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ORIGINAL PAPER

Influence of ultrasound irradiation on the adsorption of bovine serum albumin on copper

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Abstract The effect of irradiation by power ultrasound on the adsorption of proteins on copper has been investigated, using bovine serum albumin (BSA) as a model protein in pH 7 phosphate buffer solution. Open circuit potential measurements, cyclic voltammetry and electrochemical impedance spectroscopy were used to characterise the copper/solution interface. Electrochemical impedance measurements at potentials close to the open circuit potential showed that pulsed ultrasound irradiation removes the naturally formed copper oxide films in phosphate buffer solution, and that their re-formation can lead to an oxide film with different electrical characteristics. Adsorption of BSA blocks the surface, decreasing or increasing the interfacial resistance, depending on the applied potential and the oxide characteristics, as well as changing the interfacial capacitance. This study augurs well for application of the combination of electrochemical impedance plus ultrasound to other systems.

Keywords Ultrasound irradiation · Power ultrasound · Copper · Bovine serum albumin · Electrochemical impedance spectroscopy

1 Introduction

Bovine serum albumin (BSA) is the most abundant protein in the blood stream, playing an important role in many biological processes in living creatures [1, 2]. It is the principal carrier of fatty acids in circulating plasma, sequestering oxygen free radicals, deactivating various toxic lipophilic metabolites, and forming covalent adducts with various metal ions (Cu^{2+}, Ni^{2+}, Hg^{2+}, Ag^{+}, Au^{III}). It is also a reservoir of nitric oxide NO, which has been implicated in a number of important biological processes, including neurotransmission, pH blood control, and blood pressure [3].

The process of BSA adsorption on surfaces, such as on polymers or metals, has been investigated extensively, BSA being used as a model protein. For example, a recent publication reported results of the adsorption of BSA on polypyrrole, in the presence of surfactants to control the hydrophobicity of the polymer surface [4]; in other studies, adsorption on gold and platinum were investigated [5], as well as on gold modified with self-assembled alkanethiol monolayers [6, 7]. In these cases, both the quartz crystal microbalance and electrochemical impedance (or cyclic voltammetry in the case of [6]) were employed. BSA has also been suggested as a corrosion inhibitor and its effects on oxide passivation phenomena have been probed [8, 9]. Other investigations have been specifically focused on the interactions of BSA with copper, which is helpful for the understanding of biological phenomena related to covalent copper adduct formation [10–12].

Ultrasound irradiation techniques, using power ultrasound through horn probes, have been shown to be very useful for aiding in probing and reducing adsorption phenomena of organic matter [13], biomolecules [14] and even bacteria [15], as well as in investigating metallic corrosion

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phenomena [15–17]. Additionally, power ultrasound can influence the properties of oxide layers [16] and of coatings obtained by electroplating and electroless plating [18].

The combination of electrochemical impedance spectroscopy (EIS), an important tool for the study of the processes occurring at the electrode/electrolyte interface, and ultrasonic irradiation can be expected to provide important information concerning interfacial characteristics and their variation with time—electrochemical behaviour, changes in the surface properties and kinetics of adsorption processes [14].

The objective of this study was to show how EIS and other electrochemical techniques can be combined with ultrasound irradiation in order to throw light on processes occurring at metal–solution interfaces related to adsorption and specific metal–protein interactions, using copper and BSA as a model system. Interactions between these two components are regarded as important in biological systems [10]. For this purpose, BSA was allowed to adsorb on the surface of naturally oxidised pure copper immersed in pH 7 buffer electrolyte. Ultrasound irradiation was applied directly above the copper surface using a horn probe to remove the adsorbed BSA protein films and oxide film, which could then be re-formed. EIS was used to investigate the characteristics of the interface before, during and after ultrasound application, in a similar way as in previous corrosion studies [14, 16]. In general, chemical as well as physical properties, such as film adherence, presence of defects, barrier properties, inhibition, etc., influence the electrochemical response. Open circuit potential (OCP) measurements and cyclic voltammetry provide additional information and were also used in this study.

2 Experimental

2.1 Reagents and solutions

A stock solution of 1 g L^{-1} BSA in 0.1 M phosphate buffer solution pH 7 was prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate (Riedel-de-Haën, Germany). Electrochemical voltammetric and EIS experiments were performed in a glass cell containing 40 mL of solution. Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm) was used for the preparation of all solutions. Experiments were performed at room temperature in aerated solution, 25 ± 1 °C.

2.2 Electrodes

Copper disc electrodes, geometric area 0.20 cm^2, were made from copper cylinders (99.99% purity (Goodfellow Metals, Cambridge, UK) by sheathing in glass and epoxy resin and connecting the rear face to a copper wire with silver epoxy resin. Surfaces were prepared by polishing with a 1 μm diamond spray (Kemet, UK). The copper electrode was held in an inverted position with the exposed disc surface facing upwards in the electrochemical cell, to allow ultrasound irradiation. The cell also contained a Pt foil counter electrode of area 1 cm^2 and a saturated calomel electrode (SCE) was used as reference.

2.3 Instrumentation

Cyclic voltammetry was performed using a EG&G-PAR, model 273 potentiostat/galvanostat, in the potential range −0.2 to 1.0 V vs. SCE, with a scan rate of 2 mV s^{-1}.

Electrochemical impedance spectra were recorded using a Solartron 1250 Frequency Response Analyser coupled to a Solartron 1286 Electrochemical Interface (Solartron Analytical, UK), controlled by ZPlot 2.4 software (Scribner Associates, USA). A sinusoidal perturbation of 10 mV rms was applied over the frequency range 65.5 kHz–1 Hz, in 10 steps per decade, at potentials OCP −40 mV, OCP, and OCP +40 mV, with 60 s auto-integration. Extension of the spectra to lower frequencies is not shown, since longer signal acquisition times were found to be needed to give reproducible data. This was not possible during the time during which ultrasound irradiation was applied; it was decided to always use the same experimental conditions for comparative purposes.

Ultrasound irradiation was applied using a VCX-750 sonic horn (Sonic Materials Inc., USA) working at 20 kHz frequency, with a Vibracel titanium micro tip of 3 mm diameter held at a distance of 10 mm from the copper electrode surface in solution. Pulsed irradiation was employed in order to minimise heating effects of the electrode and solution. Consecutive pulses were applied of 2 s irradiation followed by a 1 s quiet period during a total of 420 s, at a power of 24.4 W cm^{-2}, the power being calibrated by the calorimetric method described in [16].

3 Results and discussion

3.1 Cyclic voltammetry

Cyclic voltammetry was carried out in the potential range −0.2 to +1.0 V vs. SCE in order to study the electrochemical behaviour of copper in phosphate buffer solution, in particular the formation of different characteristic copper oxide species (CuO, Cu_2O) and Cu^{2+} release into solution at potentials more positive than 0 V.

In Fig. 1a are shown five consecutive cyclic voltammograms in the absence of BSA. In the first cycle, it can be observed that above 0 V the current increased owing to CuO formation and dissolution to form Cu^{2+} ions. In the following cycles the current was lower and reached a steady state, suggesting some restructuring of the surface due to oxidation and reduction of copper-containing species.

The effect of adding BSA to the phosphate buffer can be seen by comparison of the cyclic voltammograms of Fig. 1b. The current values were reduced to approximately half of the initial values, indicating that the BSA molecules adsorbed on, and blocked, the copper surface, inhibiting the oxidation process and consequently decreasing the corresponding oxidation and reduction currents.

These data are in agreement with open circuit potential profiles shown in Fig. 2. In both cases, there was an initial increase of potential upon immersion of a freshly polished electrode, probably due to oxide formation. There was then a decrease until a steady state value was reached, close to +0.108 V vs. SCE, probably due to some active copper corrosion and substitution of CuO by Cu_2O species [19, 20]. In the presence of BSA in solution, there were fewer oscillations in the response and the open circuit potential reached a steady state more quickly. This can probably be ascribed to the surface being less accessible to the formation of oxide owing to the blocking by BSA.

3.2 Electrochemical impedance

EIS experiments were carried out before ultrasound irradiation, during ultrasound irradiation and afterwards, at various potentials defined with respect to the steady-state OCP of the system without BSA. A value of the OCP of +0.108 vs. SCE, for the copper electrode in 0.1 M phosphate buffer pH 7.0, was obtained at steady state. Figs. 3–5 show spectra obtained at OCP −40 mV, OCP and OCP +40 mV, i.e. at 68, 108 and 148 mV vs. SCE. Other spectra were obtained at 20 mV intervals but will not be discussed here since the objective of the study is to show the potential of the combination of techniques.

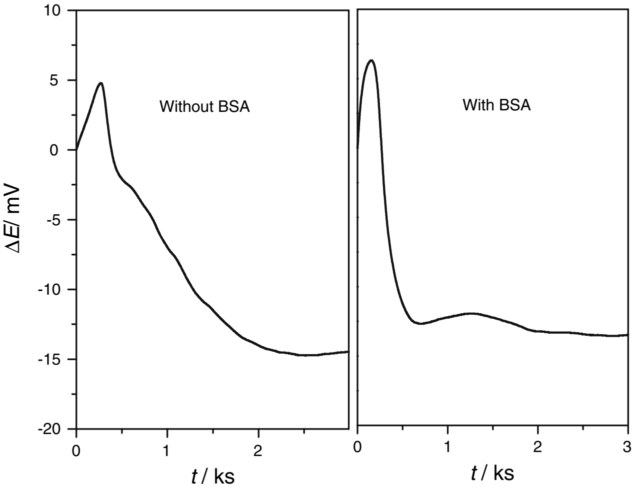
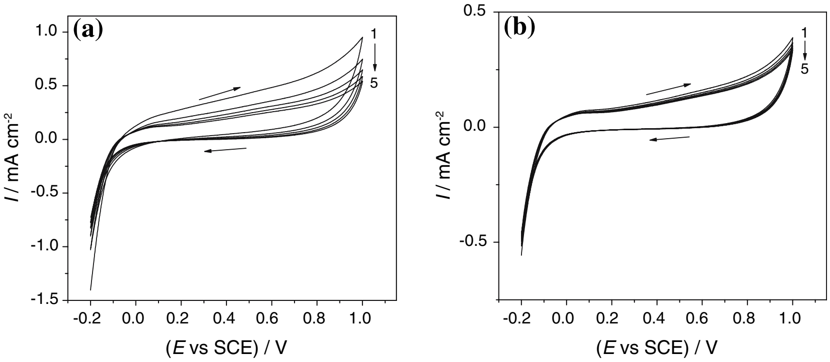


Fig. 2 Variation of open circuit potential with time in 0.1 M phosphate buffer solution without and with 1.0 g L^{-1} BSA

Fitting of the spectra was done using an electrical equivalent circuit consisting of a modified Randles circuit with two parallel *R*CPE combinations in series, as shown in Fig. 6. The constant phase element CPE = $\{(C\ i\omega)^n\}^{-1}$ was considered as a non-ideal capacitor. The CPE was found to be necessary because of the heterogeneous nature of the electrode, expressed through the exponent *n*, which was found to be approximately 0.8 in all fittings, independent of the presence or absence of BSA. The two *R*CPE elements of this circuit are taken to represent the copper (or copper oxide)-solution interface and the copper oxide/BSA film respectively. The resistance of the solution and the rest of the electrochemical cell, R_Ω, was around 15 Ω cm^2 in all experiments. The results obtained are shown in Table 1.

Other circuits were also attempted for modelling without success, including that employed in [19] for the growth of oxide films in borax solutions containing carbonate or sulphate anions between pH 7.4 and 9.2. This circuit shows processes with two time constants and has an R_2C_2 parallel

Fig. 1 Cyclic voltammograms, during five cycles, at scan rate 2 mV s^{-1}, for copper electrode in 0.1 M phosphate buffer solution pH 7.0 (**a**) in the absence of BSA (**b**) in the presence of 1 g L^{-1} BSA



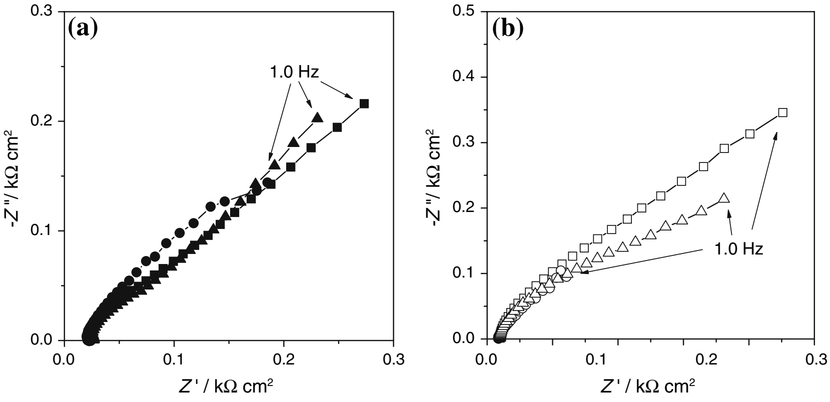


Fig. 3 Complex plane impedance spectra of copper electrode in 0.1 M phosphate buffer solution pH 7.0 at 68 mV vs. SCE: (**a**) in the absence of BSA (■) before, (●) during and (▲) after ultrasound irradiation; (**b**) in the presence of 1 g L^{-1} BSA (□) before, (○) during and (Δ) after ultrasound irradiation

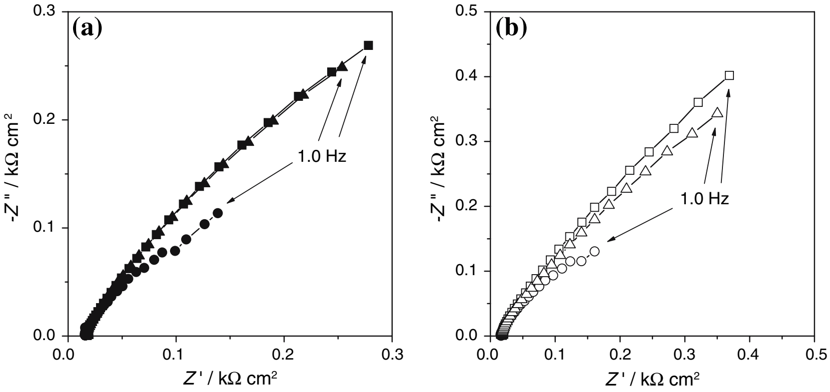


Fig. 4 Complex plane impedance spectra of copper electrode in 0.1 M phosphate buffer solution pH 7.0 at 108 mV vs. SCE: (**a**) in the absence of BSA (■) before, (●) during and (▲) after ultrasound irradiation; (**b**) in the presence of 1 g L^{-1} BSA (□) before, (○) during and (Δ) after ultrasound irradiation

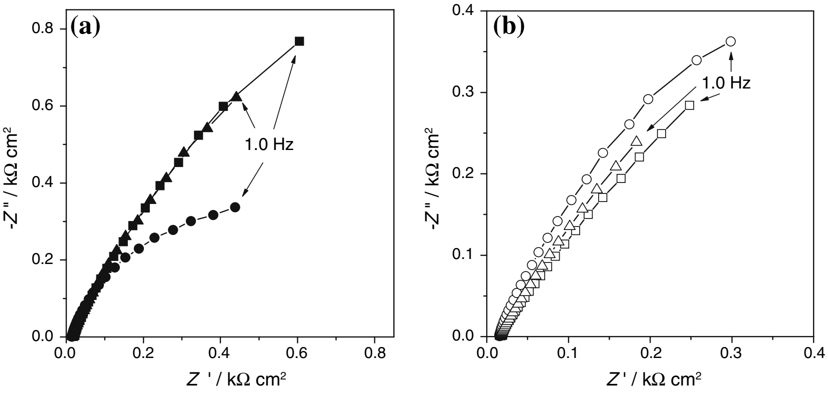


Fig. 5 Complex plane impedance spectra of copper electrode in 0.1 M phosphate buffer solution pH 7.0 at 148 mV vs. SCE: (**a**) in the absence of BSA (□) before, (●) during and (▲) after ultrasound irradiation; (**b**) in the presence of 1 g L^{-1} BSA (□) before, (○) during and (Δ) after ultrasound irradiation

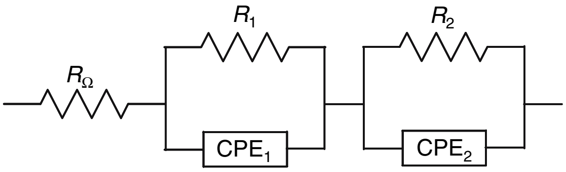


Fig. 6 Equivalent circuit used to model impedance spectra at copper electrodes in phosphate buffer, 60 mV vs. SCE, 108 and 148 mV vs. SCE

combination in series with R_1, having been used for strongly adsorbed intermediates. Other studies have not used electrical equivalent circuits.

In Fig. 3a are presented typical spectra obtained for the experiments without BSA at 68 mV. The general features of the spectra before and after ultrasound irradiation are similar, whilst those recorded during irradiation showed noticeable differences, which were reproducible between experiments. The first process occurred in the region of

Table 1 Values of *R* and *C* obtained from fitting of the spectra in Figs. 3–5, in 0.1 M phosphate buffer solution (PB) in the absence and presence of BSA in solution. The equivalent circuit is given in Fig. 6

(*E* vs. SCE) (mV)	Before irradiation				During irradiation				After irradiation			
	R_1 (Ω cm^2)	C_1 (mF cm^{-2} s^{n-1})	R_2 (Ω cm^2)	C_2 (mF cm^{-2} s^{n-1})	R_1 (Ω cm^2)	C_1 (mF cm^{-2} s^{n-1})	R_2 (Ω cm^2)	C_2 (mF cm^{-2} s^{n-1})	R_1 (Ω cm^2)	C_1 (mF cm^{-2} s^{n-1})	R_2 (Ω cm^2)	C_2 (mF cm^{-2} s^{n-1})
+68												
PB	58	0.15	795	0.69	25	0.77	577	0.91	48	0.13	826	0.80
PB + BSA	128	0.13	873	0.34	16	1.42	371	1.51	111	0.14	514	0.49
+108												
PB	38	0.97	921	0.62	73	1.34	582	1.90	35	0.64	804	0.56
PB + BSA	68	0.33	1310	0.35	55	1.19	609	1.27	67	0.29	1080	0.39
+148												
PB	45	0.69	2430	0.23	116	0.61	686	0.29	54	0.69	2210	0.25
PB + BSA	21	1.11	1340	0.62	31	0.54	1570	0.19	12	1.23	1070	0.72

high frequencies, when the curves assumed a semi circular form, and in the region of lower frequencies a second semicircle began to appear. During ultrasound irradiation the second semicircle became less evident, which can be correlated with dynamic surface changes induced by the cavitation effect, then recovering after the irradiation period. This was not the case with the interfacial capacitance, which remained higher and reflects better the permanent changes induced by ultrasound irradiation. This phenomenon of re-formation of metal oxide films having different properties, and thence a different structure, has been noted previously with carbon steel [16].

Fig. 3b shows spectra obtained in the presence of BSA in solution, the freshly polished copper electrode having been exposed to BSA, the system then being allowed to reach steady state before recording the first spectrum under the same conditions. The same general features were apparent, i.e. the high frequency semi-circle and the beginning of a second semi-circular feature at lower frequency. Analysis of the spectra shows that the interfacial resistance was higher before than after irradiation, with a partial recovery of the BSA coverage, as would be expected if changes in adsorption take place.

Additionally, in quiescent solution, the capacitance values were smaller than in the absence of BSA, once more demonstrating the important effect of BSA adsorption, particularly after irradiation. However, during ultrasound irradiation, the capacitance values increased significantly. The cavitation effect promoted by the pulsed ultrasound irradiation removed the oxide films, exposing a new surface which could then re-form temporarily during the "off" period as a heterogeneous thin film (copper oxide–copper), which led to a decrease of interfacial resistance and can be associated with a high charge separation during this time. This result is different from that obtained with aluminium or steel for which the capacitance decreased [16], showing the importance of the nature of the metal substrate and the ability to commence forming oxide films rapidly at open circuit.

The influence of BSA during the irradiation period can be seen in the *R* and *C* values, which can be attributed to the beginning of adsorption of protein during the period of time between pulses, 1 s, (the FRA response signal was averaged over a longer period of time than this). This process would have also involved specific copper–BSA interactions, more details of which will be discussed below.

At 0.108 V vs. SCE, the open circuit potential in the steady state, the spectra had a different form, as shown in Fig. 4. The values of *R* in Table 1, in the absence of BSA, under the three conditions showed small variations, although the values of *C* were higher during irradiation. At this potential the processes occurring can be summarised as some oxidation of copper to Cu^{2+} and some reduction of dissolved oxygen, so that during irradiation both processes should have been enhanced more or less equally. Therefore, the presence of BSA will block copper dissolution, leading to a higher observed resistance (R_2), although this effect should be less during irradiation, which is what is observed. In this case the values of C_1, without BSA, were significantly higher than those obtained at 68 mV.

Spectra obtained at 0.148 V vs. SCE are shown in Figs. 5a and 5b. Here, a significant amount of copper began to be oxidised to Cu^{2+} species, as can also be deduced from the potential-pH diagrams [20], which can then react directly with hydroxide ions from the solvent. This explains the semi-circle becoming more visually evident and the higher impedance magnitude in the absence of BSA, before and after irradiation. No significant permanent changes to the surface and interfacial structure caused by ultrasound were evident from the values presented in Table 1. The effect of BSA was different in that it decreased the observed interfacial resistance and that of the oxide film. By adsorbing, it appeared to prevent facile formation of copper oxide/hydroxide, so that the *R* values were proportionally lower for the complete sequence of the three experiments. Capacitance values followed the opposite trend before and after irradiation, suggesting that there were indeed some specific interactions occurring.

Rationalisation of the phenomena observed in these experiments, in 0.1 M phosphate buffer solution pH 7.0, must take into account the fact that the copper surface was covered by a thin oxide film. This oxide film is responsible for the changes in the processes seen at the different potentials, without and with BSA. It has been observed in borax solution [21], that the passive layer formed on copper has a duplex structure, being composed of an outer hydrated copper oxide film and an inner dehydrated film composed of Cu_2O, due to the thermodynamic tendency of Cu (II) oxide to be reduced to Cu (I) oxide at this potential with a surface excess of Cu (II) ions. The same tendency can be expected in phosphate buffer. Going to more positive potentials, there is a tendency for more $Cu^{2+}(aq)$ formation and thence more facile oxide film formation, particularly as CuO. This was easily seen in the increased resistance, R_2, values from 68 to 148 mV vs. SCE, even during the irradiation period, owing to the delay between pulses and the signal acquisition time being longer than one full pulse cycle since continuous irradiation was not possible owing to solution heating. The film formed afterwards had a slightly different structure to that before irradiation.

There was certainly evidence of BSA adsorption under these conditions; it is predicted, since BSA possesses acidic and basic groups and thus has a net charge. BSA's isoelectric point is around 4.7 [22], so that it has a net negative charge at pH 7.0 that can facilitate interactions with the surface at more positive potentials and with positively charged ions, such as Cu^{2+}, with partial charge transfer through adsorption on the surface. The pH of zero charge of CuO has been estimated as 9.5 [23], so that the surface should have a net positive charge in pH 7 phosphate buffer close to the OCP which will also attract the negatively charged BSA. Evidence of these phenomena has been seen in the impedance data. It was not evident in the cyclic voltammograms, although the variation of OCP with time gave an indication that BSA adsorption interferes with oxide formation by blocking the surface, so that the steady-state with respect to oxide formation is reached faster.

4 Conclusions

This study showed that ultrasound irradiation removes the copper oxide films naturally-formed in phosphate buffer solution, and that their re-formation can lead to an oxide film with different characteristics than before, owing to the freshly exposed copper surface. Adsorption of the protein BSA, studied as model protein to illustrate the approach, was found to occur, particularly as evidenced by analysis of electrochemical impedance spectra. BSA protein blocks the surface decreasing or increasing the interfacial resistance, depending on the applied potential and the oxide characteristics, as well as altering the interfacial capacitance. Reproducibility between the experiments conducted was good, which augurs well for application to other systems in the future and a better understanding of the use of proteins as corrosion inhibitors. Further work will involve characterisation of the adsorption process and the extent of adsorption using the quartz crystal nanobalance with copper-coated gold quartz crystals.

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