

Comment on "Direct Electrochemistry and Electrocatalysis of Heme Proteins Entrapped in Agarose Hydrogel Films in Room-Temperature Ionic Liquids"

A recent article by Wang et al. [Wang, S.-F.; Chen, T.; Zhang, Z.-L.; Shen, X.-C.; Lu, Z.-X.; Pang, D.-W.; Wong, K.-Y. *Langmuir* **2005**, *21*, 9260–9266] described the electrochemistry and electrocatalysis of heme proteins entrapped in agarose films in ionic liquids. We wish to comment on the experimental results and on their proposed interpretation.

Wang et al. state that "The redox potentials for hemoglobin, myoglobin, horseradish peroxidase, cytochrome *c*, and catalase (in agarose films) were found to be more negative than those in aqueous solutions".¹ The redox potentials reported for HRP, hemoglobin, myoglobin, and cytochrome *c* are –320 mV, –348 mV, –430 mV, and –522 mV versus Ag|AgCl, respectively, in agarose films¹ and –470 mV,² –62 mV,^{3,4} –160 mV,^{3,5} and 60 mV,⁶ respectively, in aqueous solution. Clearly, the statement made by Wang et al. is not correct in the case of HRP; more importantly, the large decreases in the redox potential observed with hemoglobin, myoglobin, and cytochrome *c* are indicative of substantial structural changes.

The similarity in the reported redox potentials of the very different heme proteins HRP, hemoglobin, and catalase within the agarose films measured in ionic liquids (Table 2, in ref 1) is striking. Moreover, the redox potentials reported are very close to the redox potential of immobilized heme on graphite (–350 mV⁷), and gold electrodes (–290 to –330 mV⁸). This similarity in the values of the redox potentials suggests that it is the response of the heme itself (either partially or fully removed from the protein during the immobilization process) that is being measured. The small differences in the redox potentials of the different proteins in the agarose film can be explained by the presence of different apoproteins in the modifying mixture after the heme was fully or partially removed.⁹ While it is unlikely that the covalently attached heme in cytochrome *c* is removed, the large decrease in its redox potential is indicative of a significant change in the protein structure, as it is known that the redox potential is reflective of the conformational changes in the protein structure. Recent debate on the issue of whether myoglobin retains its heme group when placed in lipid films on graphite electrodes is further evidence of the degree of uncertainty in this area.^{9–11}

Using Laviron's model,¹² the rate of electron transfer for Fe³⁺/Fe²⁺ conversion of the immobilized proteins was also reported. However, Wang et al. incorrectly applied one limiting case of

Laviron's model, where $n\Delta E \geq 200$ mV (n is the number of electrons transferred, and ΔE is the peak separation in mV), to their data where peak separations reached only 81–114 mV. In such cases where $n\Delta E \leq 200$, increases in the scan rate will not produce corresponding peak separations, and another version of Laviron's model should be used.

The establishment of direct electrochemical communication between the redox center of an immobilized protein and the electrode surface is crucial in order to be able to use such electrodes for the construction of bioanalytical and other devices such as biofuel cells and bioswitches. In many cases, it can be experimentally difficult to prove that the protein retains its intact structure; for example, graphite is often used as the electrode material, and many spectroscopic methods cannot be used because of its inherent lack of transparency. Figures 1 and 2 (ref 1) present FTIR and UV–vis spectra of films of dried hemoglobin, hemoglobin in an agarose film, and hemoglobin in an agarose film containing DMF. From the similarity of the spectra, the authors concluded that the structures of the heme proteins are virtually intact within these films. However, the UV–vis spectra alone (especially when only represented by the Soret band) cannot serve as proof of intact protein structure; visible spectra are necessary but not sufficient proof of the structural integrity of the protein. Furthermore, a comparison was not made with the spectra of aqueous solutions of hemoglobin, and, even more importantly, the spectra of the protein–agarose films were not shown after the exposure to the ionic liquid. The Soret peak maximum shifted by 2 nm for the hemoglobin–agarose–DMF film, and the intensity decreased significantly, both of which may suggest more pronounced structural changes. Indeed, Li et al. previously showed that even rather minor changes (2 nm blue shift for Mb in DMSO) in the visible spectra may be indicative of alterations in the protein conformation,¹⁴ and more spectral evidence is needed in Wang's case to prove that the protein structure is intact. The CT- and Q-bands were not provided in Wang's paper; the position and presence of these bands is also a useful indicator of protein structural changes.

FTIR, which can be used to probe protein secondary structure, also showed noticeable shifts in the amide bands of hemoglobin, mainly in the hemoglobin–agarose–DMF film, indicating that an alteration in the structure of hemoglobin may have occurred and that DMF did not strengthen "the hydrophobicity of the micro-environment, probably through the formation of hydrogen bonds between DMF and agarose or Hb". Furthermore, cytochrome *c* showed a 5 nm blue shift in comparison to the published value of 410 nm for the Soret band,⁶ indicative of a change in the protein structure or protein environment, which could be in agreement with the unusually large shift in the redox potential observed by cyclic voltammetry. Another difficulty that arises is whether it is appropriate to compare the spectra of films on glass slides to the situation of the protein within the film on glassy carbon. Carbonaceous materials adsorb proteins readily, but the immobilization of a protein by adsorption may often lead to protein denaturation or structural changes.^{7,15} In the case of a thin film, all the protein present on the electrode surface might be affected, if the protein layer is several micrometers thick (which we expect in Wang's case), then the protein in the

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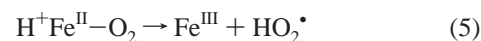
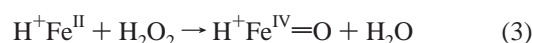
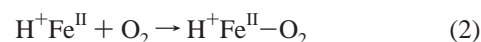
innermost layer, responsible for the electron transfer with the electrode, could be unfolded as a result of the influence of the electrode material.

A major challenge in the electrochemistry of redox proteins lies in obtaining direct electron transfer between the electrode and the protein at a bare electrode surface. Wang et al. reported that small peaks, ascribed to the $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox process, were observed even with bare glassy carbon electrodes in phosphate buffer. The value of the redox potential was not reported and compared with the E° of HRP in agarose film in ionic liquids. It does not seem plausible that these peaks can be attributed to the direct electrochemical oxidation and reduction of HRP, where the heme redox center is deeply buried in the insulating protein and polysaccharide matrix. Cyclic voltammetry of recombinant HRP containing a His-tag attached to the C-terminus ($\text{C}_{\text{His}}\text{HRP}$) revealed an E° of -220 mV at pH 6.5 versus $\text{Ag}|\text{AgCl}$ when adsorbed onto a gold electrode,¹⁶ a value that is also much higher than that reported for HRP in solution (-470 mV vs $\text{Ag}|\text{AgCl}$).² However, the electrode modified with $\text{C}_{\text{His}}\text{HRP}$ exhibited electrocatalytic reduction of H_2O_2 at $+550$ mV versus $\text{Ag}|\text{AgCl}$,¹⁷ indicative of the formation of compound I/II, in contrast to the catalytic wave reported by Wang (see discussion below). Comparative cyclic voltammograms with recHRP (without a His-tag) and native HRP showed no clear redox waves, indicating that the orientation of the adsorbed enzyme is crucial in achieving efficient direct electron transfer between the enzyme and the electrode.

Various modifying or supporting layers are often used with redox proteins in order to obtain direct electron transfer between the heme and the electrode.^{18–23} Direct electrochemistry was readily observed in these reports, with redox potentials for the $\text{Fe}^{3+}/\text{Fe}^{2+}$ conversion being similar to potentials reported by Wang et al. In many cases, water miscible organic solvents are used to prepare the electrodes. Figure 6 (ref 1) compares the response of electrodes modified with myoglobin–agarose film in the absence and presence of DMF. DMF was used to form a more uniform film of the protein and agarose on the electrode surface; the response of electrodes prepared in such a manner was significantly better. Because DMF has a higher boiling point than water (153 °C²⁴), the concentration of DMF will increase in the protein mixture as the electrode is dried. The observed improvement in the response may arise from free heme extracted from the protein as a result of the high concentration of the organic solvent. As an example, it is well-known that the heme of HRP is easily extracted from the enzyme in the presence of an organic solvent, especially in acidic conditions. Apo-HRP is readily produced by extracting the heme from the holoenzyme with butanone.²⁵ When using propanediol as a stabilizer in the adsorption of various peroxidases onto carbon electrodes, all electrodes revealed very similar E° values, indicating that the heme was extracted during the curing process as water evaporated from the enzyme–water–propanediol solution.⁷ Such electrodes also lost their ability to electrocatalytically reduce hydrogen

peroxide at high potentials (through the formation of compound I/II), and a catalytic effect was only seen close to the E° value of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox conversion. Mondal et al. studied cytochrome *c* peroxidase and found that, when the enzyme was adsorbed on carbon in an active form, electrocatalytic reduction of H_2O_2 was observed close to the E° values of compound I/II (around $+650$ mV vs $\text{Ag}|\text{AgCl}$).^{26,27} No electrocatalytic current at positive potentials was observed by Wang et al., which, if present, could be clearly detectable at $+200$ mV on Figures 10 and 11 (ref 1).

Wang et al. report the electrocatalytic reduction of *t*-BuOOH (*tert*-butyl hydroperoxide). Similar behavior was previously reported for a variety of heme-protein electrodes.^{18,20,23,28–31} However, in these reports, considering the potentials involved (around -200 to -300 mV vs $\text{Ag}|\text{AgCl}$) and the absence of catalytic current at positive potentials, the reported bioelectrocatalysis is evidently based on the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple (1) reacting with hydrogen peroxide. The product of this reaction is $\text{H}^+\text{Fe}^{\text{II}}-\text{O}_2$ (compound III) when oxygen is present (2), and $\text{H}^+\text{Fe}^{\text{IV}}=\text{O}$ (compound II) when hydrogen peroxide is present (3). Compound II is transformed to compound III by reaction with an additional molecule of hydrogen peroxide (4). Compound III is catalytically inactive, but can be reduced back to the ferric form (5),³² according to the alternative side reaction scheme



The interpretation of Wang et al.¹ and of previous reports^{18–21,23,28–31} cannot be described by the classical peroxidase cycle involving compound I and II formation. The catalytic current observed by Wang and others can be better described as a pseudocatalytic process based on the $\text{Fe}^{3+}/\text{Fe}^{2+}$ heme reaction, where the Fe^{2+} form reacts with H_2O_2 but do not involve compound I or II. The reaction scheme above also explains the very narrow linear concentration range of these peroxide biosensors (obtained at the potentials of $\text{Fe}^{3+}/\text{Fe}^{2+}$ conversion), as inactivation of the heme protein is expected under such conditions. Peroxidative enzymatic catalysis involves the formation of compounds I and II,³² whose interconversion occurs at potentials of ca. $+700$ mV versus $\text{Ag}|\text{AgCl}$ at pH 7.³³ When peroxidative enzymatic reduction is detected electrochemically, an increase in the catalytic reduction current is observed between 600 mV and 0 mV versus $\text{Ag}|\text{AgCl}$.^{34–36}

The values for the Michaelis constant, K_m , were evaluated for the oxidation of TCA and *t*-BuOOH catalysis by various proteins. However, as described previously for HRP,³² the use of the

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parameters K_m (and k_{cat}) is inappropriate for peroxidase kinetics because the peroxidase enzymatic cycle consists of three, usually irreversible, reactions, with no upper rate limitations.³² A similar situation occurs with catalases, where the enzyme can neither be saturated with its substrate hydrogen peroxide before it is inactivated, nor form a true enzyme–substrate complex preceding compound I formation.³⁷ Moreover, the reported values of K_m and I_{max} for all five proteins,¹ although inappropriate, are of the same order of magnitude, and do not by any means reflect the biological diversity of those five proteins.

In conclusion, Wang et al. have demonstrated a catalytic response using protein modified electrodes in ionic liquids.

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However, the response cannot be interpreted as peroxidative catalysis involving compounds I and II. We suggest that heme, which may be fully or partially removed from the protein, is responsible for the observed electrochemical reactions.

Acknowledgment. The authors thank the Irish Research Council for Science, Engineering and Technology and the Swedish Research Council for financial support.

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LA061336A

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