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Electrochemical activity of holotransferrin and its electrocatalysis-mediated process of artemisinin

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ABSTRACT

Holotransferrin, the iron (III) transport protein in the blood, can significantly increase the anticancer activity of artemisinin, which is isolated from the Chinese plant qinghaosu. This paper investigates the action process of holotransferrin-induced electrocatalytic reduction of artemisinin by spectroscopic and electrochemical techniques. Results show that holotransferrin(Fe(III)) is the electrochemical sites of holotransferrin, which can catalyze the reduction of artemisinin through lowering the overpotential by about 80 mV. Compared with the different electrochemical behaviors of artemisinin with apotransferrin and holoprotein (apotransferrin in the presence of Fe(III)), respectively, it demonstrates that holotransferrin(Fe(III)) plays an important role in the electrocatalytic reduction of artemisinin, which can catalyze the cleavage of the endoperoxide bridge in artemisinin. A reliable two-step process is proposed to explain that artemisinin is activated by holotransferrin(Fe(III))-mediated electrocatalytic reduction. These results can provide further information for better understanding the anticancer action of holotransferrin-conjugated artemisinin.

Artemisinin (qinghaosu, QHS) is a sesquiterpene lactone isolated from the Chinese plant (*Artemesia annua* L.) and is being widely used for the treatment of malaria.[1,2] The antimalarial activity of QHS is closely related to its endoperoxide bridge (Fig. 1).[3] Studies show that QHS exerts its antimalarial action by a two-step process involving in iron-mediated cleavage of the endoperoxide bridge and generation of free radicals.[4,5] In a malaria parasite, iron is present in the form of heme, derived from the proteolysis of hemoglobin in host cell.[6] QHS is first activated by intraparasitic heme-iron, which catalyzes the cleavage of this endoperoxide.[7–9] This is a key step in its antimalarial mechanism.[2,3] A resulting free radical intermediate then kills parasite by alkylating and poisoning the malarial protein.[5]

Recently, studies have shown that QHS reveals profound cytotoxic activity against tumor cells, especially in the presence of holotransferrin.[10–12] Holotransferrin (holo-TF), a Fe(III)-binding transferrin (Fig. 2), is a suitable ligand for targeted drug delivery, since it can be specifically recognized and taken up by transferrin receptors actively expressed on the surface of most tumor cells.[13–15] In the presence of holo-TF, dihydroartemisinin, an analogue of QHS, selectively killed molt-4-lymphoblastoid cell (a human leukemia cell line), whereas the same treatment was significantly less toxic to normal human lymphocytes.[16] Incubation with dihydroartemisinin alone was found to be less effective than in combination with holo-TF,[17,18] indicating that holo-TF played an important role in the enhancement of anticancer activity of QHS. While studies have shown that holo-TF enhances the anticancer activity of QHS,[11,18] the mechanism of such an action is far from fully understood. The anticancerous mechanisms of QHS in the presence of holo-TF seem to involve many different pathways. One is that QHS specifically kills cancer cells via a receptor-mediated endocytosis.[18] Another possible one is that holo-TF catalyzes

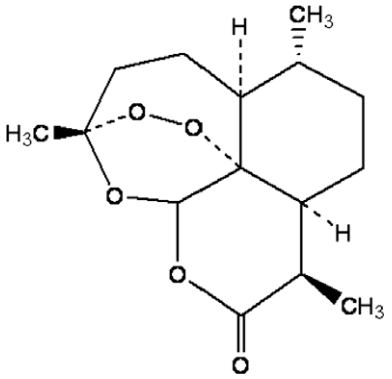

Figure 1. Chemical structure of artemisinin.

Abbreviations: QHS, artemisinin; holo-TF, holotransferrin; apo-TF, apotransferrin, holoprotein, apotransferrin in the presence of Fe(III); holo-TF(Fe(III)), holotransferrin(Fe(III)).

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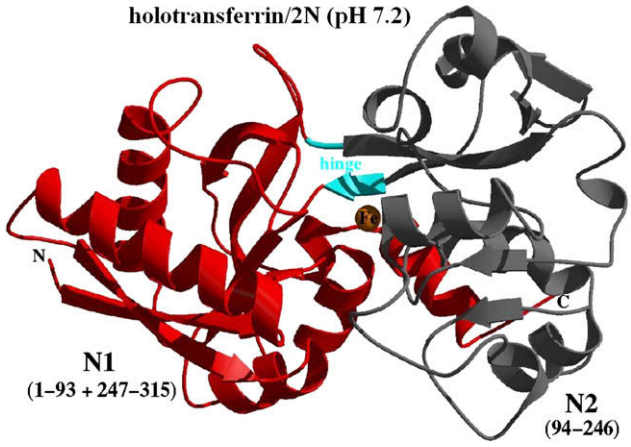


Figure 2. Ribbon structure of the N-lobe of human serum holotransferrin. (Color figure is available at http://blood.uvm.edu/lab/everse_bio.html.)

the cleavage of the endoperoxide of QHS, which will be similar to the antimalarial activity of QHS mediated by electrocatalysis of hemin. To the best of our knowledge, there are no related reports concerning whether holo-TF can induce the cleavage of the peroxide bond of QHS. Therefore, exploring the catalytic reduction of QHS by holo-TF could be an approach to uncovering the anticancer mechanism of QHS.

Electrochemical methods have been widely applied in investigating electrocatalytic effect of protein with drug because it is sensitive, rapid, and easy to use.[19,20] In electrochemical system, working electrode is considered to be suitable media for studying biocatalytic process in vitro because of its good biocompatibility and high electron-transfer rates.[8] In previous studies, the antimalarial mechanism of QHS induced by electrocatalysis of hemin or hemoglobin has been investigated by electrochemical methods.[7–9] In this paper, holo-TF-mediated activation of QHS has been investigated by electrochemistry and UV–vis absorption spectroscopy. Furthermore, a reliable electrocatalytic mechanism is proposed to elucidate that holo-TF induces cleavage of the endoperoxide bridge in QHS. These results will contribute to better understanding of the anticancer mechanism of QHS when it is incubated with holo-TF.

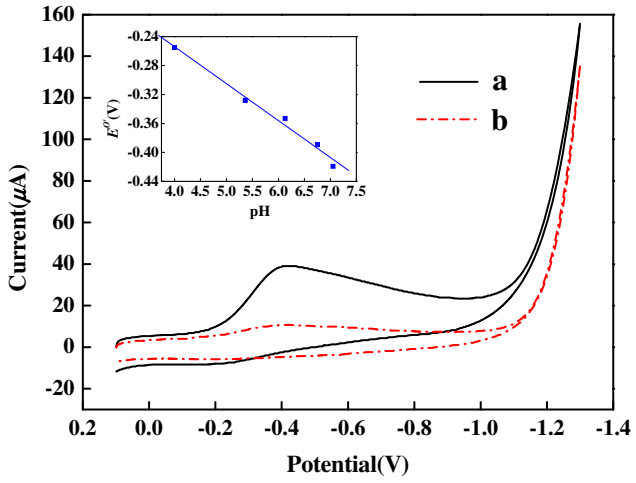


Figure 3. Cyclic voltammograms of (a) 5.0 μM holo-TF and (b) 5.0 μM apo-TF in buffer solution (0.1 M phosphate-buffered saline at pH 7.2). Inset: plots of the apparent standard potential ($E^{0\prime}$) vs pH value. Scan rate: 100 mV s^{-1}. Supporting electrolyte = buffer solution.

Electrochemical characteristics of holo-TF. Direct electrochemical behavior of holo-TF[21] was first studied with cyclic voltammetry.[22] Holo-TF showed an irreversible cathodic peak at −0.42 V at bare silver electrode (Fig. 3, curve a), attributing to the reduction of holo-TF. However, the corresponding cyclic voltammogram of apo-TF,[21] an iron-free transferrin,[14,15] showed no electrochemical response (Fig. 3, curve b). This indicates that the electroactive site of holo-TF is attributed to holo-TF(Fe(III)). The cathodic peak currents (i_p) of holo-TF linearly increased with the scan rate (v) in the range of 25–100 mV s^{-1} with regression coefficient of 0.997 ($n = 5$), which was characteristic of an adsorption-controlled process.[23] The logarithm of peak currents ($\log i_p$) vs logarithm of scan rate ($\log v$) showed linear relationship with a slope of 1.03 for cathodic currents in the range of v from 25 to 100 mV s^{-1}. This value is very close to the theoretical value of 1 expected for thin-layer electrochemical behavior,[23] indicating that the electroactive site of holo-TF, holo-TF(Fe(III)), can be reduced to holo-TF(Fe(II)) on the forward cathodic scan. The electrochemical behavior of holo-TF showed a strong dependence on the pH value of buffer solution.

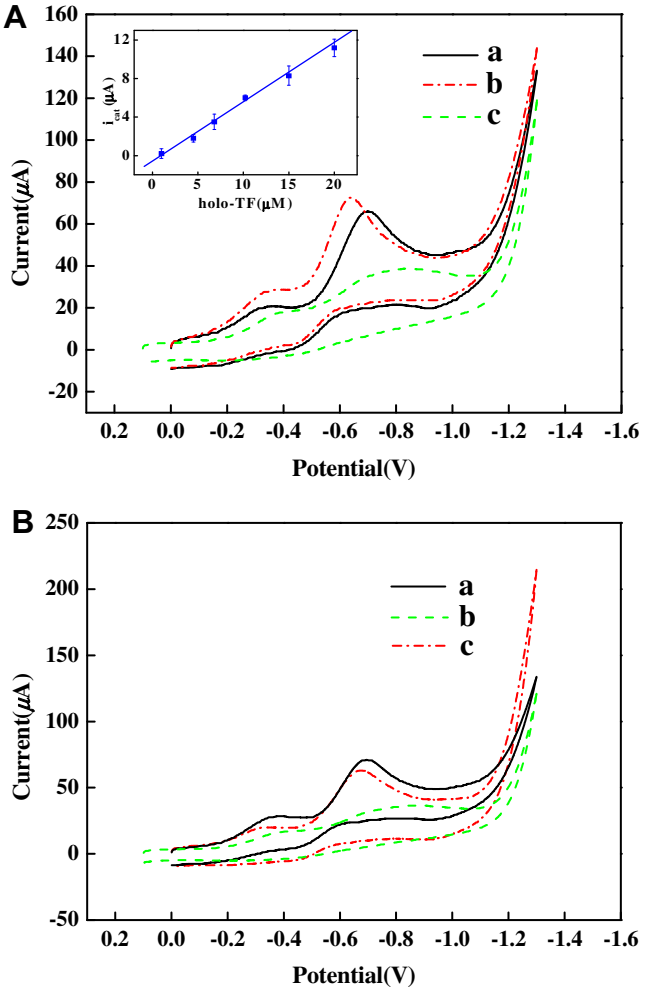


Figure 4. (A) Cyclic voltammograms of 500.0 μM QHS with (a) 0, (b) 10.0 μM holo-TF, and (c) 10.0 μM apo-TF in buffer solution (0.1 M Britton–Robinson buffer containing 50% ethanol at pH 7.2). Inset: plots of the electrocatalytic current (i_{cat}) vs holo-TF concentration. The error bars represent the standard deviation calculated form at least three independent measurements. Scan rate: 100 mV s^{-1}. Supporting electrolyte = buffer solution. (B) Cyclic voltammograms of 500.0 μM QHS (a), 50.0 μM Fe(III) in the presence of 500.0 μM QHS solution containing 10.0 μM apo-TF (b), and 10.0 μM holoprotein in the presence of 500.0 μM QHS (c) in buffer solution (0.1 M Britton–Robinson buffer containing 50% ethanol at pH 7.2). Scan rate: 100 mV s^{-1}. Supporting electrolyte = buffer solution.

With the increase of pH value, the cathodic peak potential of holo-TF shifted negatively, demonstrating that proton existed in the electron transfer process of holo-TF. In the pH range from 4.0 to 8.0, the apparent standard potential ($E^{0\prime}$) showed a linear dependence on the pH value with a slope of −51 mV/pH (inset of Fig. 3), which is reasonably close to the theoretical value of −59 mV/pH for the one-electron-transfer coupled by single proton transportation.[24] This indicates that a single proton accompanying a single electron transfer occurs between holo-TF(Fe(III)) and electrode surface. Based on the above results, the electrochemical reduction of holo-TF can be represented by the equation:

$$\text{holo-TF(Fe(III))} + H^+ + e^- \rightarrow \text{holo-TF(Fe(II))},$$

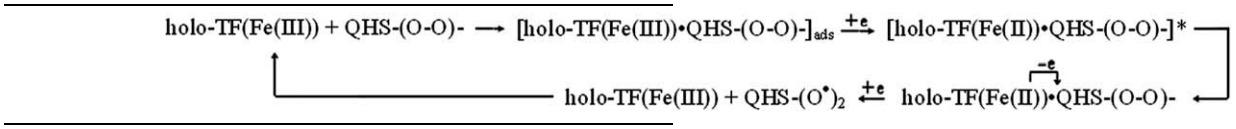
where the charges on holo-TF are omitted.

Electrocatalysis of QHS by holo-TF. Electrocatalytic effect of holo-TF towards QHS was investigated by electrochemical methods, such as cyclic voltammetry (Fig. 4). An irreversible reduction peak at −0.70 V (Fig. 4A and B, curve a) was observed for QHS solution,[25] attributing to its peroxide bond.[7,8] The gradual addition of holo-TF induced a positive shift of the cathodic peak of QHS to −0.62 V, accompanying the increase in the cathodic peak currents (Fig. 4A, curve b). This indicates that holo-TF catalyzes the reduction of QHS, resulting in the cleavage of endoperoxide bridge. Holo-TF lowered the cathodic overpotential for reduction of QHS by 80 mV, demonstrating a decrease in activation energy for this system. A plot of cathodic peak current (i_{cat}) of QHS vs holo-TF concentration was obtained with cyclic voltammetry. Here the i_{cat} value is defined as the difference between i_p in the presence of holo-TF and i_p in the absence of holo-TF. The i_{cat} values had a linear relationship with holo-TF concentration in the range of 1.0–20.0 μM with a detection limit of 0.2 μM (inset of Fig. 4A). Peak currents at −0.62 V enhanced linearly with increasing scan rates in the range of 25–100 mV s^{-1}, demonstrating that the interaction of QHS with holo-TF on electrode surface was an adsorption-controlled process. The catalytic efficiency expressed as the ratio of the reduction peak current of QHS in the presence (I_c) to the absence of holo-TF (I_d), that is I_c/I_d, decreased with the increase of scan rates (50–500 mV s^{-1}), which was also a characteristic of electrocatalytic reduction[26] of QHS by holo-TF. Fixing the concentration of holo-TF and increasing QHS concentration induced the decrease in the peak current of holo-TF, accompanying the positive shift of peak potential of QHS.[27] This further confirms that Fe(III)-binding holo-TF can catalyze the cleavage of the endoperoxide of QHS.

Control experiments were carried out to further investigate the electrocatalytic mechanism of QHS by holo-TF. Electrochemical behaviors of QHS with apo-TF, and holoprotein (apo-TF in the presence of Fe(III))[28] were investigated by cyclic voltammetry, respectively. The addition of apo-TF to the QHS solution induced that the cathodic peak of QHS negatively shifted to about −0.86 V (Fig. 4A, curve c), demonstrating that an adduct was formed between QHS and apo-TF,[24,27] correspondingly, apo-TF could not catalyze the reduction of QHS. Interestingly, after addition of Fe(III) in the QHS solution containing apo-TF, positive shift of peak potential was not observed (Fig. 4B, curve b), indicating that subsequent addition of Fe(III) could not catalyze the reduction of QHS. This is attributed to the formation of an adduct between QHS and apo-TF, which can hinder the electrocatalytic activity of Fe(III) towards the reduction of QHS. Furthermore, the effect of holoprotein on the reduction of QHS is investigated. When holoprotein was added to the QHS solution, the reductive peak potential of QHS positively shifted from −0.70 to −0.67 V (Fig. 4B, curve c), accompanying the increase in peak current. Holoprotein lowered the overpotential for the reduction of QHS by 23 mV, indicating that holoprotein could catalyze the cleavage of peroxide bridge in QHS. Compared with the interaction of QHS with apo-TF, the electrocatalytic center of holoprotein towards QHS is ascribed to holoprotein(Fe(III)). The catalytic efficiency (I_c/I_d) decreased with the increase of scan rates (50–500 mV s^{-1}), which was also a characteristic of holoprotein(Fe(III))-catalyzed reduction of QHS. Control experiments further indicate the electroactive center of holo-TF, holo-TF(Fe(III)), plays an important role in the electrocatalytic reduction of QHS.

UV–vis absorption investigation can provide a useful complement to electrochemical method. QHS alone does not show any absorption, whereas holo-TF has the absorption signal at the wavelength of 280 nm, corresponding to the chromophores of protein. Addition of QHS resulted in the decrease of the absorbance of holo-TF at 280 nm, whereas no new absorption band was observed (Fig. 5). This indicates that the interaction of QHS with holo-TF takes place by the formation of an intermediate adduct, while the iron-bound structure of holo-TF (holo-TF(Fe(III))) does not been disturbed. This is supported by our previous study that QHS only induces the interchromophoric conformation change of holo-TF, whereas the local structure of iron-binding sites cannot be intervened.[27] Compared with spectroscopic characteristics of QHS with hemin or hemoglobin,[7,8] it further demonstrates that an intermediate adduct is formed in the process of holo-TF-induced electrocatalytic reduction of QHS.

Therefore, based on electrochemical and spectroscopic results, holo-TF-mediated electrocatalytic process of QHS at electrode surface can be considered as a sequential two-step process, which can be explained as follows:



First, holo-TF interacts with QHS to form an adduct [holo-TF(Fe(III))·QHS–(O–O)–]$_{ads}$, which is adsorbed on electrode surface. Second, holo-TF(Fe(III)) obtains one electron and then forms an intermediate adduct [holo-TF(Fe(II))·QHS–(O–O)–]*, which induces

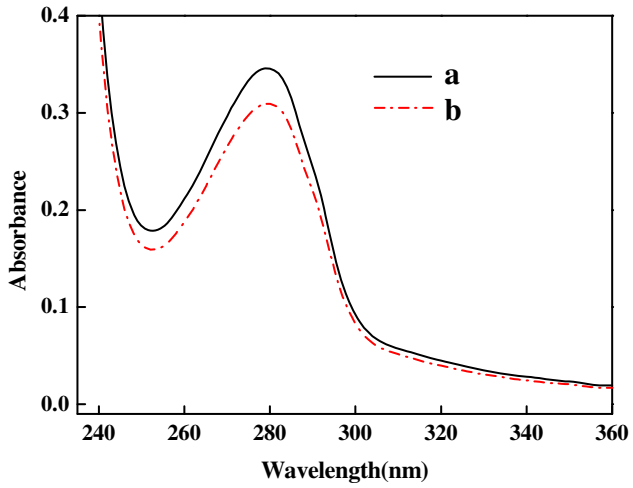


Figure 5. UV–vis absorption spectra of (a) 5.0 μM holo-TF and (b) 10.0 μM QHS in the presence of 5.0 μM holo-TF in 0.1 M phosphate-buffered saline (pH 7.2).

the cleavage of the peroxide bond in QHS and then generates QHS–$(O^{\cdot})_2$ free radicals. These indicate that QHS is activated by holo-TF(Fe(III))-mediated electrocatalytic reduction, which is similar to previous reports of the antimalarial mechanism of QHS.[7–9]

In summary, electrochemical and spectroscopic data show that QHS can be reduced via holo-TF-mediated electrocatalysis by lowering the overpotential. It allows us to propose a potential pathway to explain the anticancer mechanism of QHS in the presence of holo-TF. Holo-TF-conjugated QHS initially can be efficiently taken up by cancer cells via receptor-mediated endocytosis,[14,15] subsequently, QHS is activated by the cleavage of the endoperoxide with holo-TF-induced catalysis. Since holo-TF is a natural component in the blood, it is expected that the electrocatalytic reduction of QHS by holo-TF can remain longer so that it can kill cancer cells more effectively. This may also help to explain the previous reports of the combined incubation in QHS and holo-TF can significantly increase the anticancer activity of QHS.[11,16] These results indicate that holo-TF(Fe(III))-mediated cleavage of the peroxide bond of QHS is likely to be one of the anticancer mechanisms, which provides a new understanding of the anticancer mechanism of holo-TF-conjugated QHS.

Acknowledgments

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21. Purified human holotransferrin (holo-TF, purity >98%, iron content: 1100–1600 μg/g) and apotransferrin (apo-TF, purity >98%, iron content <0.005%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The purity and iron-load of the protein were checked by an SDS–polyacrylamide gel electrophoresis and a polyacrylamide–urea gel electrophoresis, respectively.
22. Cyclic voltammetric measurements were performed on a CHI660A electrochemistry workstation (CH Instruments Co., USA). The electrochemical cell consists of a three-electrode system, a silver electrode (with 3 mm diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire as the counter electrode. All solution was deoxygenated using highly pure nitrogen for 15 min and the nitrogen atmosphere was maintained over the solution during the measurements.
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28. Holoprotein (apotransferrin in the presence of Fe(III)) was obtained in the presence of 20 mM biocarbonate when apo-TF was allowed to interact with $FeCl_3$. Residual free ions in solution were removed by dialysis of the solution using a cellulose membrane (MWCO = 3500) against nanopure water overnight.