Investigation of the Antioxidant Property of Ascorbic Acid

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The antioxidant property of ascorbic acid (AA) was demonstrated during the process of electron transfer (ET) and the corresponding effects of AA were visibly affected by using porphyrin zinc with various substitutes as oxidants. As electron receptors, porphyrin zinc molecules obtained the electron that came from the electron donor AA, which was perfectly convenient for the simulated antioxidant reaction. The results of the obtained kinetics data showed that the antioxidant property of AA may be affected owing to the difference of the oxidants. Experimental results showed that thin-layer cyclic voltammetry (TLCV) and scanning electrochemical microscopy (SECM) were applied as investigating means to evaluate the rate constant of electron transfer achieved and expressed concerns about the simplicity and reliability, and they were also more vigorous methods to study the simulated biomembrane.

1. Introduction

Ascorbic acid (AA) is an essential component in the diet of humans and a small range of other mammals, and also a typical long used pharmaceutical agent. The convincing evidence was admitted that the antioxidant action enables AA to protect tissues and cells against oxidative damage by producing free radicals and reactive oxygen-derived species. Owing to the fact that many studies seem to reveal that these protective effects may be a result of the intake of antioxidants, more attention is being focused on potentially antioxidant substances, such as carotenoids and AA.[1−10] Recently, it was demonstrated that AA is needed for the activation of movement of the animals and also was suggested as an inhibitory neurotransmitter. Despite the fact that there have not been results in vivo studies in this field yet, the intensive studies of AA in biology are contributable to the fact of the excellent antioxidant activity. Oxygen free radicals are continuously formed as a byproduct of cellular respiration and present a potential threat to cellular functions, which may cause cell damage through the peroxidation of membrane lipids, and compromise cellular transport functions. The deleterious effects of these free radicals are counterbalanced by the intracellular antioxidant defense systems, to which ascorbic acid may contribute.[11−17] Of course different free radicals around AA which present various surroundings will affect antioxidant effects of AA. Therefore, the zinc porphyrins with various substitutes were chosen for the studies at the simulated biomembrane. The advantage of using zinc porphyrins as the detected molecule was that they may offer different electrochemical conditions for the simulated antioxidant process.

Porphyrin derivatives play a key role in essential biological processes such as photosynthesis, dioxygen transport, and storage. Complexes of many metals with various porphyrins have been extensively studied to understand the biosynthetic formation and biological activity of natural compounds. From the perspective of coordination chemistry, such complexes have been used in a variety of applications as models for biological electron transport in biomembranes and other investigations.[18−27]
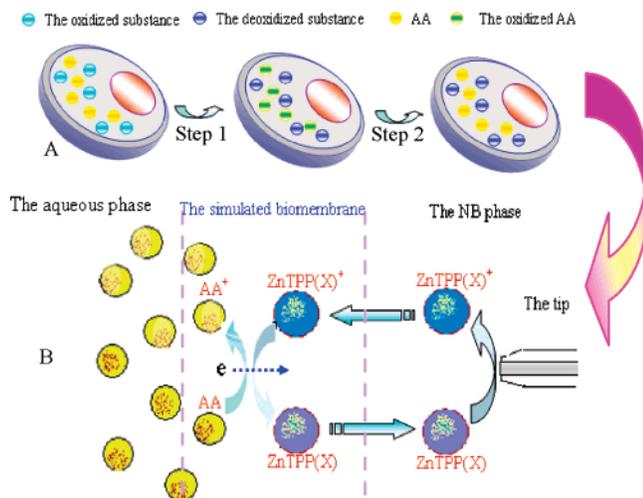
Metalloporphyrins adsorbed on the surface of graphite or other inert electrodes are widely used as electrocatalysts for the electrochemical reduction or oxidation of a variety of important molecules.[28−32]

Considerable interest has been emerging and remarkable developments can be seen in the field of charge transfer at the liquid−liquid interface.[33−39] The liquid−liquid interface is the simplest and most promising model for understanding charge-transfer processes in biological systems, which include not only ion transport but also electron transport. Electron transfer at the liquid−liquid interface is fundamentally important for understanding the energy conversion in biomembranes.[40,41] As we all know, AA is involved in many metabolic processes where it acts as an electron donor, and in fact, the essential element of an antioxidant reaction is electron-transfer process. To investigate the effect of antioxidants on a human cell, zinc porphyrins with various substitutes were selected as electron receptors, whose oxidations replacing free radicals and oxygen-derived species in a cell can be deoxidized. Due to these facts, our interest and recent efforts have been concentrated on the antioxidant property of AA during the cross-phase electron-transfer kinetic process at the simulated biological membranes by electrochemical investigation. Combining this with the electrochemical technology, the rate constants at the interface and the effect of different substitutes of metalloporphyrin on the antioxidant property of AA are discussed. A lower concentration of AA was applied to better simulate the content of AA in the human body. The obtained kinetics properties of the AA and porphyrins zinc systems at the liquid−liquid interface were fit enough to offer a possible contribution to biology and clinic medical research.

2. Experimental Section

2.1. Chemicals. Lithium chloride (LiCl) (Shanghai Runjie Chemical Reagent Co. Ltd, China), sodium perchlorate (NaClO$_4$) (AR, Beijing Chemical Reagent Co. Ltd, China), and ascorbic acid were of the highest available purity. Various pretreatments did not alter their behavior so that they were usually used as received from commercial sources and without further purification. Nitrobenzene (NB) (Shanghai Chemical

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SCHEME 1: (A) The Simplified Antioxidant Process of AA in the Cell and (B) the Corresponding Reaction at the Electrode
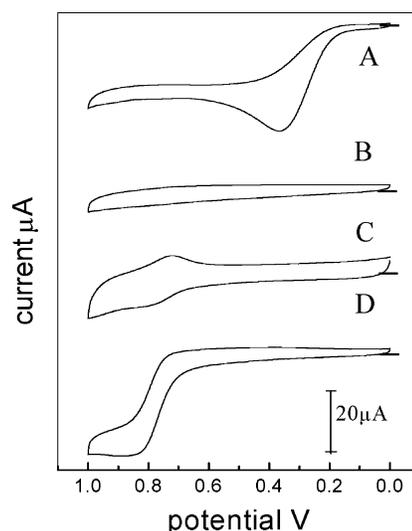

Figure 1. Voltammetric observation of electron transfer between AA and ZnTPP(OH). (A) Cyclic voltammogram for 3 mM AA at an uncoated EPG electrode: supporting electrolyte, 0.1 M NaClO$_4$ + 0.1 M LiCl. (B) Repeat of part A after the electrode surface was covered with 1.5 μL of NB. (C) Cyclic voltammogram with the electrode covered with 1.5 μL of NB containing 1 mM ZnTPP(H) and supporting electrolyte (0.01 M TBAClO$_4$). The aqueous solution contained only supporting electrolyte (0.1 M NaClO$_4$ + 0.1 M LiCl). (D) Repeat of part C with 10 mM AA present in the aqueous phase; 1.5 μL of NB containing 1 mM ZnTPP(H) and supporting electrolyte (0.01 M TBAClO$_4$). Scan rate, 5 mV s^{-1}.

Reagent Co. Ltd, China) was distilled under reduced pressure before use. Tetrabutylammonium perchlorate (TBAClO$_4$) was synthesized with tetrabutylammonium chloride (TBACl) (AR, Aldrich) and 5,10,15,20-tetraphenylporphyrin zinc (ZnTPP), methyloxy-5,10,15,20-tetraphenylporphyrin zinc (ZnTPP(OCH$_3$)), and monohydroxyl-5,10,15,20-tetraphenylporphyrin zinc (ZnT-PP(OH)) were prepared with the same method reported elsewhere,[34] and zinc porphyrins were also synthesized in our lab.[42] All chemicals were analytical grade or better. Special precautions were taken to deal with NB and other hazardous chemicals when necessary. Aqueous solutions with various concentrations were prepared from distilled water with LiCl and NaClO$_4$ as supporting electrolytes, while organic solutions were prepared from NB with TBAClO$_4$ as the supporting electrolyte.

2.2. Apparatus and Procedures. All the electrochemical experiments were performed with a CHI-900 workstation. A three-electrode configuration (tip, auxiliary, and reference electrodes) was applied as in other experiments. The working electrodes were pyrolytic graphite electrodes (EPG) in the thin-layer cyclic voltammetry (TLCV) method with 0.32 cm^2 of the edges of the graphite planes prepared. The EPG were pretreated and the resulting surface was moderately hydrophobic as indicated by the failure of a drop of water to spread across its surface. The process employed for introducing thin layers of organic solvents on a graphite electrode surface was as follows: 1.5 μL of NB solution containing the reactant of interest was introduced to the EPG surface that was in an upside-down position and the organic phase spread spontaneously across the electrode surface. The electrode was then turned over and immediately immersed in the aqueous phase. A schematic depiction of the electrochemical cell adopted was described previously.[38,39,43−45] Supporting electrolytes and reactants were introduced into the NB phase before the thin layer was placed on the electrode surface.

While in the scanning electrochemical microscopy (SECM) operation, the measurements were performed with a commercially available SECM instrument (CHI 900, CH-instrument Co. Ltd, Austin, USA). The working electrode-UME was placed in the upper phase (NB solution) throughout all the measurements, and the feedback model was applied to record the electrical signals at the tip from the redox species at the interface. The corresponding preparation and further treatment of the UME tip were described previously.[46−48] High purity nitrogen was

used for deaeration. All the experiments were conducted at the laboratory temperature (22 ± 2 °C).

3. Results and Discussion

To demonstrate the antioxidant property of AA, the ITIES was used as a simulated biological membrane to detect the process. Scheme 1 shows the simplified antioxidant process of AA in the cell. In Scheme 1A, at first the produced free radicals and reactive oxygen-derived species in the cell were deoxidized by AA. During this process AA was oxidized and the free radicals and reactive species resume their physiological function (step 1). Then the oxidized AA can be regenerated with the assistance of relative enzyme action and protect tissues and cells against oxidative damage again (step 2). The intricate reactions of step 1 could be simulated by the corresponding reaction at the electrode as shown in Scheme 1B. During the simulated reactions, zinc porphyrins with various substitutes were chosen play the role of free radicals or oxygen-derived species in the cell. Zinc porphyrin was oxidized at the electrode and then diffused to the liquid−liquid interface, which was regarded as the simulated membrane. As electron receptors, the oxidized porphyrin zinc received the electron offered from AA and regenerated its original form at the liquid−liquid interface. Corresponding reactions at the electrode are shown as follows: ZnTPP(X)$^+_{(NB)}$ + AA$_{(w)}$ → ZnTPP(X)$_{(NB)}$ + AA$^+_{(w)}$ (X = H, OCH$_3$, or OH).

3.1. Evaluated with the TLCV Method. When redox-active reactants are present in the aqueous solution beneath the thin layer of organic solvent in cells, electron transfer between reactants located in the two phases may occur and the reaction rate will be monitored electrochemically. Figure 1 is a set of current−potential responses obtained with AA and ZnTPP(OH). Dipping the graphite electrode (EPG) into an aqueous solution of 3 mM AA with 0.1 M NaClO$_4$ and 0.1 M LiCl as supporting electrolyte, before the thin layer of organic solvent was placed
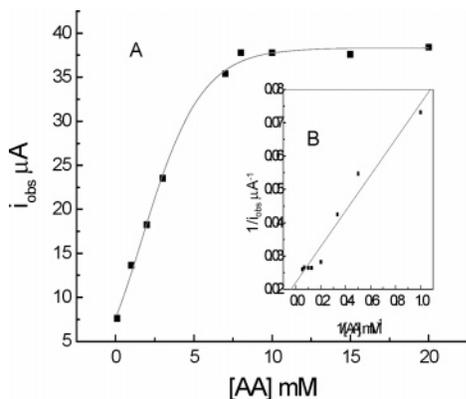
Figure 2. (A) Variation of the steady-state currents (i_{obs}) such as that in Figure 1D with the concentration of AA in the aqueous phase; 1.5 μL of NB phase contained 1 mM ZnTPP(H). (B) Reciprocal observed currents vs [AA]$^{-1}$ based on part A.

Figure 3. Effects of AA with various concentrations on the shape of the SECM current$-$distance curves. In all experiments reactants in the NB were constant and the concentrations of AA were such that the following values of Kr ($Kr = C_W/C_O$) resulted: (a) 15, (b) 10, (c) 2, (d) 0.1, and (e) 0.

on the surface of the graphite electrode, the irreversible cyclic voltammetric response of AA was observed on a bare graphite electrode as Figure 1A shows. After a thin layer of NB was placed on the electrode surface to separate it from the aqueous solution, no voltammetric current was observed for the oxidized response because any form of AA ions could no longer reach the electrode surface (Figure 1B). Figure 1C exhibited a clear, reversible cyclic voltammogram obtained when 1 mM ZnTPP-(OH) and 0.01 M TBAClO$_4$ supporting electrolyte was dissolved in the NB before the thin layer was formed on the electrode, while the aqueous solution contained only supporting electrolyte without any AA present. The reversible response near 0.77 V corresponds to the apparent half-wave potential of the ZnTPP-(OH)$^+$/ZnTPP(OH) couple. Thus, we could obtain the difference between the formal potentials of the two reactants, ΔE, which is one of the factors working on the driving force of the bimolecular reaction at the liquid$-$liquid interface. The hydrophobic porphyrin zinc molecules dissolved in the thin layer of organic solvent can be reduced and then diffused back to the electrode surface, and an antioxidant reaction may occur at the liquid$-$liquid interface when AA presented in the aqueous phase. When AA was added to the aqueous phase, repeating the operation of Figure 1C, an anodic plateau was obtained at the potential where ZnTPP(OH)$^+$ was reduced to ZnTPP(OH) as Figure 1D shows. Thus, the simulated antioxidant process was formed at the interface, where the cross-phase electron was derived from the oxidation of AA and seized by ZnTPP(OH)$^+$, which can be deoxidized as before.

The usually applied method to obtain the rate constant of the bimolecular reaction at the liquid$-$liquid interface was that values of i_{obs} were measured at several concentrations of one phase and with no change of the opposite phase.[38,39,46$-$48] In the manipulation, with a stable concentration of ZnTPP(OH) in the NB phase, concentrations of AA in the aqueous phase were increased from 0.1 to 20 mM (Figure 2A). The fitted curve showed that i_{obs} would become independent of the sufficiently high concentration of AA in the aqueous phase ($>$10 mM). That is, the concentration of ZnTPP(OH) in the NB phase becomes negligibly small and the corresponding anodic plateau current, i_D, is limited by the diffusion of ZnTPP(OH) and ZnTPP(OH)$^+$. By using the equations in refs 38 and 39, a plot of i_{obs}^{-1} versus C_{AA}^{-1} is presented in Figure 2B. The diffusion current (i_D) of the reacted system can be obtained and Table 1 shows the corresponding rate constants.

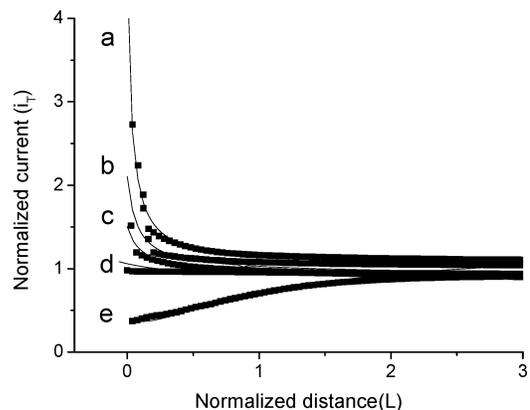The analogical reacted system of AA and ZnTPP(H) (or ZnTPP(OCH$_3$)) was studied in the same method, as shown in

the Supporting Information (Figures 1$-$4). In Table 1, it is seen that the larger driving force was obtained for ZnTPP(OCH$_3$) and ZnTPP(OH) systems, and they present bigger rate constants than the other system. This may be due to the different substitutes of the porphyrins zinc. Because the electron-withdrawing substitutes $-$OCH$_3$ (or $-$OH) will make the number of electrons in the porphyrin ring less than in porphyrin ring of ZnTPP(H), and they certainly will need a more positive potential to be oxidized, so the larger apparent half-wave potentials were exhibited subsequently. The most convincing evidence was offered by the calculated result of electron affinities of the three reactants in NB.[48] As the table shows, a greater driving force resulted in a faster rate constant. Different reactive surroundings offered by various substitutes affect the rate constant of the antioxidant reaction, and inversely, the rate constant also presented the magnitude of antioxidant capability of AA. As expected, the above results were also confirmed by the following experiments.

3.2. Appraised with SECM. The mechanical stability of the liquid$-$liquid boundary and the accuracy and reproducibility of the data obtained at two immiscible electrolyte solutions (ITIES) were studied by SECM i_T vs d curves. Furthermore, application of ITIES with fixed and adjustable interfacial potentials enables SECM measurements to be carried out over a wide range of driving forces without the limitation of the potential window.[49$-$60] In Figure 3 and the Supporting Information (Figures 5 and 6) are three sets of feedback curves of the reacted system at interface with various Kr ($Kr = C_W/C_O$); they were 15, 10, 2, 0.1, and 0, respectively (solid squares are the experimental data). At first the tip was positioned in the NB phase at a sufficiently positive potential at which metalloporphyrins can be oxidized at the UME tip and then diffused from the UME to the interface.[49] When the tip was close to the interface, tip-generated porphyrin cations received the electron derived from AA and regenerated the original porphyrins molecules in NB, thus the simulated antioxidant process was accomplished. The regenerated metalloporphyrin molecules diffused back to the tip being oxidized again as cations, and then the steady-state current was enhanced and positive feedback was obtained at the tip consequently. If no AA existed in the aqueous phase, there would not be sufficient electrons offered to deoxidize ZnTPP(H)$^+$ (or ZnTPP(OCH$_3$)$^+$ or ZnTPP(OH)$^+$) and then the molecules of porphyrin zinc cannot be regenerated at the interface. So the substrate surface only blocked mediator diffusion to the tip and appeared as an insulator and negative

TABLE 1: Parameters Obtained for the Reacted Systems at ITIES

reactant in NB[a]	E_w^f (mV)[c]	E_{NB}^f (mV)[b]	overall driving force (mV)	k_{TLCV} (cm s^{-1} M^{-1})	k_{SECM} (cm s^{-1} M^{-1})
ZnTPP(H)	710	310	400	0.57	0.076−2.315
ZnTPP(OCH$_3$)	740	310	430	0.60	0.148−5.544
ZnTPP(OH)	770	310	460	0.89	0.480−8.620

[a] Supporting electrolyte: 0.01 M TBAClO$_4$ and 1 mM metalloporphyrins. [b] Apparent formal potential of the reactant redox couple in the NB vs Ag/AgCl electrode in the H$_2$O phase. [c] Formal potential of AA in the H$_2$O phase vs a saturated Ag/AgCl electrode as measured by cyclic voltammetry at the bare EPG electrode in the H$_2$O phase. Supporting electrolyte: 0.1. M NaClO$_4$ + 0.1 M LiCl.

feedback current was observed. As presented, the magnitude of the enhanced steady-state currents at the interface depended on the concentration of AA, as the concentrations of AA decreased in aqueous the tip currents fell and the current feedback changed from positive to negative correspondingly.

Typical calculation approaches and theories conformed to the previous report.[48] A series of bimolecular reaction rate constants were obtained (0.076−2.315, 0.148−5.544, and 0.48−8.62 cm s^{-1} M^{-1}, respectively), which showed a good agreement with the value obtained by TLCV. Comparing the rate constant data listed in Table 1 shows that values of *k* obtained by TLCV were involved in the range of a series of values of *k* received by SECM, which means that the two different methods could be combined and TLCV could be used as a simple method to detect the process of electron transfer at the liquid/liquid interface. From the results, we proposed that the various substitutes have effects on the antioxidant property of AA. That is to say, the antioxidant property of AA is sensitive to the surrounding situation in the human body. So it is feasible to choose a more suitable and comfortable surrounding to keep the antioxidant property of AA working well.

4. Conclusions

In this work, simulated biologic systems were successfully used for detection of the antioxidant property of AA at ITIES under experiment conditions, and yielded good-quality voltammograms with rate constants. To our knowledge, this study is the first to investigate electron transfer between AA and zinc porphyrins with various substitutes at a simulated biological membrane. Due to the different influence of the substitutes on the electron receptors, they excellently simulated the free radicals and reactive oxygen-derived species, which seized an electron and resumed its original form. The effective heterogeneous phase rate constants for antioxidant reaction were sensitive to the changes of the driving force, which increased as the driving force increases. On the other hand, the magnitude of the rate constant accounts for the capability of AA. That is to say, as an indispensable substance in the human body, different conditions around AA molecules can affect the antioxidant property of AA during the antioxidant function to different extents. Thus, a comparatively more beneficial circumstance should be offered to keep the AA working well and demonstrate a more excellent antioxidant property in the physiological process. In addition, both applied technologies should be suitable for the investigation at simulated biomembranes and further biological investigation.

Supporting Information Available: Figures giving the voltammetric observation of electron transfer between AA and ZnTPP(H) and ZnTPP(OCH$_3$) and the variation of the steady-state currents with the concentration of AA in the aqueous phase, as well as the effects of AA with various concentrations on the shape of the SECM current−distance curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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