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Cu(III)-Polypeptide complexes exhibiting SOD-like activity

Review Article

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Summary. The SOD-like activity of Cu(III)-complexes with polypeptides poly-L-lysine and poly-L-glutamic acid respectively was investigated. The Cu(II)-polypeptide complexes were first oxidized by K_2IrCl_6 to give the corresponding Cu(III)-compounds.

The oxidation of Cu(II) and the corresponding Cu(II)/Cu(III) potential was evaluated by cyclic voltammetry (c.v.), UV-Vis and EPR spectroscopic (r.t.) experiments. Spin trapping EPR spectra were also conducted to confirm the formation of the superoxide radical. The SOD-like activity of each Cu(III)-complex was proved using the nitro blue tetrazolium (NBT) method slightly modified.

Keywords: Amino acids – SOD-like activity – Cu(III)-Poly-L-lysine – Cu(III)-Poly-L-glutamic acid

Introduction

It is well known, that Cu_2Zn_2SOD (superoxide dismutase, SOD), is one of the metalloproteins which catalyzes the proton dependent dismutation of superoxide anions into dioxygen and hydrogen peroxide (Mc Cord and Fridovich, 1969). The activity of this enzyme involves a cyclic Cu(II)/Cu(I) redox process of the copper(II) ion held at the active center of SOD.

In recent years, extensive research work has been done on synthetic analogs of SOD (Cao et al., 1997; Ramadan and El-Naggar, 1996; Bonomo et al., 1994 and references therein). In the framework of these efforts, the coordination structures and redox potentials of each Cu(II) ion in the synthesized copper complexes, considered to possess SOD-like activity, were found to play a significant role.

So far, all the synthetic SOD-models were Cu(II)-complexes with various biological important ligands: (Cao et al., 1997; Ramadan and El-Naggar, 1996; Bonomo et al., 1994 and references therein).

Herein we report for the first time, the superoxide scavenging activity of Cu(III)-complexes with poly-L-lysine and poly-L-glutamic acid as ligands and evidence that these Cu(III) complexes possess SOD-like activity.

Materials and methods

Chemicals

Poly-L-lysine and poly-L-glutamic acid and the SOD enzyme(BeSOD) were all products of Sigma, Germany. All other chemicals used were from Aldrich. Double distilled water, free of CO_2, was used throughout the experiments.

Potentiometry

The fully deprotonated complexes of Cu(II) are formed at pH > 10. Solutions of the copper(II)-complexes ($3 \cdot 10^{-5}$ M) were prepared by the reaction of $Cu(NO_3)_2$ with the polypeptides in 10% excess.

The potentiometric measurements were performed by using a Crison BU 2030 microautoburette equipped with a TT 2050 microcomputer, an ST 2038 microstirrer (all from Crison) and an Epson LX-400 recorder. The potentiometric titrations were carried out at 25 ± 0.1°C and I = 0.1 M KNO_3 as ionic strength. Glass electrode calibration, the ionic product of water K_w, the purity of KOH and all the ligands tested, were all performed by the program GRAN (Gran, 1952), whereas the calculations pertaining to the determination of the pK_a values of the ligands and the glass electrode calibration, were performed repeatedly, using the computer program ACBA (Arena et al., 1979).

Cyclic voltammetry

Cyclic voltammetry was performed at 25°C, using freshly prepared copper(II)-polypeptide solutions, with $[Cu]^{2+}$ at a concentration of $5 \cdot 10^{-3}$ M and I = 0.1 M $NaClO_4$, as ionic strength. A three-electrode system, consisting of a carbon working electrode, a platinum wire auxiliary electrode and a saturated KCl calomel reference electrode was used. Voltammograms were generated using a BANK Electronics instrument and recorded on a LINSEIS LY1400 X-Y recorder, at a scan rate of 100 mV·s^{-1}. The E^0 values were determined as the midpoint between the peak potentials, with an accuracy of ±5 mV. The E^0 values are given in terms of standard electrode potentials versus NHE.

UV-Vis spectroscopy

The redox-pH profiles were obtained over a pH range of 4.5–10 and for wavelengths ranging from 200–800 nm. Equimolar concentrations of copper(II)-polypeptide and $[IrCl_6]^{2-}$ at a concentration of $2.5 \cdot 10^{-4}$ M were left to react and their UV-Vis spectra were recorded at various time intervals as follows:

(i) After mixing the solutions of the Cu(II)-complex and $[IrCl_6]^{2-}$
(ii) After the addition of the O_2^- generating solution (see below).

Stock solutions of $[IrCl_6]^{2-}$ were prepared freshly before each set of experiments. The redox equilibrium was measured by the disappearance of Ir(IV) peak at λ_{max} = 490 nm (ε = 4,100 M^{-1} cm^{-1}) and simultaneously by the appearance of Cu(III) peak at 365 nm (Bossu et al., 1977).

Superoxide generation

For the generation of O_2^- ions, the method of the alkaline solution of hydrogen peroxide and acetone was used (Sichel et al., 1991). Mixing together 200 μl of KOH 30 mM, 200 μl

of H_2O_2 100 mM and 100 μl of acetone (spectrophotometric range reagent), a starting solution was obtained which was divided in two 250 μl portions. Copper(II)-complex solution and water were added to equal portions, respectively.

EPR spectroscopy

First-derivative X-band EPR spectra were recorded on a conventional spectrometer (Varian E-9). The settings were: Microwave frequency = 9.438 GHz, microwave power = 20 mW, modulation intensity = 0.63 mT, gain = 10^5, time constant = 500 ms, scan time = 500 sec.

Assay for SOD-like activity

The nitro blue tetrazolium method (Beauchamp and Fridovich, 1971) slightly modified was used for the determination of the SOD-like activity of the two Cu(III)-polypeptide complexes.

Results and discussion

Potentiometric curves for both ligands show that copper(II) has the tendency to form complexes at 2:1 (ligand:metal) molar ratio, rather than 1:1. At Fig. 1 the potentiometric curves of Cu(II)-poly-L-Lys, as recorded by UV-Vis, are shown. This is in accordance with findings from other groups, that the number of deprotonated peptide functional groups is a critical factor for the thermodynamic stabilization of the Cu(III) state (Bossu et al., 1977).

Analysis of the potentiometric data with the aid of the computer program ACBA (Arena et al., 1979) revealed the following dissociation constants: For poly-L-Lys, $pK_1 = 2.7$ $pK_2 = 9.2$ whereas for poly-L-Glu, $pK_1 = 2.4$ $pK_2 = 9.6$. The pK_1 values refer to carboxyl group (—COOH), whereas the pK_2 values to amino group ($—NH_2$), both at the site of metal coordination.

From cyclic voltametric studies, the E^0 values of the Cu(II)/Cu(III) couples versus NHE were evaluated as follows: For the $[CuL_2]$ type complexes, and for L = poly-L-Lys, $E^0 = 0.6$ V whereas for L = poly-L-Glu, we have $E^0 = 0.63$ V (Fig. 2). This is in accordance with already published results (Bossu et al., 1977), showing that Cu(III) can exist in solution as a complex with the above ligands in 1:2 (metal:ligand) molar ratio.

In addition, the Cu(II)/Cu(III) couple potentials E^0, have for every complex similar values to other peptide ligands (Bossu et al., 1977). That means, that the coordination to the Cu(II) center is made by the deprotonated peptide and the NH_2 groups present, for both polypeptide ligands (Scheme 1).

The SOD-like activity of the Cu(III) complexes was also monitored and analyzed by UV-Vis spectroscopy. After mixing the solution of the Cu(II) complex with the one of the oxidizing agent $[Ir^{IV}Cl_6]^{2-}$, the characteristic peak of maximum absorption for Ir(IV) at $\lambda_{max} = 490$ nm was gradually decreasing with time, while simultaneously a new absorption at $\lambda_{max} = 365$ nm appeared, which is characteristic of Cu(III) (Bossu et al., 1977), (Fig. 3a–b). After addition of the superoxide radical generating solution, Cu(III) is reduced to Cu(II) again, which is shown by the disappearance of the $\lambda_{max} = 365$ nm peak (Fig. 3c).

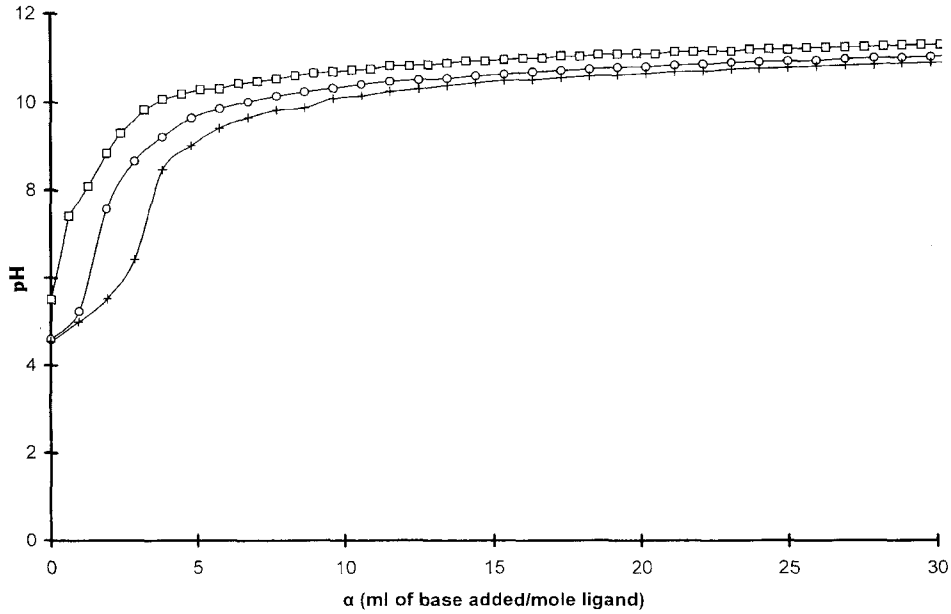

Fig. 1. Potentiometric curves of the system: Cu^{2+}-poly-l-lysine. $[Cu^{2+}] = 3 \times 10^{-5}$M. ○, Ligand/$Cu^{2+}$ 1:1 molar ratio; +, Ligand/Cu^{2+} 2:1 molar ratio; □, Poly-L-Lys

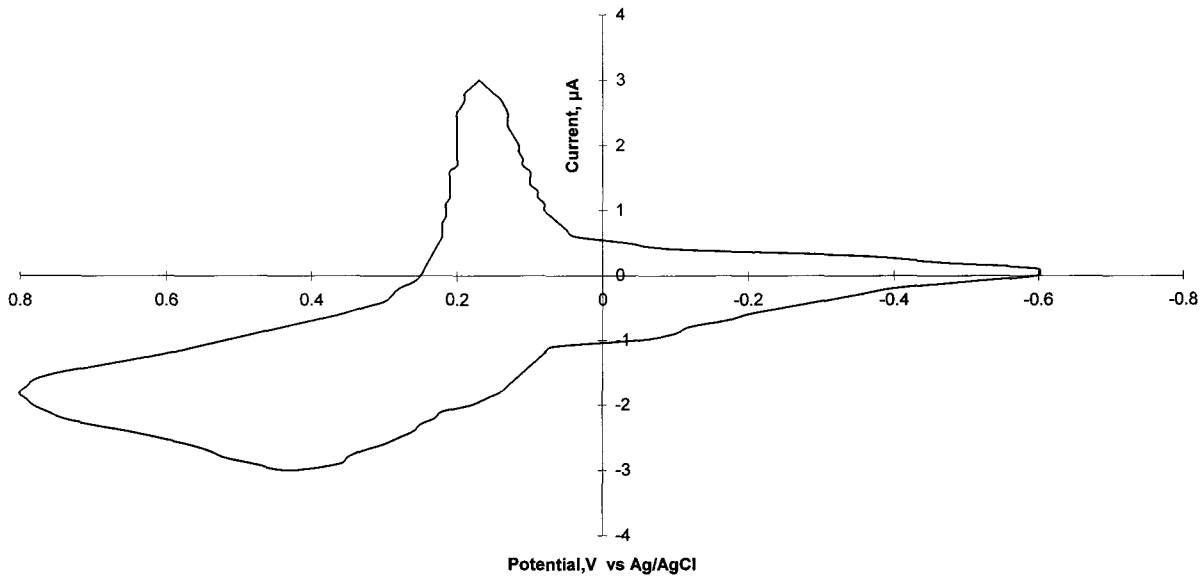

Fig. 2. Cyclic voltammogram of $[CuL_2]$ in aqueous solution at a graphite electrode: L = poly-l-lysine, $[CuL_2] = 5 \times 10^{-3}$M, I = 0.1 M $NaClO_4$, scan rate = 100 mVs^{-1}. $E^0 = 0.7$ V vs NHE

$$[Cu^{II}L_2]^0 + [Ir^{IV}Cl_6]^{2-} \Leftrightarrow [Cu^{III}L_2]^+ + [Ir^{III}Cl_6]^{3-} \quad (a)$$

$$[Cu^{III}L_2]^+ + O_2^- \rightarrow [Cu^{II}L_2]^0 + O_2 \quad (b)$$

Scheme 1

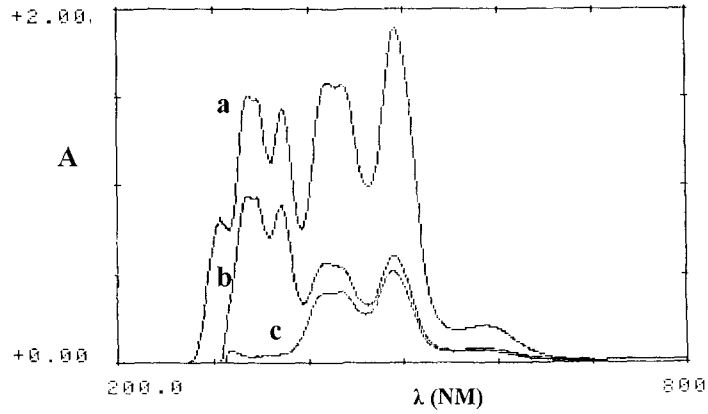

Fig. 3. UV-Vis spectra for the monitoring of the Cu(II) oxidation and the O_2^- scavenging activity of Cu(III). **a**) $[Cu^{II}L_2]$ with $[Ir^{IV}Cl_6]^{2-}$ at the molar ratio of 1:1. $[Cu^{II}L_2] = 2.5 \times 10^{-4}$M), 50$\mu$l, **b**) the same solution after 15 min, **c**) after addition of the O_2^- generating solution

From the EPR spectra, it can be concluded, that Cu(II), in the form of its (1:2) complex with the above ligands, is oxidized by $[Ir^{IV}Cl_6]^{2-}$ almost entirely (Fig. 4a–b). The disappearance of the characteristic peak of Cu(II) after mixing with the $[Ir^{IV}Cl_6]^{2-}$ solution is profound. In spin-trapping experiments, the spin-adduct of O_2^- with the spin trap DMPO (5,5-dimethyl-1-pyrroline-N-oxide) is formed, having the formula: DMPO-OOH. Figure 5a shows the characteristic multiple-peak signal (with $a_H = a_N = 14{,}25$ G) indicative of the DMPO-OOH adduct (Britigan et al., 1986). Cu(III)-complex with either poly-L-Lys or poly-L-Glu quenched the observed signal, showing SOD-mimicking activity (Fig. 5b).

Mixing the O_2^- generating solution with a solution containing the [Cu(III)-L_2] complex, (L = poly-L-Lys or poly-L-Glu), the characteristic signal of Cu(II) shows that the reduction of Cu(III) by the superoxide ion O_2^- has occurred (Fig. 6a). When the spin trap DMPO is added to the above solution, the characteristic peaks of the DMPO-OOH spin-adduct begin to form, whereas the Cu(II) signal is absent showing that Cu(III) is unaffected (Fig.

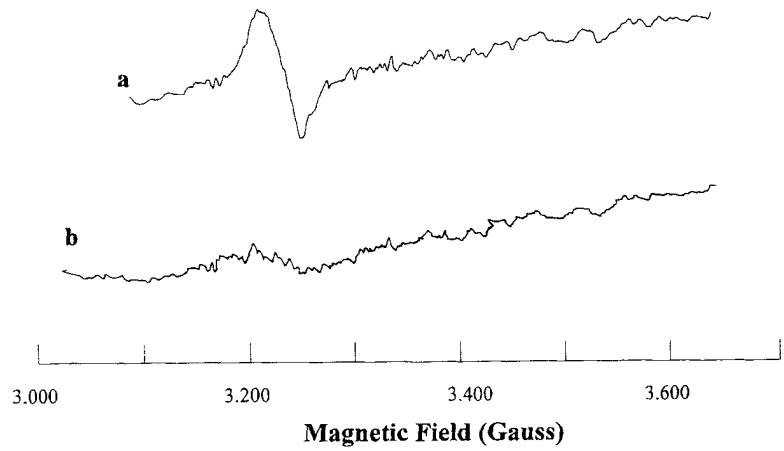

Fig. 4. EPR spectra at room temperature of **a**) [Cu(II)-(poly-L-Lys)$_2$] (2.5×10^{-4}M), 50μl, **b**) [Cu(II)-(poly-L-Lys)$_2$] after mixing with 50μl of $[IrCl_6]^{2-}$ solution, of equimolar concentration

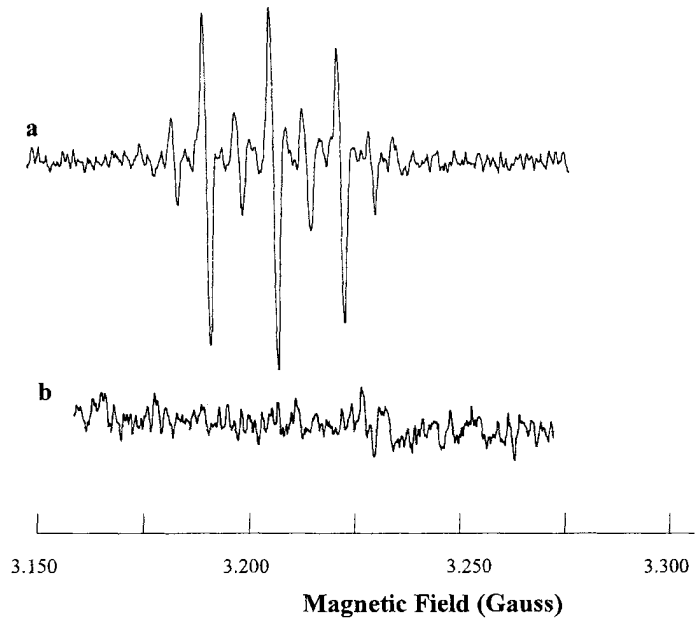

Fig. 5. EPR spectra at room temperature of O_2^- taken: **a**) immediately after superoxide generation and spin trapping with DMPO (50mM). The peaks correspond to the spin adduct: DMPO-OOH, **b**) after mixing of the previous solution with equal volume (50μl) of a Cu(III) solution (2.5×10^{-1}M)

6b). All the above EPR data support the findings from the UV-Vis study, already mentioned.

From the above discussion, it is evident, that the Cu(III) complexes mimic the SOD activity through redox cycles, comprising the Cu(II)/Cu(III) interconversion (Scheme1).

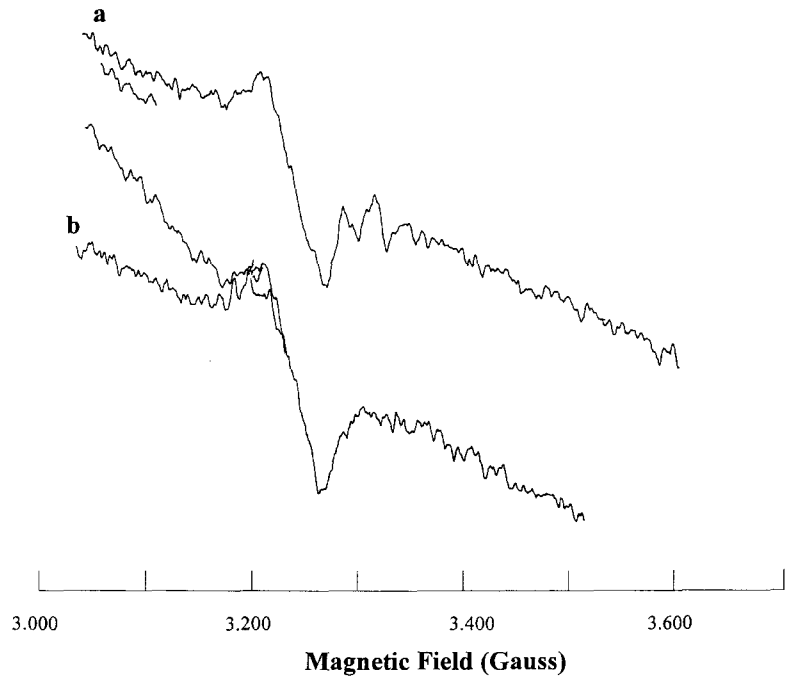

Fig. 6. EPR spectra at room temperature of O_2^- taken: **a**) after mixing of [Cu(III)-(poly-L-Lys)$_2$] (2.5×10^{-4}M) solution with the O_2^- generating solution (in 1:1 volume ratio), **b**) after mixing of the previous solution with the spin trap DMPO (50mM) solution

The SOD-like activity of the Cu(III)-polypeptide complexes, was also confirmed through the use of the procedure of Beauchamp and Fridovich, 1971 with a slight modification. All available assays of superoxide dismutase are indirect and depend upon its ability to scavenge O_2^- from reaction mixtures and thus to inhibit reactions caused by O_2^-. It is known that O_2^- reduces nitro blue tetrazolium (Beauchamp and Fridovich, 1977) and is used to assay SOD which inhibits the formation of blue formazan. Superoxide radical anions were generated by the system suggested by Sichel et al. (1991) as described above. The O_2^- ions were detected by UV-Vis spectrophotometry following the reduction of nitro blue tetrazolium (NBT) to blue formazan. The latter gives a characteristic peak at $\lambda_{max} = 560$nm. When Cu-Zn-SOD is added to the system (100U/ml) the formation of formazan is inhibited and the absorbance at $\lambda_{max} = 560$nm disappears. The same effect has the addition of the Cu(III)-polypeptide complexes to the above system, showing complete inhibition of NBT reduction. Figure 7 shows the UV-Vis data obtained from the above superoxide generating system with NBT and Cu(III)-Poly-L-Lys/NBT. These data support the SOD-like activity of the two Cu(III)-complexes.

In conclusion, we have shown that these Cu(III) poly-L-Lys and Cu(III)-poly-L-Glu complexes are formed at 1:2 (metal:ligand) molar ratio and possess SOD-like activity. Further work is necessary, to elucidate the structure of the above polypeptide-Cu(III) complexes and to dissect the specific chemical environment created by these ligands around copper(III) ions.

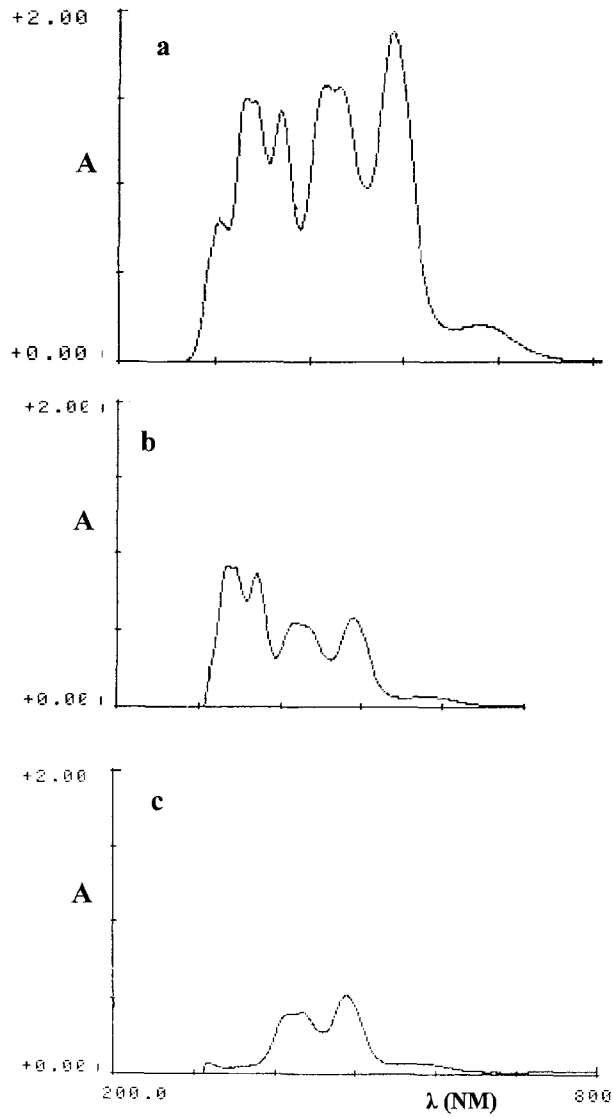

Fig. 7. UV-Vis spectra for the NBT assay. **a**) Taken after mixing the superoxide radical generating solution with NBT ($200\mu mol \cdot dm^{-3}$), **b**) taken after mixing the superoxide radical generating solution with the solution of Cu(III) (2.5×10^{-4}M), **c**) taken after addition of NBT ($200\mu mol \cdot dm^{-3}$) at the previous system (**b**)

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