**Electrocatalytic detection of ascorbic acid using N,N,N',N'-tetramethyl-
para-phenylene-diamine (TMPD) mediated oxidation at unmodified gold
electrodes; reaction mechanism and analytical application**

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Abstract

The redox mediator N,N,N',N'-tetramethyl-para-phenylene-diamine (TMPD) is shown to catalyse oxidation of ascorbic acid (AA), which is otherwise known to exhibit irreversible electrode kinetics and produce deposits on the electrode surface. The mechanism of the catalysed reaction is reported and inferred to follow the sequence

Kinetic parameters are deduced from the modelling of cyclic voltammetry as a function of voltage scan rate. Using TMPD, AA can be oxidized at a low overpotential where direct electrochemical oxidation is minimal, and products are formed in solution. Consequently, electrode fouling is avoided and a reliable electrochemical signal can be recorded on unmodified gold electrodes, allowing the quantitative analysis of AA in even complex media without pre-treatment of the samples. The presented method offers high sensitivity, with a sufficient limit of detection for the detection of AA in beverages, without interferences from others reactants during cyclic voltammetry measurements. The applicability of the technique is demonstrated on commercial orange juice, offering a straight forward, inexpensive and precise alternative to other AA determination strategies.

Keywords: Electrochemical detection, Cyclic Voltammetry, Ascorbic Acid, Catalytic Reaction, N,N,N',N'-tetramethyl-para-phenylene-diamine

1. Introduction

Epidemiological studies suggest a variety of health benefits associated with ascorbic acid (AA), a hydrosoluble six-carbon lactone, also known as vitamin C. Although its protective role against medical conditions, such as cardiovascular disease, stroke, and cancer in general, remains inconclusive, there is evidence for its function as powerful antioxidant in biological systems in vitro.[1] As a reducing agent, AA donates two electrons, preventing other substances from being oxidized. Following the loss of a first electron, the formed ascorbyl radical, also known as ascorbate (AA^{+*}), exhibits a half-life of 10^{-5} seconds and is therefore known to function as radical scavenger[2,3], as it is more stable and less reactive than other, potentially harmful free radicals. Following the loss of a second electron, dehydroascorbic acid (AA^{2+}) is rapidly metabolized.[4,5]

Unlike most mammalian species, humans are not able to synthesize AA from glucose; hence its uptake from food sources[6], mainly vegetables[7], is essential. Its medical importance, but also its ability to protect oxidizable constituents, such as compounds related to taste, colour and flavour in beverages[8], results in the addition of AA in the food industry, and to animal feed, pharmaceuticals and cosmetics.[9,10] The AA concentration in food sources lies in the order of mg per litre or kilogram[11,12], whereby climate, the method of harvest, storage and processing conditions may impact its content.[13] Hence, reliable and accurate analytical quantification methods for agricultural and food products are needed. Furthermore, it is desirable to accurately monitor AA concentrations in body liquids and tissues. Although AA in excess is usually excreted from the body, in some individuals its immoderation can lead to health issues, such as gastric irritation or renal problems.[14]

The successful detection of AA in foods and biological fluids has been carried out using methodologies, including titration[15], chromatography[16], HPLC[17,18], spectroscopy[19], chemiluminescence[20] and electrochemistry.[21] Due to difficulties, such as poor

reproducibility and electrode fouling, electrode modifications sparked high interest among the electrochemical society. However, most strategies involve expensive materials and overall complicated and time intense electrode modification procedures, such as the use of nanoneedles[22], nanoparticles[23], nanosheets[24], nanotubes[25], nanowires[26] or alloys[27] and can take hours, in some cases even days until reliable analysis data can be collected.[22] With the use of redox active mediators, drawbacks, as stated above, can be avoided. The direct electrochemical oxidation of AA exhibits a large overpotential, and results in electrode fouling. By introducing a redox mediator, indirect detection of the analyte can be achieved with high precision.[28] The mediator undergoes a heterogeneous reaction at the electrode, followed by homogeneous redox recycling with the analyte. This catalytic reaction, labelled as an EC' reaction, causes an amplification of the measured current, seen in cyclic voltammetry. Furthermore, a redox mediator can catalyse the oxidation of AA closer to its equilibrium potential, while reaction products are formed in solution and not at the electrode surface. EC' reactions have been extensively studied previously and are well documented.[29,30] A clear understanding of the mechanism helps in analyzing the electrochemical response and makes detection more reliable.

Herein, we report the reaction mechanism and quantitative analysis of AA with N,N,N',N'-tetramethyl-para-phenylene-diamine (TMPD). The presented work represents a precise, straight-forward, and inexpensive method, demonstrated for the detection of AA in commercial orange juice.

2. Theory and Simulation

The EC' mechanism is well established and in its simplest form is defined by the following scheme:

The voltammetric behaviour has been characterized for cyclic voltammetry at macro- and microelectrodes.[30–32] Commonly, the catalysis equilibrium is assumed to lie completely on the side of the products, in which case the response of the electrochemical system is characterized by the electron transfer kinetics of the couple A/B and the irreversible catalysis reaction rate constant k_{cat} . In reversible electrochemical systems, cyclic voltammograms show an increase and shift in peak current in presence of the catalysis, which both become more pronounced at higher catalysis rate, and concentrations of X. In the specific case where X is not in excess, and the catalysis reaction is fast, a split wave may be observed, at which the current peak splits into a first peak, in which X is consumed, and a second peak corresponding to the normal transformation of A.[32]

In the following, we discuss the use of the mediator redox couple A/B for an overall two-electron oxidation. In the reported study, the standard one electron EC' reaction mechanism as presented in equations (1) and (2) lead to insufficient currents, therefore the reaction scheme had to be extended in its complexity to allow for the possibility of the two electron oxidation of AA. One possible reaction mechanism sees the oxidized mediator B directly catalysing both electron transfer steps in an EC'C' type mechanism[33]:

Where $k_{cat'}$ and $k_{cat''}$ are the reaction rate constants for the first and second electron transfer catalysis steps, respectively, and $K_{cat'}$ and $K_{cat''}$ are the corresponding equilibrium constants. Alternatively, the second electron transfer step might be induced by disproportionation of the

singly oxidized catalysis product X' . Consequently, in this mechanism the A/B couple catalyses directly only the first step:

In addition to the electron transfer kinetics of the A/B couple, these two-electron electrocatalysis mechanisms are defined by the two catalysis equilibria: the forward rate constants $k_{cat'}$ and $k_{cat''}$, and the equilibrium constants $K_{cat'}$ and $K_{cat''}$ in case of the EC'C' mechanism; or the forward catalysis and disproportionation rates $k_{cat'}$ and k_{disp} , and the catalysis and disproportionation equilibrium constants $K_{cat'}$ and K_{disp} for the second mechanism. Whereas in the EC'C' mechanism an increase in either of the catalysis rate constants or concentration of X leads to higher peak currents and larger potential shifts in cyclic voltammetry, similar to the standard EC' mechanism, the effect of kinetic parameters in the electrocatalysis-disproportionation mechanism cannot be understood as intuitively, because it connects two oxidation-reduction cycles.

The commercial simulation package DigiSim[®] [34–38] was used to model the above processes and evaluate the different mechanism possibilities. DigiSim[®] models the heterogeneous electron transfer steps for the Butler-Volmer equation and so the TMPD/TMPD^{*+} couple is characterized by a transfer coefficient, and a standard electrochemical rate constant as well as the formal potential E_f^0 . The fitting procedure applies reasonable initial parameter estimates and uses the Digisim internal automated fitting procedure, which minimizes the residual difference between simulation and experiment. Visual fine adjustment of parameters was subsequently performed to give more weight to peak value, position and curve shape in the fitting.

3. Experimental

3.1. Chemical reagents

All chemicals were purchased from Sigma-Aldrich, if not indicated otherwise. TMPD (99%) and D-isoascorbic acid were used as received. Phosphate buffered saline (PBS) solution consists of 8 g sodium chloride ($\geq 99\%$), 0.2 g potassium chloride ($\geq 99\%$), 1.44g sodium phosphate dibasic ($\geq 99\%$), 0.24 g potassium phosphate dibasic ($\geq 99\%$) and was completed to 1L using ultrapure water with a resistivity not less than $18.2 \text{ M}\Omega \text{ cm}$ at 25°C (Millipore water purification system). Orange juice was purchased from Innocent juices as “Innocent – pure squeezed fruit, nothing else”.

3.2. Electrochemical Measurements

Electrochemical measurements (cyclic voltammetry) were carried out using a high-performance modular potentiostat (PGSTAT302N, Autolab, UK,). Experiments were conducted inside a temperature controlled Faraday cage. A gold macroelectrode (Alvatek Ltd, UK) with a radius of 1.5 mm functioned as working electrode and was polished prior to experiments using a water-alumina mix (1.0, 0.3 and $0.05 \mu\text{m}$, 30 seconds for each grade) on microcloth polishing pads (Buehler, USA).[39] In all experiments, a standard calomel electrode and a platinum mesh were employed as reference and counter electrodes, respectively.

4. Results and Discussion

In this section, we first establish a reaction mechanism for the oxidation of AA with TMPD^{*+} , which results from the fitting of theoretical voltammetric curves to experimental cyclic voltammetry data with varied scan rates and concentration ratios of AA to TMPD. We then demonstrate that a limit of detection (LOD) can be achieved, suitable for the detection of AA

in beverages. Finally, as a proof of concept, we present the quantitative analysis of AA content in orange juice.

4.1. Reaction Mechanism

The electrochemical behaviour of TMPD was analysed using cyclic voltammetry. Fig. 1a shows cyclic voltammograms, recorded in 0.5 mM TMPD in PBS buffer solution at scan rates varying from 10 to 100 mV s⁻¹. TMPD exhibits two sequential electron transfer steps with oxidation peaks at 0.025 and 0.425 V vs. SCE (Fig. S1), in agreement with literature.[40] The presented work focuses on the first oxidation step, as shown in Fig. 1a. Theoretical curves were fitted to experimental data using the DigiSim[®] simulation software for cyclic voltammetry (Basi).[34–38] A diffusion coefficient D_{TMPD} of 6.52 x10⁻⁶ cm² s⁻¹ was determined using the Randles-Ševčík method, in good agreement with the literature value of 6.43 x10⁻⁶ cm² s⁻¹[41], and was fixed in the DigiSim[®] program. Fig. 1b shows the linear behaviour of the TMPD peak current as a function of the square root of scan rate, whereby details about the calculation of D_{TMPD} can be found in the supporting information. To determine the Butler-Volmer kinetic parameters of TMPD at the macroelectrode, the redox reaction

was simulated and a fitting of the concentration-independent standard electrochemical rate constant k_s as well as the standard electrode potential E^0 was conducted. Without changing the overall shape or position of the simulated curves, the initial concentration ratio of TMPD to $TMPD^{*+}$ was adjusted for each scan (assuming between 0.27 and 0.18 mM $TMPD^{*+}$ in solution prior to the electrochemical measurement), due to its rapid oxidation by atmospheric oxygen. This allowed an offset correction for the presented curves. A capacitive contribution between 2 x10⁻⁶ F and 6 x10⁻⁶ F was included to the fitting. The standard electrochemical rate

constant k_s was determined to be equal or larger than 0.1 cm s^{-1} , *i.e.* reaching the fully reversible limiting case, at which currents are limited by thermodynamics and diffusion rate, and the resulting theoretical voltammograms (Fig. 1, dotted lines) are in good agreement with the experimental measurements (full lines). A standard electrode potential E^0 of 0.018 V vs. SCE was determined.

Ascorbic acid shows a strongly pH dependent behaviour during voltammetry, at which the two electron steps during oxidation are resolved above pH 8. Slow electrode kinetics result in irreversible voltammetric behaviour, while it is also known that the doubly oxidized product dehydro-ascorbic acid can partially dissociate to form 2,3-diketogluconate.[42] We observe here the reported behaviour of slow electrode kinetics (Fig. S2) and electrode fouling over subsequent voltammetry cycles. Due to these intrinsic properties, ascorbic acid is difficult to quantify electrochemically directly. A suitable redox mediator, such as TMPD, may be able to overcome such issues.

To understand the reaction mechanism and kinetics, AA in concentrations of 1.0 and 1.5 mM were added to the TMPD solution. AA concentrations were chosen in excess of TMPD (2:1 and 3:1), to ensure stability of experimental measurements and reduce uncertainties related to AA concentrations in the simulations. TMPD in solution is oxidized by oxygen to its radical cation TMPD^{*+} , which becomes apparent by its characteristic blue colour. Upon addition of AA in excess to TMPD, the solution decolorizes as TMPD^{*+} is reduced. In the presence of AA, TMPD reacts homogeneously with AA, resulting in an enhanced anodic current signal of TMPD while the initial oxidation peak at 0.3 V vs. SCE (Fig. S2) shifts cathodically (Fig. 2a). The scan rate dependence of the recorded voltammograms showed quickly that a regular EC' mechanism could not satisfactorily describe the system. The applicability of different mechanisms could be compared to experimental concentration and scan rate dependence of the TMPD/AA system with the help of Digisim[®]. In this manner, the mechanisms introduced

earlier were investigated. While neither the EC'C' nor the EC'-disproportionation mechanisms exhibit excellent fits on their own, a combination of two catalysis steps and singly oxidized ascorbic acid disproportionation show best agreement:

This is in agreement with the known redox potentials of TMPD and AA under these conditions[43], and the reported disproportionation of the singly oxidized ascorbic acid.[44] No adjustment of the initial TMPD concentration was necessary, as AA fully reduces all TMPD in solution. Kinetic parameters for the AA/TMPD homogeneous reactions (7)-(9) were found to be: for the first catalysis step (7) equilibrium constant $K_{cat'} = 20$, rate constant $k_{cat'} = 5 \times 10^5$ L/(mol s), for the second catalysis step (8) equilibrium constant $K_{cat''} = 500$, rate constant $k_{cat''} = 1.4 \times 10^4$ L/(mol s), and for the disproportionation (9) equilibrium constant $K_{disp} = 25$, rate constant $k_{disp} = 3 \times 10^2$ L/(mol s). A diffusion coefficient of AA (D_{AA}) of 5.7×10^{-6} cm² s⁻¹ was taken from literature.[45] In order to obtain kinetic rates, the Butler-Volmer model was applied, using the fixed parameters: temperature = 298.2 K, semi-infinite diffusion, planar geometry of the electrode with an area of 0.07 cm², $E^0 = 0.018$ V, $\alpha = 0.5$, potential steps = 0.001 V, noise level = 0 A, and an expanding grid factor of 0.05. The same parameters were used for simulating the TMPD/TMPD^{*+} electrode reaction kinetics in the absence of AA.

As it can be seen in Fig. 2a, simulated voltammograms of TMPD (dotted lines) in the presence of AA are in good agreement with the experimental measurements (full lines). The resulting comparison of experimental (black) and simulated (red) peak current values of the voltammograms are presented in Fig. 2b, where the same determined kinetic reaction

parameters were applied at all scan rates. Herein it is proposed that the enhanced TMPD peak current can be used for the detection of AA in complex media, such as beverages.

4.2. Limit of Detection

In order to evaluate the applicability of the proposed system for the detection of AA in commercial food and beverage products, the limit of detection (LOD) needs to be identified. Five independent cyclic voltammetry measurements in 0.5 mM TMPD in PBS were conducted, and their average is shown as full black curve in Fig. 3. An error bar has been added to the oxidation peak, representing three times the standard deviation (STD) of the peak current, corresponding to a confidence level of about 99.9 %.[46] All peak current values recorded below the value of three times the STD added to the average peak current of TMPD are said to fall within the noise level and defined as non-detectable. Different concentrations of AA have been added to TMPD and a level of detection (LOD) of 30 μM was identified. This LOD is suitable for the analysis of foods and beverages, as their AA content usually lies in the order of milli or micromolar.[26] Furthermore, the LOD of AA using TMPD is competitive with literature methods[21], but more importantly, it offers a much simpler and less time consuming procedure.

4.3. AA Detection in Orange Juice

As a proof of concept and to demonstrate the straight forward methodology, we investigated the AA content in commercially available pure orange juice. Fig. 4a presents cyclic voltammetric measurements in orange juice (black) and orange juice in the presence of 0.5 mM TMPD (full potential range can be seen in Fig. S3). A clean oxidation peak of TMPD can be seen with no other interferences at this potential, allowing direct quantitative analysis of AA. A calibration curve was established, showing a linear relationship between AA

concentration and recorded electrochemical signal (Fig. 4b). Five independent measurements of the orange juice sample were taken and the small STD of the observed peak current for these measurements (Fig. 4 inset) demonstrates the robustness of the system and its precision. The obtained value of AA in orange juice of 1.24 mM is in good agreement with the average manufacturer specification of 1.4 mM.

5. Conclusions

The presented work demonstrates a simple, precise and inexpensive electrochemical detection of AA using TMPD as redox mediator. This strategy allows the quantitative analysis of AA in complex media without pre-treatment of the samples, even juices containing pulp. The reaction mechanism of AA with TMPD involves the reversible heterogeneous conversion of TMPD to TMPD^{+*} at the macroelectrode, the homogeneous two-step oxidation of AA by TMPD^{+*} to AA^{+*} and AA^{2+} in solution, as well as a disproportionation reaction of two AA^{+*} to AA and AA^{2+} . With the use of TMPD as redox active mediator the overpotential of AA can be shifted closer to its equilibrium potential, avoiding deposition of reaction products at the macroelectrode. With a LOD of 30 μM , this system is well suited for the detection of AA in complex media, such as beverages. The successful analysis of AA in orange juice is reported, demonstrating the applicability and precision of the presented study. A reduction of the LOD using microelectrodes could be considered. For future applications to body fluids, interferences, such as dopamine and uric acid should be investigated.

Acknowledgements

S.K. thanks the support from the European Commission under the Marie Curie Programme (grant number 702009). The contents reflect only the authors' views and not the views of the European Commission.

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Figure Captions

Figure 1: Electrochemical analysis of TMPD by cyclic voltammetry. a) Experimental (full lines) and simulated (dotted lines) cyclic voltammograms of a 3-mm gold electrode in 0.5 mM TMPD. b) Linear relationship of the peak current and scan rate (v) for the extraction of the TMPD diffusion coefficient following the Randles-Ševčík method.

Figure 2: a) Experimental (full lines) and simulated (dotted lines) cyclic voltammograms of 0.5 mM TMPD and two concentrations of AA. b) Representation of experimental (black) and simulated (red) peak currents as a function of the square root of scan rate for cyclic voltammograms of TMPD (Figure 1) and AA (a).

Figure 3: Level of detection (LOD) determination by cyclic voltammetry in 0.1 mM TMPD and various concentrations of AA. Full line (black) represents the average of 5 independent voltammograms in 0.1 mM TMPD. Error bar indicates 3x the standard deviation of the peak current in the absence of AA.

Figure 4: AA detection in orange juice. a) Cyclic voltammograms of commercially available pure orange juice and orange juice containing 0.5 mM TMPD. b) Calibration curve for the electrochemical detection of AA. Error bar indicates the standard deviation of five independent measurements of the sample.