberculosis infection. Future work involves exploring whether only two markers out of the reported four[3] are

medically useful along with exploring breath sample collection and a pre-concentration system.

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