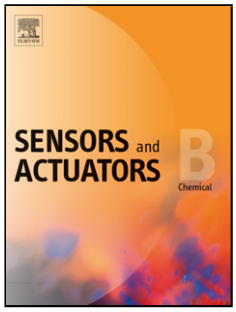

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Understanding Electroanalytical Measurements in Authentic Human Saliva Leading to the Detection of Salivary Uric Acid

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Highlights

- General features of electroanalysis in human saliva are investigated.
- Microelectrodes are shown to be suitable for measurements in saliva.
- Ferrocenemethanol and ferrocyanide oxidations were studied in undiluted saliva.
- Dissolved oxygen was detected in undiluted saliva.
- A method for the detection of salivary uric acid was developed.

Abstract

The electroanalytical responses of several redox-active species are investigated in authentic human whole saliva. First, we show that ferrocenemethanol **(**$FcCH_2OH$**)** and ferrocyanide ($[Fe(CN)_6]^{4-}$) display well-defined voltammetry in undiluted saliva at a carbon microdisc electrode without the addition of supporting electrolyte. Second, we demonstrate that dissolved oxygen is detectable in saliva. The ferrocenemethanol oxidation is shown not to be altered by the medium apart from the steady-state currents which change according to the viscosity of the solvent. In contrast, the voltammetry of ferrocyanide oxidation and oxygen reduction in authentic saliva are significantly different from those in synthetic saliva or aqueous electrolyte. It is demonstrated that the electrode is partially blocked by organic molecules or other electrochemically inert species when placed in authentic saliva samples. The distortion in voltammetry thus reflects the different surface sensitivity of the two

processes. Ohmic drop is proved to be minimal at microelectrodes even without added electrolyte. Microelectrodes are thus suitable for use in electrochemical analysis of saliva samples. Finally, we demonstrate that uric acid which is a potential biomarker for gout, oral cavity cancer and other several diseases can be directly detected in authentic saliva at a carbon microelectrode with the interference of ascorbic acid and dopamine of no more than 10%.

Keywords

Ferrocenemethanol; Ferrocyanide; Oxygen; Uric acid; Saliva; Voltammetry

1 Introduction

Saliva is a complex bodily fluid consisting of a variety of diagnostically useful components [1], many of which show significant and strong correlations with blood concentrations [2]. Saliva specimens can be easily collected via non-invasive means, are readily available and easily stored at lower temperatures than blood samples [3, 4]. Certain metabolites also display greater stability in saliva than blood plasma, potentially reducing errors arising from *in-vitro* artefacts [5, 6]. Consequently, saliva analysis may be preferred over traditional blood measurements in particular at point-of-care sites and for long-term self-monitoring purposes.

To facilitate the use of saliva as a diagnostic auxiliary, inexpensive and reliable analytical assays are required. In this work, electroanalytical methods are studied, developed and validated for applications in human saliva samples. *Authentic* saliva however is a complex medium and the electroanalytical performance of many redox-active species can be significantly different from their behaviour in standard aqueous electrolytes. Even *synthetic* saliva, as will be demonstrated, can be a poor model medium. Potential issues surrounding electrochemical measurements in authentic saliva include the fouling of electrode surface, the variation between different samples in terms of their viscosity and hence the diffusion coefficients of the analytes, as well as the presence of dissolved interfering molecules. [2]

Herein, the deviation of electrochemical responses in authentic saliva from those seen in other inorganic electrolytes is investigated using the standard redox probes: ferrocenemethanol/ferroceniummethanol and ferrocyanide/ferricyanide. Both systems are often used as redox mediators in biosensors [7-9]. The understanding of these redox processes in authentic saliva is thus highly important for accurate measurements in biological

and medical samples. Analysis of the voltammograms at a microdisc electrode reveals the physical origins underlying the altered responses in authentic saliva. Importantly using the two redox processes above, we demonstrate that well-defined voltammetry can be obtained directly in *undiluted* authentic saliva without any pre-treatment or modification of the samples. Similar studies were performed for the electrochemically and chemically irreversible redox process of oxygen reduction to further demonstrate the contrasts between authentic and synthetic saliva.

With understanding of the characteristics of electroanalytical measurements in authentic saliva, this work then moves on to the detection of uric acid in saliva samples. Salivary uric acid has displayed potential as a biomarker for gout [10], renal disease [11], oral cavity cancer [12, 13], obesity, hypertension and metabolic syndrome [14, 15] as well as type 2 diabetes [16]. Its concentration has further been used in the monitoring of the efficiency of treatment of chronic gout arthropathy [17] and hyperuricemia [18], and in the estimation of oxidative stress produced by smoking and periodontitis [19, 20]. Significant positive correlations between the concentrations of uric acid in saliva and blood have been established [14, 18]. Detection of salivary uric acid has been made using microfluidics [21, 22], capillary electrophoresis [10, 11], colorimetry [23], spectrophotometry [24], chromatography [25] and biosensors [26]. A number of electrochemical methods are available for the detection of uric acid [27-32]. However, only few methodologies have been validated with authentic saliva and in those cases the saliva samples are greatly diluted in inorganic buffers [33]. In this work, an electroanalytical method for the detection of uric acid is developed and applied to authentic whole saliva without requirement for dilution or any modification of the samples via the use of a carbon microdisc electrode.

2 Experimental

2.1 Chemical reagents and instrumentation

All chemical reagents were used as received without further purification: ferrocenemethanol ($FcCH_2OH$, 97%, Aldrich), potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$, ≥99%, AnalaR NORMAPUR), uric acid (≥99%, Sigma-Aldrich), L-ascorbic acid (99%, Sigma-Aldrich), dopamine hydrochloride (Sigma-Aldrich) and potassium chloride (≥99%, Sigma-Aldrich). All solutions were prepared using deionized water (Millipore) with a resistivity of 18.2 MΩ cm at 25°C.

Synthetic saliva prepared according to DIN 53160-1 was obtained from Synthetic Urine e.K., Eberdingen, Germany. The synthetic saliva contains Mg^{2+}, Ca^{2+}, K^{+}, Cl^{-}, CO_3^{2-} and HPO_4^{2-} ions. It has the ionic strength of ca. 43 mM and pH of ca. 6.8. Note that the synthetic saliva contains only some of the inorganic consituents of the authentic saliva, and many synthetic formulae often exclude organic components, proteins or other polypeptides present in the real samples, including the commercial synthetic saliva used herein.

Authentic saliva samples were kindly donated by healthy volunteers. Consents from the participants were obtained prior to the collection of samples and all local guidelines regarding the work with human saliva were followed. The samples were collected using a Salivette® (Sarstedt, Germany) [2]. Swabs were given out to participants to chew for 1 min. The Salivettes containing the chewed swabs were then centrifuged at the speed of 1000g for 2 min to extract the saliva samples. Authentic saliva consists of 99% water, inorganic species (Na^{+}, K^{+}, Ca^{2+}, Cl^{-}, HCO_3^{-}, PO_4^{3-}, HPO_4^{2-}, Mg^{2}+, SCN^{-}, NH_3, etc.), organic substances (uric acid, creatinine, glucose, cholesterol, lactate, etc.) and a variety of proteins such as enzymes, mucus and glycoproteins. [2] The ionic strength of authentic saliva is ca. 50-100 mM [34].

Electrochemical experiments were performed with a μAutolab Type III potentiostat (Utrecht) using a standard three electrode setup in a Faraday cage thermostated at 25 °C. A carbon microdisc (33 μm diameter, BASi) and a platinum mesh were used as working and counter electrodes respectively. A leakless Ag/AgCl (in 3.4 M KCl, eDAQ) was used as a reference electrode in order to prevent contamination of the samples by chloride ions, which could otherwise affect the responses measured [35].

2.2 Ferrocenemethanol and ferrocyanide oxidation

Synthetic or authentic saliva samples containing 1.0 mM ferrocenemethanol ($FcCH_2OH$) were prepared by directly dissolving 0.5 mg of $FcCH_2OH$ (s) into 2.30 mL of the samples. Cyclic voltammetry of $FcCH_2OH$ oxidation was first scanned anodically before being reversed back in the potential range of -0.2 - 0.8 V at the scan rate of 10 mV s^{-1}.

Similar experiments were performed with ferrocyanide ($[Fe(CN)_6]^{4-}$). The samples containing 1.0 mM ferrocyanide were prepared by dissolving 1.0 mg of $K_4[Fe(CN)_6]\cdot 3H_2O$ (s) in 2.37 mL of synthetic or authentic saliva samples.

2.3 Oxygen reduction

Cyclic voltammetry of oxygen reduction in synthetic or authentic saliva samples was recorded by scanning the potential cathodically from 0.2 V to -1.2 V, then reversed back to 0.2 V at the scan rate of 10 mV s^{-1} in the absence and presence of atmospheric oxygen (~0.3 mM dissolved O_2) [36]. When needed, saliva samples were deoxygenated by a strong flow of nitrogen gas into the samples for 5 min.

2.4 Uric acid, ascorbic acid and dopamine oxidation

Cyclic voltammetry of uric acid, ascorbic acid and dopamine oxidation in synthetic or authentic saliva was recorded by scanning the potential anodically from -0.2 V to 1.0 V, then reversed back to -0.2 V at the scan rate of 10 mV s^{-1}. In authentic saliva specimens, standard addition method was used to determine the levels of the relevant species in the samples.

3 Results and discussion

Herein, the characteristics of electrochemical measurements in authentic saliva are first investigated. An assay for the detection of uric acid is then developed and validated in authentic undiluted saliva specimens.

3.1 Investigations of the electrochemical behaviour in *authentic* vs. *synthetic* saliva

The electrochemical performances of several redox-active species are investigated in authentic saliva and the results compared with that in synthetic saliva and standard electrolytes. First, the ferrocenemethanol/ferroceniummethanol and ferrocyanide/ferricyanide redox systems are studied. Oxygen reduction is then investigated.

a) Ferrocenemethanol ($FcCH_2OH$) oxidation

The oxidation of 1.0 mM ferrocenemethanol ($FcCH_2OH$) in 0.10 M KCl solution, in synthetic saliva and in authentic saliva was investigated at a carbon microdisc electrode at a scan rate of 10 mV s^{-1}. The use of microdisc electrodes minimizes the contribution of capacitative currents and allows under favorable conditions the measurement of the electron-transfer

kinetics of the redox couple. The resulting cyclic voltammograms presented in Figure 1 display steady-state behaviour with the steady-state currents (mean ± standard deviation) of 6.4 ± 0.1, 6.6 ± 0.1 and 4.2 ± 0.1 nA in 0.10 M KCl, synthetic saliva and authentic saliva respectively. From these mass-transport limited currents and using equation 1 [37], the diffusion coefficients of $FcCH_2OH$ in the differing media were determined to be $8.1 \pm 0.1 \times 10^{-10}$ (comparable to literature [38, 39]), $8.3 \pm 0.1 \times 10^{-10}$ and $5.3 \pm 0.1 \times 10^{-10}$ $m^2\ s^{-1}$ respectively.

$$I_{ss} = 4nFcDr_e \quad (1),$$

where I_{ss} is the steady-state current, n is the number of electron transferred per molecule and F is the Faraday constant (96,485 C mol^{-1}). c and D are the bulk concentration and the diffusion coefficient of the redox-active species respectively. r_e is the radius of the electrode determined using $[Ru(NH_3)_6]^{3+}$ reduction [40] to be 20.5 ± 0.1 μm (SI).

Diffusion coefficient (D) varies approximately with the inverse of the solution viscosity (η) for sufficiently large molecules according to the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r} \quad (2),$$

where k_B is the Boltzmann constant, T the absolute temperature and r the radius of the diffusing particle.

The viscosity of synthetic saliva is approximated to be similar to that of pure water at 25 °C. Authentic saliva showed a variation in viscosity, with the average being ca. 1.1-1.3 times higher than that of pure water at 25 °C [41]. The higher viscosity of authentic saliva is due to the presence of mucin and other macromolecules [42]. The lower diffusion coefficient observed in authentic saliva as compared with synthetic saliva is thus consistent with the higher viscosity of the former.

For one-electron transfer systems, as in the case of $FcCH_2OH$ oxidation, the rate of electron transfer can be analyzed using mass-transport corrected Tafel analysis. In the irreversible limit (eqn. 3) [37], the plot of $\ln\left(\frac{I_{ss}}{I} - 1\right)$ against E gives the slope of $\frac{\alpha F}{RT}$ for a one-electron transfer process and hence the experimental transfer coefficient (α) can be determined. In the reversible limit (eqn. 4) [43], the electrochemical flux is controlled by the Nernst equation, and hence no kinetic information can be obtained. In this reversible limit, a plot of $\ln\left(\frac{I_{ss}}{I} - 1\right)$ against E gives the slope of a constant value of $\frac{F}{RT}$, hence yielding an

experimentally determined *apparent* transfer coefficient of 1 (equalling the number of electrons transferred).

$$\textbf{Irreversible limit:} \quad \ln\left(\frac{I_{ss}}{I} - 1\right) = \frac{\alpha F}{RT}\left(E - E_f^0\right) + \ln\left(\frac{-I_{ss}}{FA_e k^0 c}\right) \qquad (3),$$

$$\textbf{Reversible limit:} \quad \ln\left(\frac{I_{ss}}{I} - 1\right) = \frac{F}{RT}\left(E - E_f^0\right) + \ln\left(\frac{D_{\text{reduced}}}{D_{\text{oxidized}}}\right) \qquad (4),$$

where I_{ss} and I are the steady-state and transient currents respectively. R is the molar gas constant (8.314 J mol^{-1} K^{-1}). E and E_f^0 are the applied potential and formal potential respectively. A_e is the geometric surface area of the electrode and k^0 the rate of electron transfer. D_{reduced} and D_{oxidized} are the diffusion coefficients of the reduced and oxidized species respectively.

For the oxidation of $FcCH_2OH$, the experimentally determined apparent transfer coefficient (α) was 1.06 ± 0.04 in all the electrolytes and real samples studied, indicating the electron transfer is electrochemically reversible under all conditions; see inlay in Figure 1. The voltammograms of $FcCH_2OH$ oxidation are the same to within 2 mV in all media with the half-wave potentials ($E_{½}$) at 0.226 ± 0.002 V (vs. Ag/AgCl).

In this section, we have thus demonstrated that well-defined voltammetric responses of $FcCH_2OH$ can be obtained in undiluted unmodified authentic saliva samples, and that the responses observed were electrochemically reversible. However, the magnitude of the steady-state currents varies according to the viscosity of the samples. The $FcCH_2OH/FcCH_2OH^+$ system is thus recommended for use as a redox mediator in sensors for saliva applications, but a calibration curve of $FcCH_2OH$ steady-state currents vs. concentrations of the target analytes should be obtained directly in the saliva samples to prevent errors from the variation in the viscosity of the medium.

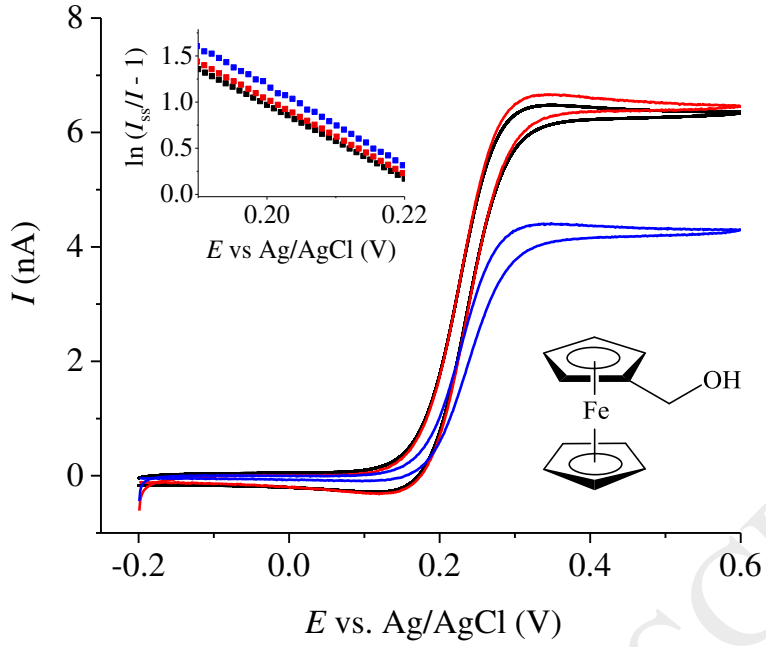

Figure 1: Cyclic voltammetry of 1.0 mM ferrocenemethanol ($FcCH_2OH$) oxidation in 0.1 M KCl (black), synthetic saliva (red) and authentic saliva (blue) at a carbon microdisc working electrode; scan rate of 10 mV s^{-1}. The inlay shows the results of mass-transport corrected analyses. The chemical structure of ferrocenemethanol is given on the right-hand side of the figure.

b) Ferrocyanide ($[Fe(CN)_6]^{4-}$) oxidation

The oxidation of 1.0 mM ferrocyanide ($[Fe(CN)_6]^{4-}$) in 0.10 M KCl, in synthetic saliva and in authentic saliva was next investigated at a carbon microdisc electrode (scan rate of 10 mV s^{-1}). Figure 2a shows the resulting voltammograms. The steady-state currents were 5.4 ± 0.1, 5.1 ± 0.2 and 4.5 ± 0.2 nA, corresponding to the diffusion coefficients of $6.8 \pm 0.1 \times 10^{-10}$ (comparable to literature [44, 45]), $6.4 \pm 0.3 \times 10^{-10}$ and $5.7 \pm 0.3 \times 10^{-10}$ in 0.10 M KCl, synthetic and authentic saliva respectively. The slower diffusion in authentic saliva is consistent with the more viscous nature of the sample as discussed above. However, in authentic saliva, the oxidation of $[Fe(CN)_6]^{4-}$ is found to be voltammetrically distorted with the oxidation requiring the applications of higher over-potentials ($E_{½}$ = 0.25 V) as compared with 0.10 M KCl and synthetic saliva ($E_{½}$ = 0.22 V). The apparent transfer coefficient (α) of $[Fe(CN)_6]^{4-}$ oxidation in authentic saliva is 0.8 ± 0.1, lower than the value of 1.0 ± 0.1 in 0.10 M KCl and synthetic saliva and thus suggests the more electrochemically *irreversible* nature of the oxidation in authentic samples; see inlay in Figure 2a.

The above voltammetry was conducted directly in undiluted saliva samples. To further elucidate the origin of the relatively slow kinetics in authentic saliva, a carbon microdisc electrode was first immersed in authentic saliva samples under open circuit potential. The electrode was then transferred to a solution containing only 1.0 mM $[Fe(CN)_6]^{4-}$ and 0.10 M KCl where cyclic voltammetry was again recorded. The resulting voltammograms are shown in Figure 2b and demonstrate that the redox process becomes more *irreversible* as the electrode was immersed in authentic saliva for longer time prior to the oxidation of $[Fe(CN)_6]^{4-}$ in standard 0.10 M KCl solutions; $E_{½}$ of 0.22, 0.29 and 0.32 V after 0, 1 and 5 min immersion respectively. This is an indication that the exposure of electrode to authentic saliva results in the adsorption of electrochemically inert components (such as organic molecules, proteins, etc.) or the assemble of charged species on the electrode surface. The former effectively decreases the electroactive surface area of the electrode [46]. The latter hinders the mass transport of $[Fe(CN)_6]^{4-}$ ions to the electrode due to electrostatic repulsion between negatively charged species and hence alters the electrochemical responses observed.

Similar experiments were performed for ferrocenemethanol (electrically neutral) and no changes in the voltammograms were observed from prolonged exposure of the electrode in authentic saliva samples. The hypothesis of the negatively charged species on the electrode surface was further tested using the positively charged redox probe, hexaammineruthenium (III) ($[Ru(NH_3)_6]^{3+}$). The cyclic voltammograms of $[Ru(NH_3)_6]^{3+}$ reduction in both authentic and synthetic saliva are fully reversible with the electron transfer coefficient of 1.0 within ±5% error, see SI. Therefore out of the three redox systems tested, ferrocenemethanol and hexaammineruthenium (III) which are electrically neutral and positive respectively did not show the difference in the reversibility of the voltammetry in authentic saliva as compared with synthetic saliva. On the other hand, the voltammograms of the negatively charged ferrocyanide ($[Fe(CN)_6]^{4-}$) become more irreversible in authentic saliva. The results are thus not inconsistent with the proposed hypothesis of the negatively charged species on the electrode surface.

The difference in the effects of authentic saliva on the oxidation of ferrocyanide from that of ferrocenemethanol or hexaammineruthenium also likely reflects the inner-sphere *vs.* outer-sphere electron transfer behaviours of the processes. Although ferrocyanide is often considered to be a good redox probe, the electron transfer is known to exhibit surface sensitivity [47] and is perhaps best viewed as a fast inner-sphere redox process. The

characteristics of these redox processes in authentic saliva, in particular the decrease in the steady-state currents due to the increased viscosity and the change in the electron transfer kinetics of ferrocyanide oxidation, should be considered when employed as standard redox probes in real saliva samples.

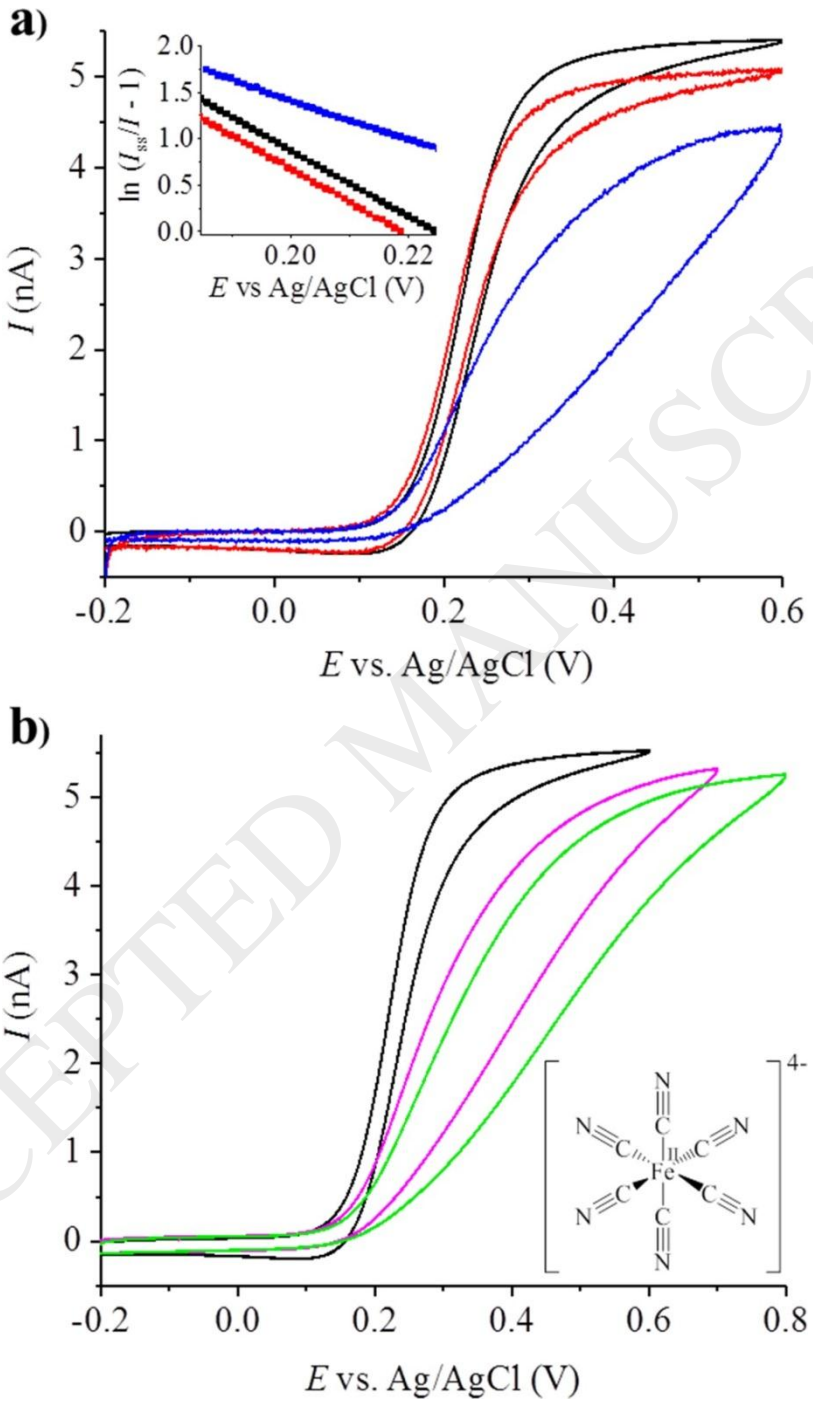

Figure 2: Cyclic voltammetry of 1.0 mM ferrocyanide ($[Fe(CN)_6]^{4-}$) oxidation **a)** performed directly in 0.1 M KCl (black), synthetic saliva (red) and authentic saliva (blue) with the inlay showing the results of mass-transport corrected Tafel analyses; **b)** in 0.1 M KCl after the carbon microdisc working electrode was immersed in authentic saliva for 0 min (black), 1 min (pink) and 5 min (green). Scan rate of 10 mV s^{-1}.

c) Oxygen reduction

The voltammetric response of oxygen reduction at a carbon microdisc electrode was investigated in authentic saliva in comparison with synthetic saliva. In this section, two types of synthetic saliva were investigated: commercial and homemade. Both have the same nominal composition. The reasons for using two different synthetic saliva samples will be discussed later below.

The concentrations of dissolved oxygen in both authentic and synthetic saliva are assumed to be close to the value of ~0.3 mM in air-saturated water at 25 ºC under atmospheric pressure [36]. At this concentration, the diffusion-limited steady-state current of a 2-e^- reduction of O_2 to H_2O_2 ($n = 2$) at the microdisc electrode radius 20.5 ± 0.1 μm is predicted (eqn. 1) to be -9.5 nA, using the diffusion coefficient of oxygen in water at 25 ºC of 2.0×10^{-9} m^2 s^{-1} [48].

In the case of homemade-synthetic saliva and authentic saliva, the reduction of oxygen reached the cathodic current of ca. -10 nA close to the predicted steady-state value before the onset of solvent breakdown interfered with the measured responses at more negative potentials; see SI. Compared with the homemade-synthetic samples, higher over-potentials were required to reduce oxygen in authentic saliva.

Interestingly, we noticed a *peak* in the oxygen reduction voltammogram in the *commercial*-synthetic saliva, as opposed to the steady-state behaviour normally expected at a microdisc electrode; see Figure 3. The peak-shaped response indicates the more difficult reduction of oxygen at very negative potentials, possibly due to interactions with unknown species in the commercial-synthetic saliva. The abnormal results in synthetic saliva further highlight the importance of the validations of any developed assays in *authentic* saliva samples.

Mass-transport corrected Tafel analysis (eqn. 3) reveals the experimental apparent transfer coefficients (α, determined using the current range of 15-50% of the steady-state currents) of oxygen reduction in homemade-synthetic, commercial-synthetic and authentic saliva to be 0.27, 0.31 and 0.22 respectively. The relatively impeded electrode kinetics in authentic saliva

is likely a result of the adsorption of organic molecules or other electrochemically inert components onto the surface of the electrode as demonstrated in the previous section. The partial blocking of the electrode surface is thought to possibly decrease the available adsorption sites for O_2 or its reduction products, and hence decreases the rate of the electron-transfer process.

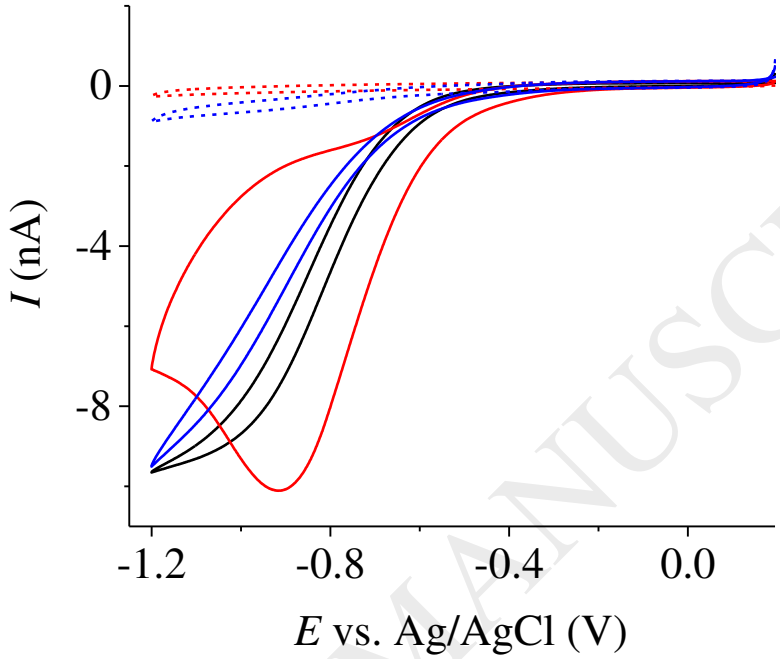

Figure 3: Cyclic voltammetry of commercial synthetic saliva (red), homemade synthetic saliva (black), and authentic saliva (blue) in the absence (dashed lines) and presence (solid lines) of dissolved ~0.3 mM oxygen at a carbon microdisc working electrode; scan rate of 10 mV s^{-1}.

3.2 Salivary uric acid detection

In the previous sections, the electrochemical behaviour of often used redox markers has been investigated in authentic saliva demonstrating that electrochemical measurements can be made using a microelectrode without the addition of supporting electrolyte. Importantly, due to the small magnitude of the currents (<10 nA) passed at the microelectrode the requirement of using added excess supporting electrolyte is completely relaxed [49, 50]. The ionic strength of human authentic saliva is 44.6 ± 11.6 mM [51]. The fact that the voltammetric response of ferrocenemethanol is found to be essentially unaltered apart from a change in the associated diffusion coefficient provides strong evidences that the voltammetric distortions observed for the other molecular species arises due to sensitivity of the redox processes to the electrode surface, and not from issues associated with ohmic drop. In the absence of

ferrocenemethanol results, the observed distorted voltammograms for the other molecular probes might be erroneously ascribed as relating to ohmic drop effects through the solution.

Having demonstrated the ability to perform electroanalysis directly in authentic saliva samples, in this section, we next focus on the detection of salivary uric acid which is a potential biomarker for gout [10], oral cavity cancer [12, 13] and other several diseases [14-16].

The cyclic voltammetry of authentic saliva sample at a carbon microdisc electrode shows an oxidative peak at ~0.7 V (Figure 4, inlay). The oxidation at this potential likely corresponds to uric acid (UA), ascorbic acid (AA) or dopamine (DA); see SI. However in authentic human saliva, AA and DA are present at very low concentrations of ca. 1.4 ± 0.3 μM [52] and < 0.5 nM [53] respectively, compared with the concentration of ca. 200-300 μM of UA [54-56]. Consequently, when measured using the carbon microdisc electrode, UA will be the dominant contributor to the electrochemical current with the interferences from AA and DA contributing no more than ~10%.

Figure 4 presents the cyclic voltammograms of 120 μM UA added to synthetic and authentic saliva at a carbon microdisc electrode at the scan rate of 10 mV s^{-1}; see SI for the voltammograms of AA and DA. The oxidation of UA (as well as AA and DA) has been reported to be a 2-electron transfer process [57]. The steady-state current for UA oxidation in synthetic saliva was 1.4 ± 0.1 nA at a microdisc electrode radius 20.5 ± 0.1 μm, corresponding to the diffusion coefficient of $7.4 \pm 0.5 \times 10^{-10}$ m^2 s^{-1}. The half-wave potential ($E_{½}$) was observed at 0.47 ± 0.01 V and the apparent transfer coefficient (α) determined using eqn. 3 to be 0.48 ± 0.02 for UA oxidation in synthetic saliva.

In authentic saliva, the oxidation wave of UA is shifted from that of synthetic saliva to a more positive potential by ~100 mV likely due to partial blocking of the electrode as previously discussed. The sloping of the oxidative currents at high potentials is due to the earlier onset of solvent decomposition. In the determination of UA concentrations in authentic saliva, the steady-state currents may be influenced by the variation in the viscosity between different samples and possibly also the differing effects of molecular interferences. The method of standard additions was thus employed. The standard additions of UA to authentic saliva samples give linear responses of the peak currents ($I_p = I_{0.7\,V} - I_{0.2\,V}$) as a function of concentrations with the sensitivity of 8.8 ± 0.8 pA μM^{-1}. In the particular sample studied, the

concentration of UA was determined to be 192 ± 7 µM (mean ± standard deviation). However, it should be noted that there is also a possible ca. 10% error as a result of the AA and DA interferences. The levels of salivary UA measured are in good agreement with the range of independent values reported in literature [54-56]. The limit of detection ($3s_B/m$) of UA in the authentic saliva samples studied is 20 µM.

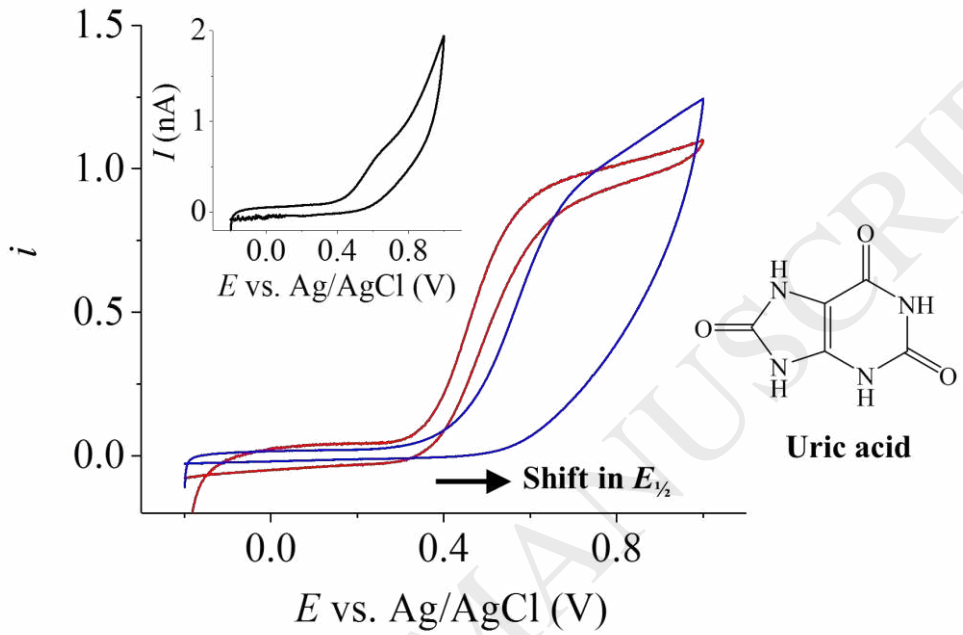

Figure 4: Normalized cyclic voltammograms of 120 µM uric acid oxidation in synthetic saliva (red) and authentic saliva (blue) showing the shift in the half-wave potentials ($E_{½}$). The inlay is the voltammogram of blank, unmodified authentic saliva. Carbon microdisc working electrode; scan rate of 10 mV s^{-1}. The chemical structure of uric acid is also given in the figure.

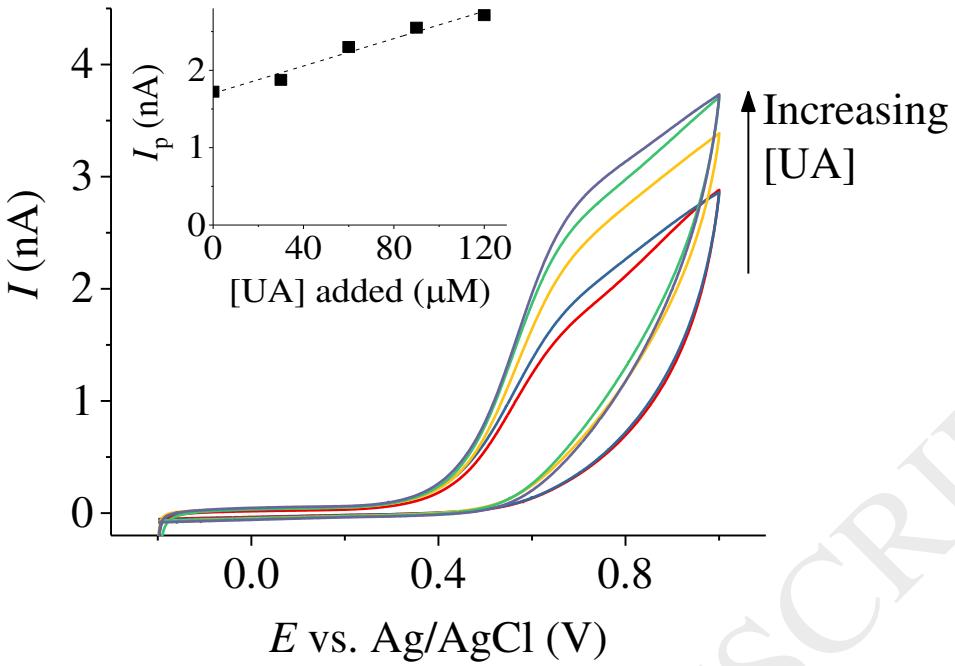

Figure 5: Cyclic voltammetry of increasing concentrations of uric acid added to authentic saliva samples at a carbon microdisc working electrode at the scan rate of 10 mV s^{-1}. The inlay shows the peak currents measured ($I_p = I_{0.7\ V} - I_{0.2\ V}$) as a function of concentrations added.

4 Conclusions

Steady-state voltammograms were observed at a carbon microdisc electrode for the oxidation of ferrocenemethanol (FcMeOH) and ferrocyanide ($[Fe(CN)_6]^{4-}$) in authentic human saliva without pre-treatment or modification of the samples. A clear electrochemical response of oxygen reduction in authentic saliva was also identified. In the case of FcMeOH, the responses are electrochemically *reversible* in authentic saliva, synthetic saliva and aqueous KCl electrolyte, although the steady-state currents are ~20-30% smaller in authentic samples due to their higher viscosities. In contrast, the voltammograms of $[Fe(CN)_6]^{4-}$ oxidation and O_2 reduction become more *irreversible* in authentic saliva probably as a result of partial blocking of the electrode by organic interferences, reflecting the surface sensitivity of the two redox processes. We have thus demonstrated the different electrochemical behaviours in authentic saliva of the two common redox probes as well as highlighted the importance of the validation of any developed assays in *authentic* saliva, as opposed to synthetic saliva. Importantly, we developed an electroanalytical method via the use of a carbon microdisc electrode for the detection of salivary uric acid. The method has been directly validated in authentic saliva specimens and is suitable for salivary diagnostic applications. The ability to perform electrochemical analysis directly in authentic saliva samples without the need for

added electrolyte is important, due to the small currents passed at microelectrodes and hence lower ohmic distortions, these electrodes are more suitable for direct use in real world media.

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