ELECTROCHEMICAL AND EPR INVESTIGATION OF SPIN PROBES ACETOXYMETHOXYCARBONYL- AND CARBOXY-PROXYL

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ABSTRACT
Redox properties of one hydrophobic spin probe 3-(acetoxymethoxy)carbonyl-2,2,5,5-tetramethyl-pyrrolidine-N-oxyl (AM-CxP) and corresponding water-soluble compound, 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-N-oxyl (3-CxP) were evaluated using electrochemical and EPR methods. The electrochemical behavior of two spin probes was investigated by cyclic voltammetry in phosphate buffer solution. The voltammograms of both compounds exhibited a single redox couple related to nitroxyl radical. EPR spectroscopy revealed that 3-CxP is less susceptible to reduction by Ascorbate than AM-CxP, nevertheless, both spin probes exhibit a great potential for *in vivo* applications.

INTRODUCTION
Nitroxide spin probes are stable free radical compounds widely used in biological systems in conjunction with electron paramagnetic resonance (EPR) spectrometry and imaging. Their application can provide a wealth of information regarding the redox and thiol status *in vivo* and *in vitro*, pH, and oxygen concentration [1]. Depending on the functional groups, different spin probes may exhibit diverse properties such as stability towards oxidation/reduction, cell, tissue, and blood-brain barrier (BBB) permeability. For instance, hydrophobic spin probes are more likely to pass through cell membranes and BBB, contrary to the charged spin probes [2]. Thus, obtaining the information from within the cell may represent a challenge, due to the difficulties of *in vitro* or *in vivo* administration of the hydrophobic spin probes, owing to their poor water solubility. Herein, we evaluate the redox properties of one such probe, 3-(acetoxymethoxy)carbonyl-2,2,5,5-tetramethyl-pyrrolidine-N-oxyl (AM-CxP), and the corresponding compound, 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-N-oxyl (3-CxP), obtained upon cleavage of the ester bond by intracellular esterases, studied by cyclic voltammetry (CV) and EPR.

METHODS
Electrochemical experiments were performed in a three-electrode glass cell. The working electrode was a glassy carbon electrode (GCE) with a diameter of 3 mm, the reference electrode was Ag/AgCl in 3 M KCl, while a platinum rod served as the counter electrode. GCE was polished on cloth with alumina paste, thoroughly washed out with deionized water, and cleaned with deionized water in an ultrasonic bath. The electrochemical measurements were performed using the Autolab electrochemical workstation (Autolab PGSTAT302N, Metrohm-Autolab BV, Netherlands). The method of cyclic voltammetry was used with a scan rate of 20 mVs^{-1}, unless otherwise stated. 0.1 M phosphate buffer at a pH of 7.4 was used as a supporting electrolyte, and the concentrations of both probes were 1.25 mM for electrochemical experiments.

EPR study of the kinetics of ascorbate-induced reduction of spin probes AM-CxP and 3-CxP was performed in the presence and absence of glutathione (GSH), whose role is to scavenge ascorbyl radical (Asc•) produced during oxidation of ascorbate (Asc). The concentration of both spin probes

in the final sample was 200 µM, while Asc and GSH were present at 100 mM and 50 mM, respectively. The EPR spectra of the samples were recorded at room temperature using a Bruker Elexsys II E540 spectrometer operating at X-band (9.85 GHz) with the following settings: modulation amplitude 1 G; modulation frequency 100 kHz; microwave power 10 mW; scan range 75 G; scan time 15 s. The spectra were recorded and analyzed using the Xepr software (Bruker BioSpin Germany).

RESULTS AND DISCUSSION

Cyclic voltammetry was used to investigate the electrochemical behavior of two spin probes, 3-CxP and AM-CxP. The obtained cyclic voltammograms (Fig. 1a) showed a pair of peaks that can be ascribed to the following process [3]:

$$>N\text{-}O^{\bullet} + e^{-} \leftrightarrow >N\text{-}O^{-} \quad (1)$$

The values of half potential ($E_{1/2}$) were 0.70 V and 0.59 V, for AM-CxP and 3-CxP, respectively.

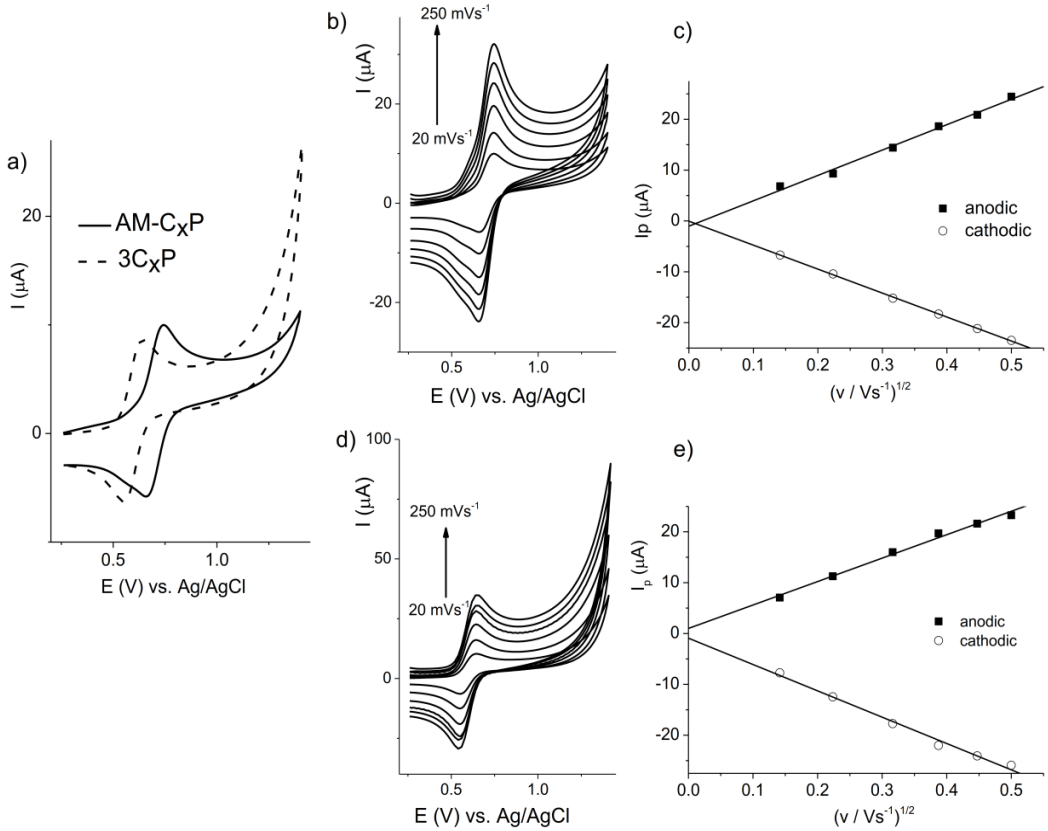

Figure 1. a) Cyclic voltammograms of 1.25 mM 3-CxP and 1.25 mM AM-CxP recorded at scan rate of 20 mVs^{-1} in 0.1 M phosphate buffer pH 7.4. Cyclic voltammograms recorded at different scan rates of 1.25 mM AM-CxP (b) 1.25 mM 3-CxP (d). Plot of peak currents versus square root of scan rate for 1.25 mM AM-CxP (c) 1.25 mM 3-CxP (e).

The nature of the electrode process occurring on the GCE surface was studied by varying scan rates in the range of 20 to 250 mVs^{-1}. The obtained voltammograms are presented in 1b and 1d. Peak currents (anodic and cathodic) showed linear behavior with the square root of scan rate (Fig. 1c and 1d), for both investigated probes, indicating a diffusion-controlled current system. The plot of the logarithm of peak current versus the logarithm of scan rate (not shown) showed linear behavior with

slopes of 0.51 and 0.48 for AM-CxP and 3-CxP, respectively. The theoretical value of 0.5 is expected for a diffusion-controlled process [4]. Peak-to-peak separations of 66 mV, as well as I_a/I_c ratio equal to 1 obtained for AM-CxP and independence of peak potential on scan rate, indicated reversible behavior of this probe. However, peak-to-peak separation of 81 mV obtained for 3-CxP, together with a slight shift of peak potential with the increase of scan rate and ratio $I_a/I_c<1$, indicated quasi-reversible behavior of this probe. The dependence of peak current on the square root of scan rate is given by Randles-Ševćik equations for reversible and quasi-reversible processes [5]:

$$I_p^{rev} = \pm 0.446\, nFA_{real}C\sqrt{\frac{nFDv}{RT}} \quad (2)$$

$$I_p^{quasi} = \pm 0.436\, nFA_{real}C\sqrt{\frac{nFDv}{RT}} \quad (3)$$

where n is the number of electrons in the electrochemical reaction, I_p is the peak current of forward process, F is the Faraday constant (C mol^{-1}), v is the applied scan rate (V s^{-1}), R is the universal gas constant, T is the temperature (K), A_{real} is the electroactive area of the electrode (cm^2) and D is the diffusion coefficient (cm^2 s^{-1}). The electroactive area of the electrode was determined from Rendles-Ševćik equation for a reversible process applied to the data obtained from cyclic voltammograms recorded in 1 mM $Fe(CN_6)^{3-/4-}$ solution in 0.1 M KCl at scan rates 20-200 mVs^{-1}. The obtained A_{real} was calculated to be 0.074 cm^2, a little higher than geometric surface area.

The plot of peak current versus $v^{1/2}$ was linear over the whole range of investigated scan rates for both spin probes and the obtained equations were:

I_{pa} = -7.41*10^{-7} + 4.93*10^{-5} $v^{1/2}$ (R^2 = 0.999) for AM-CxP (4)

I_{pa} = 9.98*10^{-7} + 4.61*10^{-5} $v^{1/2}$ (R^2 = 0.989) for 3-CxP (5)

Using equations (2) and (3) diffusion coefficients for AM-CxP and 3-CxP were determined to be 3.25*10^{-6} cm^{2}s^{-1} and 3.96*10^{-6} cm^{2}s^{-1}.

EPR was used to study the stability of both spin probes towards reduction by Asc (Fig. 2).

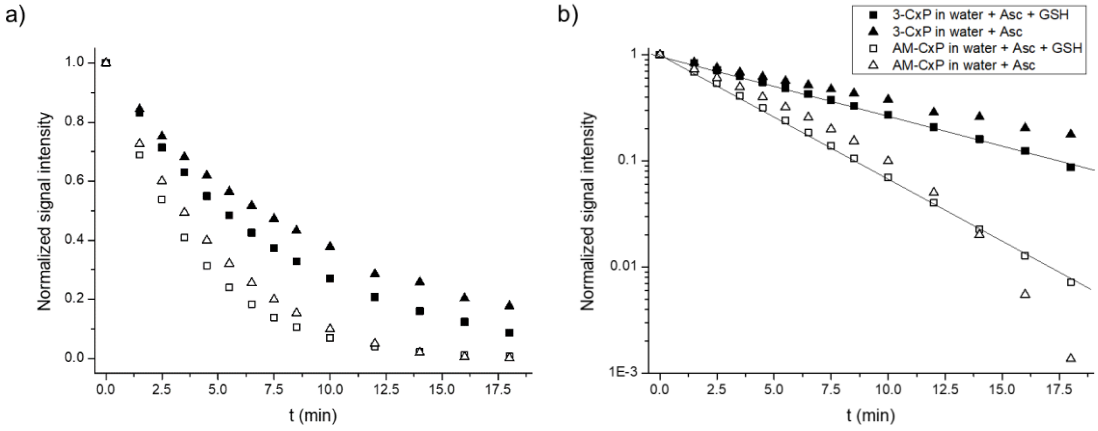

Figure 2. Reduction kinetics of 200 μM AM-CxP and 3-CxP by Asc (100 mM) in the presence and absence of GSH (50 mM) presented on linear (a) and semi-log graph plot (b).

It can be clearly observed that there is a difference in the reduction kinetics for both spin probes in the presence and absence of GSH, which can be ascribed to the more complex mechanism of

nitroxide reduction in the presence of ascorbyl radical. This conclusion is corroborated by a non-linear trend of reduction kinetic curves in semi-log plots obtained in the absence of GSH (Fig 2b).

Furthermore, it is evident that 3-CxP is less susceptible to reduction by Asc compared to AM-CxP, which may be explained by the repulsion between negatively charged ions of 3-CxP and Asc that arise from the dissociation of these two compounds in an aqueous solution [6]. Despite the greater stability of 3-CxP versus AM-CxP, given that the concentration of Asc was 500 times greater than that of AM-CxP, this probe can be considered fairly stable, and thus exhibits a great potential for *in vivo* applications.

CONCLUSION

Cyclic voltammetry of 3-CxP and AM-CxP probe indicated a single one-electron diffusion-controlled process representing the redox reaction of nitroxyl radical to deprotonated hydroxylamine. 3-CxP probe showed reversible behavior, while AM-CxP probe showed quasi-reversible behavior. According to the values of half-wave potentials, 3-CxP probe has a lower affinity for electrons (i.e. tendency to be reduced) than AM-CxP spin probe. EPR measurements show that commercially available 3-CxP is less susceptible to reduction by Asc compared to hydrophobic AM-CxP, possibly because of the repulsion between negatively charged ions of 3-CxP and Asc. On the other hand, spin probe AM-CxP can be considered fairly stable exhibiting a great potential for *in vivo* applications especially for evaluating redox behavior from within the cells.

Acknowledgment

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REFERENCES

[1] V.V. Khramtsov in: Nitroxides – Theory, Experiment and Applications, A. Kokorin (Ed.), InTechOpen, Rijeka, Croatia, 2014.
[2] G. Bačić, A. Pavićević, F. Peyrot, Redox Biol., 8 (2016) 226.
[3] V. D. Seńa, I. V. Tikhonov, L I. Borodin, E. M. Pliss, V. A. Golubev, M. A. Syroeshkin, A. I. Rusakov, J. Phys. Org. Chem., 28 (2015) 17.
[4] J. Wang, Analytical Electrochemistry. 2. New York: Wiley-VCH; 2000. p. 37.
[5] A. Bard, L. Faulkner, Electrochemical Methods: Fundamentals and Applications; John Wiley & Sons, Inc: New York, NY, USA, 2001.
[6] F. Vianello, F. Momo, M. Scarpa, A. Rigo, Magn. Reson. Imaging, 13 (1995) 219.