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Study of lipid peroxidation and ascorbic acid protective role in large unilamellar vesicles from a new electrochemical performance

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ABSTRACT

In this contribution an electrochemical study is described for the first time of lipid peroxidation and the role of antioxidant on lipid protection using large unilamellar vesicles (LUVs). In order to simulate the cell membrane, LUVs composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were used. A vesicle-modified electrode was constructed by immobilizing DOPC LUVs onto carbon paste electrodes (CPEs). Lipid peroxidation was studied electrochemically by incubating the vesicle-modified electrodes with hydroxyl (HO•) radicals generated via the Fenton reaction. Oxidative damage induced by HO• was verified by using square wave voltammetry (SWV) and was indirectly measured by the increase of electrochemical peak current to $[Fe(CN)_6]^{4-}$ which was used as the electrochemical label. Ascorbic acid (AA) was used as the antioxidant model in order to study its efficacy on free radical scavenging. The decrease of the electrochemical signal confirms the protective key role promoted by AA in the prevention of lipid peroxidation in vesicles. Through microscopy, it was possible to observe morphologic modification on vesicle structures after lipid peroxidation in the presence or absence of AA.

1. Introduction

Oxidant compounds (radicals and non-radicals) are a consequence of normal aerobic metabolism, being continuously generated in living cells (in the inner and outer mitochondrial membrane) and in several metabolic pathways in mammalian cells (microsomal electron transport) [1]. These strong oxidants are able to induce damage in cells by reacting with biomolecules, namely proteins, lipids, DNA and carbohydrates, amongst others, causing a negative effect on intra- and extracellular signal transmission [2].

Although the oxidative lesions in DNA are the primary risk factor for gene mutations, which play a key role in carcinogenesis and aging [3,4], currently, lipid peroxidation is considered as the main molecular mechanism involved in oxidative damage to cell structures and in the toxicity process that leads to cell death [5].

Lipid peroxidation is the oxidative damage induced by free radicals to the hydrophobic parts of the biological cellular membrane [6], and involves three distinct steps: i) initiation; ii) propagation iii) termination [7]. The initiation step occurs when free radicals (R•) (namely reactive oxygen species (ROS), such as $^1O_2$; $O_2•^-$ or HO•) react with a lipid substrate (LH) or when a breakdown of a pre-existing lipid hydroperoxides (LOOH) takes place caused by transition metals (Eq. (1)). In both cases a highly reactive lipid radical (L•) is generated [8]. The second phase, in the propagation of lipid peroxidation (Eqs. (2) and (3)), molecular oxygen reacts quickly with L• to produce the lipid peroxyl radical (LOO•). LOO• has the ability to remove a hydrogen atom from DNA and proteins, producing a lipid hydroperoxide (LOOH). In the termination phase, two lipid radicals react to produce a non-radical species (Eqs. (4)–(6)) [9]. Amongst the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene, which are the commonly measured end products of lipid peroxidation [10].

Initiation

$$LH + R^{\cdot} \rightarrow L^{\cdot} + RH \quad (1)$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (2)$$

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$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (3)$$

Termination

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow LOOL + O_2 \quad (4)$$

$$L^{\cdot} + L^{\cdot} \rightarrow L{-}L \quad (5)$$

$$LOO^{\cdot} + L^{\cdot} \rightarrow LO{-}OL \quad (6)$$

To counteract and prevent the oxidative damage induced by these compounds, living organisms have developed complex endogenous and exogenous antioxidant systems. According to Laguerre et al. (2010) a biological antioxidant is a compound which, when present at low concentration compared to an oxidizable substrate, protects (by itself and through its oxidation products) that substrate from oxidation, and ultimately protects the organism from the harmful effects of oxidative stress (Eqs. (7)–(9) [11]. Endogenous antioxidants include enzymes, small molecular compounds, and cofactors [12,13]. Additional protection can be provided by exogenous antioxidant compounds, which are present in foodstuffs and beverages.

$$L^{\cdot} + HX\ (\text{antioxidant}) \rightarrow LH + X^{\cdot} \quad (7)$$

$$LOO^{\cdot} + HX \rightarrow LOOH + X^{\cdot} \quad (8)$$

$$X^{\cdot} + X^{\cdot} \rightarrow X{-}X \quad (9)$$

Several procedures have been reported for the evaluation of the effects of radicals and antioxidants on cellular biomolecules, namely based on UV–vis spectrometry, chemiluminescence, chromatography and electrochemistry [14–16]. Amongst these, the use of electrochemical devices is seen to be the best of all because interactions between radicals, antioxidants and biomolecules are based on electron transfer reactions which can be easily monitored by the methodology [1]. Hu and collaborators used amperometric techniques at platinized carbon fiber electrodes to monitor and characterize the quantity of radicals (e.g. NO•, $ONOO^{-}$, NO_2, H_2O_2 and $O_2^{\bullet -}$) released by MG63 osteosarcoma cells. The electrochemical procedure proved that the malignant bone formation ability of osteosarcoma cells was related to the specific high production of NO• associated with a small production of $O_2^{\bullet -}$.

Some reports indicate the use of voltammetric techniques for the visualization of the oxidative damage induced by radicals such as hydroxyl, sulfate and superoxide at DNA layers immobilized on gold or carbon surfaces [2]. However, as far as we know, there are no electrochemical studies concerning the effect of hydroxyl radicals on phospholipid bilayers present in the large unilamellar vesicles (LUVs), as cellular membrane simulation, which can be adsorbed onto transducer surfaces.

This work describes, for the first time, the construction and optimization of a vesicle modified electrode designed to measure lipid peroxidation induced by hydroxyl radicals (HO•) generated via the Fenton reaction (Eq. (10)).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO{-} + HO^{\cdot} \quad (10)$$

The vesicle-modified electrode consisted of an adsorptive immobilization of LUVs of 1,2-dioleoil-sn-glicero-3-fosfatidilcoline (DOPC), onto a carbon paste electrode (CPE) surface. The antioxidant ascorbic acid (AA) was evaluated for its protective effect against $HO^{\bullet}$ radicals on the vesicles immobilized on the CPE. Electrochemical measurements were carried out by using square wave voltammetry (SWV) and cyclic voltammetry (CV): dynamic light scattering (DLS) and transmission electron microscopy (TEM) were also used in order to observe the morphologic changes after vesicles peroxidation. This study offers a basic understanding of lipid peroxidation and the protective role of antioxidants using electrochemical and microscopic techniques.

2. Experimental

2.1. Chemical and solutions

The lipid 1,2-di-oleoyl-sn-glycero-3-phosphatidylcholine (DOPC in chloroform solution) was obtained from Avanti Polar Lipids, Inc. (Alabaster Al, USA) and used without further purification. Ascorbic acid. (AA), phosphate buffer saline (PBS) pH 7.4, iron (II) sulfate heptahydrate, hydrogen peroxide (30%, w/v), potassium ferrocyanide, ethylenediamine tetraacetic acid (EDTA), l(+)-Ascorbic acid were from Merck. All chemicals employed were used without further purification.

The Fenton mixture (generation of hydroxyl radical) was prepared by mixing Fe^{2+}: EDTA: H_2O_2 (1 mol l^{-1}: 2 mol l^{-1}: 40 mol l^{-1}) in a molar ratio of 1:2:40 [17]. EDTA was added for solubility reasons. All solutions were prepared with water purified with a Direct-Q (Millipore) system.

2.2. Instrumentation

An Autolab PGSTAT 30 potentiostat, controlled by GPES 4.8 software, was employed for the cyclic (CV) and square wave voltammetry (SWV) measurements. Cyclic voltammograms were obtained at 100 mV s^{-1}. The characteristic parameters used to obtain square wave voltammograms were SW amplitude (ΔE_{SW}) 0.025 V; the staircase step height (ΔE_s) 0.005 V and the frequency (f) 20 Hz. A carbon paste electrode (CPE) composed by activated carbon (50%), ricine oil (30%) and nafion (20%) was used as working electrode (geometric area = 0.030 cm^2). The CPE electroactive area was calculated from convoluted CVs [18].

The following mathematical expression was applied for this calculation

$$I_{L,con} = nFAD^{1/2}C^{*} \quad (11)$$

where $I_{L,con}$ is the convoluted limiting current, n is the number of electrons exchanged by the ferrocyanide compound (n = 1), F is the Faraday constant (96,464 C mol^{-1}), A is the electroactive surface area (0.0525 cm^2), D is the diffusion coefficient of the electroactive species (in this case 7.6×10^{-6} cm^2 s^{-1}) [19] and C is the molar concentration of redox specie (4.0×10^{-6} mol cm^{-3}).

The unmodified carbon paste was introduced into the well of a Teflon electrode body and its surface was smoothed against plain white paper while slight manual pressure was applied to the electrode. Unless otherwise stated, after each experiment, the carbon paste was discarded and a new electrode surface was freshly prepared. The counter electrode was a Pt foil of large area (2 cm^2) and a Ag/AgCl wire was used a pseudo reference electrode and periodically monitored against a saturated calomel electrode.

The diameters of the DOPC vesicles, namely the large unilamellar vesicles (LUVs) were determined by dynamic light scattering (DLS) by using a Malvern 4700 analyser with a goniometer, a 7132 correlator, and an argon-ion laser operating at 488 nm. All measurements were made at a scattering angle of 90° at a temperature of 20 ± 0.1 °C.

Transmission electron microscope (TEM) micrographs was obtained using a Siemens (Germany) Jeol – Jem 1200 EX II (Elmiskop 101) P at 20–120 kV with a Megaview-II Docu camera and SIS NT Docu software.

Approximately 20 μl of the vesicle solution was placed on Parafilm®, and then a carbon coated copper grid was placed over the drop. After 30 min, the modified grid was rinsed and placed onto a drop (20 μl) of Fenton solution during 5 and 15 min in the absence of ascorbic acid, and 30 min in the presence of it. Finally, the grids were rinsed and dried under reduced pressure and stained with phosphotungstic acid 0.1% as contrast prior to placement in the microscope.