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ORIGINAL PAPER

Use of silver nanoparticles as an electron transfer facilitator in electrochemical ligand-binding of haemoglobin

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Abstract Silver nanoparticles have an activity for high intensity electron transfer. They can facilitate the electron transfer from the redox centre of a protein, as a high volume molecule, to the electrode surface. In this study, silver nanoparticles were deposited on the surface of a graphite carbon electrode in the 1 V potential region. Deposition of silver nanoparticles, with a diameter between 70 and 150 nm, was observed on the graphite electrode by transmission electron microscopy (TEM). The results demonstrated that the fine redox waves of haemoglobin could be achieved after modification of the graphite electrode by silver nanoparticles. The cathodic and anodic peaks of haemoglobin were at –135 and +375 mV vs. Ag/AgCl, respectively. The effect of guanosine 3′,5′-triphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP) on the structure of haemoglobin was investigated. It was observed that GTP shifts the cathodic and anodic peaks positively, indicating the transfer of the haem group to the surface of protein as a reflex of easier oxidation and reduction, while GDP and GMP do not show this behaviour. GTP binds with haemoglobin, while GDP and GMP do not.

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1 Introduction

Metal and semiconductor nanoparticles have received considerable attention in recent years. They have unique chemical, electrical and optical properties due to their size-dependent properties and quantum-size effect. They are very promising for practical applications in diverse fields such as electronic nanodevices, molecular catalysts, multifunctional reagents and biosensors [1–6]. Nanoparticles are very different from their bulk materials in their electronic, optical and catalytic properties originating from their quantum-scale dimensions [2, 7]. Similarly, nano-sized Ag is different from its bulk counterpart. Nano-sized materials have attracted much attention in the bioelectrochemical field because of their extraordinarily fascinating application in microelectronics, catalysis, molecular identification and chemical and biochemical sensors [1–7]. Since proteins are huge molecules, electron transfer from the redox centre of a protein to the surface of an electrode is a problem. Numerous efforts have been made to improve the electron transfer characteristics using mediators, promoters and direct electron transfer for haemoproteins [8–16]. Recently, the use of nanoparticles to facilitate electron transfer from the redox centre of the protein to the electrode surface has improved [1, 5, 7]. Electrochemical studies of haemoproteins may provide information regarding the electron transfer and structural characteristics of proteins [17].

Some reports have used electrochemical methods for the investigation of folding or unfolding of proteins as

well as the effects of ligand binding and inorganic solvents on the structure of some haemoproteins such as cytochrome c, myoglobin and haemoglobin [17–24]. Moosavi-Movahedi et al. reported that electrochemical methods can be used to determine the structural changes during the transition from unfolded cytochrome c to the folded structure [14]. Peng et al. investigated some of the structural properties of haemoglobin in the presence of ATP and other materials using electrochemical methods [15]. Dayer et al. investigated the structural changes of haemoglobin in the presence of different concentrations of sodium dodecyl sulfate (SDS) and showed that the electrochemical investigation of the folding and unfolding of haemoglobin by SDS gives the same results as those obtained using fluorescence and turbidity methods [18].

Haemoglobin is a hetero-tetramer, consisting of two alpha and two beta subunits. Each polypeptide chain contains a prosthetic haem group that cooperatively binds and releases oxygen [25, 26]. Haemoglobin undergoes major tertiary and quaternary conformational changes as it equilibrates between a low-affinity deoxy T-state and a high-affinity oxy R-state [27]. The globin structure affects the redox potential of the haem site of haemoglobin and protects it from rapid oxidation and allows for reversible oxygen binding. Therefore, the redox potential of haemoglobin is sensitive to alterations in the globin surrounding the active haem [25, 28]. The redox potential of the active site is an important parameter in describing the propensity of the site to donate or accept electrons, allowing us to gain insight into how effectors, materials and drugs can induce alteration in the equilibrium between the oxidized and the reduced forms of the protein, which leads to reversible oxygen binding [15].

It is well known that guanosine 3′,5′-triphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP) are very important factors in the differentiation of erythroid cells, stimulating them to synthesize haemoglobin and other proteins [29]. These three compounds have a primary role in the activation and deactivation of certain cellular proteins, such as kinases and G-proteins [30]. Thus, investigations of the effects of these materials on structural changes in haemoglobin and its affinity for oxygen are also very important.

Tamburrini et al. reported that the oxygen affinity of haemoglobin was dramatically reduced in the presence of GTP [31]. However, there are two still unanswered issues, namely, how GTP affects haemoglobin and what effects GDP and GMP have on the structure of haemoglobin. Here we report, for the first time, an investigation of the binding effects of the ligands GTP, GDP and GMP, in solution, on the structural changes of haemoglobin using a nanosilver modified graphite electrode.

2 Experimental section

2.1 Instrumentation

Transmission electron microscopy (TEM) was performed with a Hitachi HU-12A instrument (Japan, Hitachi). All electrochemical measurements were carried out with a potentiostat/galvanostat (model 263A, EG&G PAR, USA). A multiblock heater (Lab-Line Instruments, Barnstead International, Iowa, USA) was employed to control the temperature during the electrochemical measurements.

2.2 Reagents

Silver nitrate ($AgNO_3$), adult human haemoglobin (Sigma code: H7379), GTP, GDP, GMP, guanosine and 3-(*N*-morpholino) propanesulfonic acid (MOPS) (99%) were purchased from Sigma. Double distilled water was used in all the experiments. Stock solutions were stored at 4°C.

2.3 Preparation of nanosilver modified graphite electrode

Before modification, the bare graphite electrode (2.0 mm in diameter) was polished successively with Al_2O_3 (particle sizes: 0.3 and 0.05 μm) slurry on emery paper and sonicated in HNO_3, acetone and double distilled water for 10 min. The nanosilver particles, from 1 mM silver nitrate solution, were initially electro-deposited (at 1.0 V for a deposition time of 30 s) on the graphite electrode. The potential of 1.0 V was applied by using a two-electrode cell with a working graphite electrode as the cathode and another graphite electrode as the anode.

2.4 Electron microscopy of nanoparticles

After deposition of silver nanoparticles on the surface of the graphite electrode, scrapings from the electrode surface were mounted on 400 mesh Formvar/carbon-coated copper grids and then viewed by TEM operating at 80 kV.

2.5 Electrochemical measurements

We employed a single-compartment voltammetric cell equipped with a platinum rod auxiliary electrode, a saturated Ag/AgCl reference electrode (Metrohm & Co., Leinfelden-Echterdingen, Germany) and a silver nanoparticle modified graphite electrode with a disk diameter of 2.0 mm as the working electrode (Azar Electrode Co., Orumiah, Iran). The whole experiment was performed at 25 ± 0.5°C and test solutions were de-aerated by bubbling with high purity nitrogen for at least 30 min before starting the experiments.

3 Results and discussion

3.1 Transmission electron microscopic images

Figures 1(a) and (b) shows TEM images of silver nanoparticles that had been deposited on the graphite electrode surface at a potential of 1 V. Electrochemical silver deposition is induced only if the voltage is high enough to drive the reduction of the silver ions at the cathode [32]. Meanwhile, water oxidation is occurring at the anode, giving the following net reaction [32]:

$$2Ag^{+} + H_2O \rightarrow 2Ag^{0} + \frac{1}{2}O_2 + 2H^{+}$$

The TEM images show that 70–150 nm diameter nanosilver particles had been deposited on the graphite surface.

3.2 Effect of nanosilver on the electron transfer from haemoglobin to the surface of electrode

Figure 2(a) shows a typical cyclic voltammogram (CV) of the bare graphite electrode. Figure 2(b) shows a cyclic voltammogram of a nanosilver modified graphite electrode in a solution of 0.01 M MOPS buffer, while Fig. 2(c) shows a cyclic voltammogram of a nanosilver modified graphite electrode in a solution of 30 μM haemoglobin in 0.01 M MOPS buffer at pH 7.0. The cathodic and anodic peaks are at –135 mV and +375 mV vs. Ag/AgCl, respectively. From these findings, the formal potential was calculated as $(E_c + E_a) / 2 = 120$ mV vs. Ag/AgCl; cathodic and anodic peaks were not observed using the bare graphite electrode. This shows that nanosilver acts as a facilitator of electron transfer from the redox species of haemoglobin to the electrode surface and vice versa. These results are in line with previous work that explains the behaviour of nanoparticles as the facilitators of electron transfer [9]. No redox behaviour was seen for other materials (GTP, GDP and GMP) used in ligand binding studies under the same conditions (data not shown).

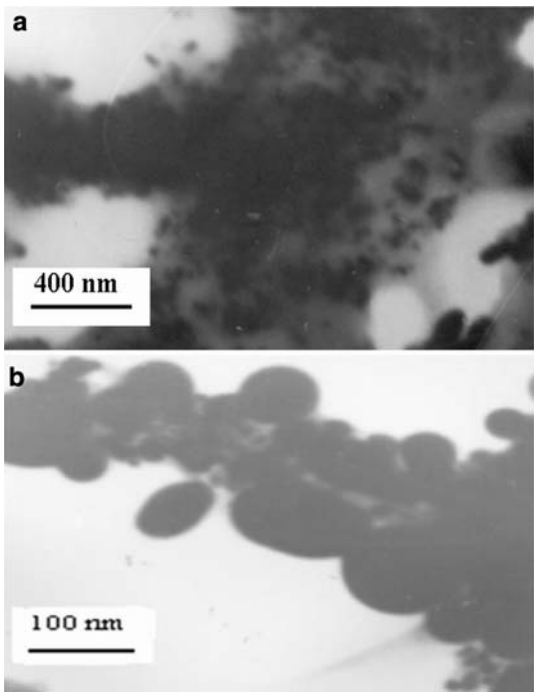

Fig. 1 TEM images of silver nanoparticles at different magnifications

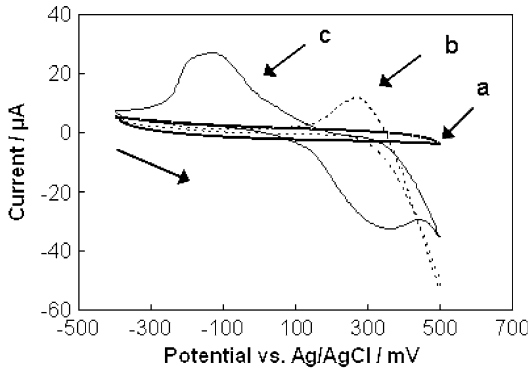

Fig. 2 Cyclic voltammograms using (**a**) the graphite electrode in 0.01 M MOPS buffer, (**b**) nanosilver modified graphite electrode in 0.01 M MOPS buffer and (**c**) nanosilver modified graphite electrode in the presence of 30 μM haemoglobin in 0.01 M MOPS buffer (scan rate 100 mV s^{-1})

3.3 Dependence of peak current on the scan rate

A linear dependence of anodic and cathodic peak currents on the square root of the scan rates is shown in Figs. 3(a) and (b). This behaviour is similar to a diffusion-controlled redox process [33], but no decrease in peak current was observed after repeated cycles of this experiment when the electrode was removed from the haemoglobin solution and dipped in the solution without haemoglobin (data not shown). These findings indicate that the haemoglobin is adsorbed onto the nanoparticle surface. Thus, the reason for the observed linear dependence of anodic and cathodic peak currents on the square root of scan rate, $v^{1/2}$ is that the redox process is controlled by the diffusion of counter-ions to maintain electro-neutrality on the electrode surface. Other possible causes are the resistance of the material or an electron transfer process occurring by an electro-hopping mechanism [34, 35].

3.4 GTP effect

Figure 4 shows the cyclic voltammetric effects of different GTP concentrations in a haemoglobin solution using a

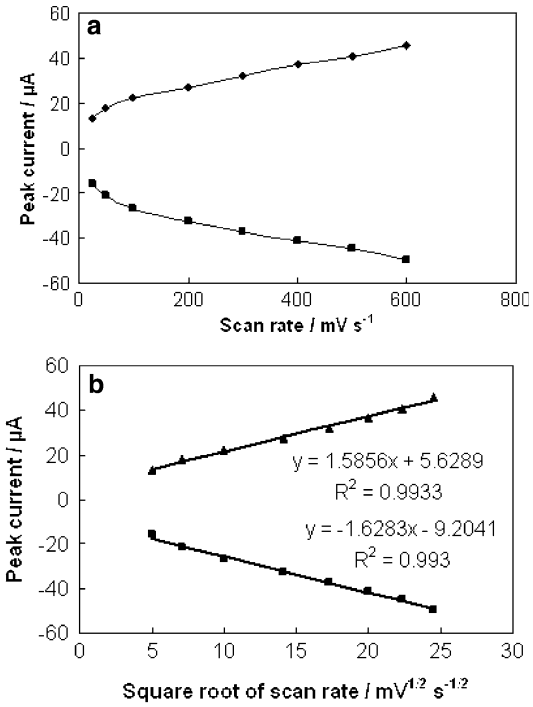

Fig. 3 Relationship of cathodic and anodic peak currents for 30 μM haemoglobin in 0.01 M MOPS buffer with (**a**) scan rate (ν) (**b**) square root of scan rate ($\nu^{1/2}$)

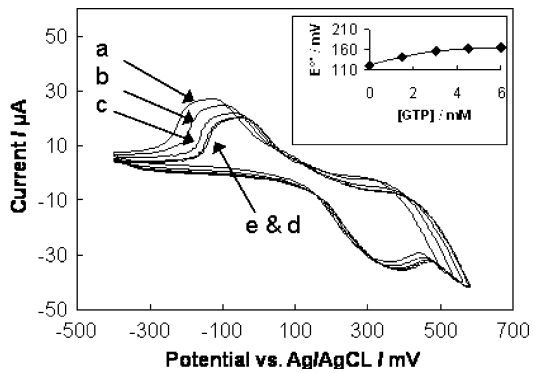

Fig. 4 Cyclic voltammograms of haemoglobin in the presence of (**a**) 0, (**b**) 1.5, (**c**) 3.0 (**d**) 4.5 and (**e**) 6.0 mM GTP, respectively (scan rate 100 mV s^{-1}). Inset shows the plot of the GTP concentration versus the formal potential

nanosilver modified graphite electrode. The inset of Fig. 4 shows the relationship between E°′ and [GTP]. The cathodic peak of haemoglobin shifts 50 mV while the anodic peak shifts 17 mV in the positive direction with the GTP concentration ranging from 0 to 4.5 mM. Here we see almost no CV changes for haemoglobin above 4.5 mM GTP. CV studies also showed no shift in both the cathodic and anodic peaks in the presence of 4 mM guanosine (data not shown). These results indicate that only the phosphate part of GTP can interact with haemoglobin. Peng et al. studied the effect of ATP on haemoglobin. They observed that 3 mM ATP shifts only the cathodic peak with no shift in the anodic peak and a concentration of 7 mM ATP only shifts the anodic peak. Thus, at low concentrations (3 mM), ATP stabilizes the reduced state of haemoglobin, and at high concentrations (7 mM) it strongly binds preferentially to the ferrous state over the ferric state [15]. However, in our study, we saw cathodic and anodic positive shifts of haemoglobin at GTP concentrations less than 4.5 mM, after which equilibrium was achieved. GTP facilitated both reduction and oxidation, so its effect on the structure of haemoglobin was to transfer haem to the surface of the protein. The work of Peng et al. [15] and our previous work [36] showed that 30 μM concentration of 2,3-diphosphoglycerate (DPG as a typically allosteric effector to haemoglobin) can cause a positive shift in cathodic peak near 45 mV. Our present work shows that a high GTP concentration (4.5 mM) is required for its binding to haemoglobin; therefore, GTP has low affinity to haemoglobin compared to that of DPG.

3.5 GDP and GMP effects

The effects of GDP (Fig. 5) and GMP (Fig. 6) on haemoglobin were also studied by cyclic voltammetry. These ligands induced almost no differences in the CV properties of haemoglobin even at concentrations of GDP and GMP as high as 6 mM. It seems that the ligand binding affinity between haemoglobin and GDP/GMP is extremely low. At pH 7.0, GDP and GMP cause a slight shift, however GTP leads to a 50 mV shift in the cathodic peak. Therefore, some GTP-specific binding sites exist in haemoglobin that do not interact with GMP and GDP. Peng et al. reported that the structure of globin controls the redox potential of the haem site of haemoglobin; thus the redox potential of haemoglobin is sensitive to alterations in the globin surrounding the active haem [15]. Change in the redox po-

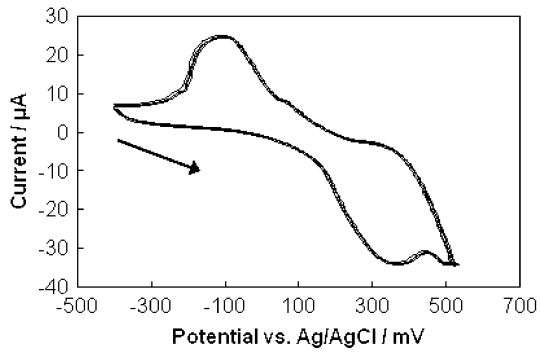

Fig. 5 Cyclic voltammograms of haemoglobin in the presence of different concentrations of GDP at a scan rate of 100 mV s^{-1}. Specific conditions delineated in the text of the Experimental section

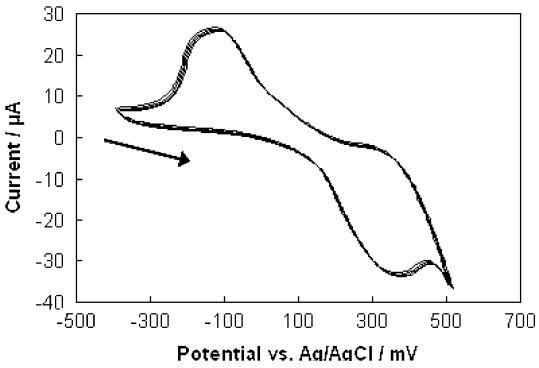

Fig. 6 Cyclic voltammograms of haemoglobin in the presence of different concentrations of GMP at a scan rate 100 mV s^{-1}. Specific conditions delineated in the text of the Experimental section

tential of haemoglobin in the presence of GTP means that the GTP-specific binding sites have been bound, causing structural change in the haemoglobin. Cathodic and anodic peaks of the haemoglobin shift to more positive potential, which indicates transfer of the haem group to the protein surface as a reflex of easier oxidation and reduction. Such changes in redox potential or the structure of haemoglobin are not observed with GDP and GMP. Similarly, Peng et al. demonstrated an analogous phenomenon with ADP and AMP, which induced only a slight shift in cathodic peak at pH 7.0; but ATP led to a 40 mV shift. They demonstrated the existence of ATP-specific sites on haemoglobin and the lack thereof for ADP and AMP [15].

The use of electrochemical methods to observe the protein–nucleotide interaction is relatively new. To date, Li et al. used pyrolytic graphite (PG) disk electrode with the haemoglobin entrapped on a membrane consisting of egg-phosphatidylcholine (egg-PC) [15]. Other examples include the use of an iodide modified silver electrode [36] and our present study. Furthermore, the use of a nanosilver modified graphite electrode in this study results in well-defined peaks for haemoglobin, better than the method of Peng et al., and this method is more stable than the use of the iodide modified silver electrode. The binding interactions will be different for similar materials with different symmetries and structures, especially in ligand binding. GTP, with its three phosphates, can bind with haemoglobin but GDP and GMP cannot. Such is the result of the work of Pomponi et al., in which the haemoglobin of different animals showed different ligand binding properties because of differences in symmetry of ligand binding sites [25].

4 Conclusions

We have shown, using a nanosilver modified graphite electrode combined with CV, that the ligand GTP affects haemoglobin as a ligand, while GDP and GMP do not. GTP affects the structure of haemoglobin at high concentrations, which shows that there are multiple receptor sites on haemoglobin for this ligand. GDP and GMP do not affect the cathodic and anodic peaks of haemoglobin indicating the lack of affinity of haemoglobin for these materials.

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