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Effective protection of biological membranes against photo-oxidative damage: Polymeric antioxidant forming a protecting shield over the membrane

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ABSTRACT

We have prepared a chitosan polymer modified with gallic acid in order to develop an efficient protection strategy biological membranes against photodamage. Lipid bilayers were challenged with photoinduced damage by photosensitization with methylene blue, which usually causes formation of hydroperoxides, increasing area per lipid, and afterwards allowing leakage of internal materials. The damage was delayed by a solution of gallic acid in a concentration dependent manner, but further suppressed by the polymer at very low concentrations. The membrane of giant unilamellar vesicles was covered with this modified macromolecule leading to a powerful shield against singlet oxygen and thus effectively protecting the lipid membrane from oxidative stress. The results have proven the discovery of a promising strategy for photo protection of biological membranes.

1. Introduction

The development of robust strategies of skin protection against excess of light absorption is a dynamic field of research. It is clear that filter-only strategies are not enough, since visible light also may promote damage on the skin tissue. Few molecules actually absorb UVA and visible radiation (derivatives of flavins, porphyrins, and melanin) and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is accomplished mostly by photosensitization. It is well known that photosensitization reactions involve light absorption by a photosensitizer and excited state reactions in which part of the energy absorbed is transferred to surrounding molecules either by electron transfer (type I) or energy transfer to oxygen (type II) reactions [1]. Type II reactions lead to formation of singlet oxygen ($^1O_2$), which is the main species involved in skin and hair damage due to UVA and visible irradiation [2].

Biological membranes contain unsaturated phospholipids and $^1O_2$ reacts with acyl chain double bonds to form hydroperoxides, which are the initial species leading to a full extent peroxidation [3]. Due to the hydrophilic character of the hydroperoxide, this group migrates to the bilayer surface increasing the area occupied by the lipid (Scheme 1). Indeed, the lipid peroxidation promotes a variety of physiologic and pathologic processes in the context of diseases related to oxidative stress [4].

Giant unilamellar vesicles (GUVs) are membrane mimics that allow direct measurements of membrane damage. We and others have used GUVs to study lipid photosensitization [5], membrane permeabilization [6], increase in area per lipid [7,8], formation of lipid domains [9] and the molecular disorder which yields to transient pores on the membrane [10]. Other authors have reported that anti-oxidant agents may reduce physical and molecular changes in membranes [11].

Here, we report studies using GUVs coupled to photophysical measurements to understand the factors affecting the protection of membranes against photoinduced $^1O_2$. As a protecting molecule we have used gallic acid (GA) (Scheme 1), which is a standard antioxidant known to deactivate $^1O_2$ [12]. Methylene blue (MB) has been employed as $^1O_2$ producer by photo excitation in aqueous solution.

Previous studies have shown the advantages of chitosan in providing specific features to liposomes and GUVs [13]. It has been shown that chitosan interacts with and irreversibly covers the vesicle membrane. Besides, chitosan is a reactive macromolecule and its modification to produce beneficial characteristics has been accounted. For instance, Curcio et al. [14] bonded antioxidant molecules as GA in a free-radical induced grafting reaction. The antioxidant and cytotoxicity properties of GA labeled chitosan have been described in cellular systems and

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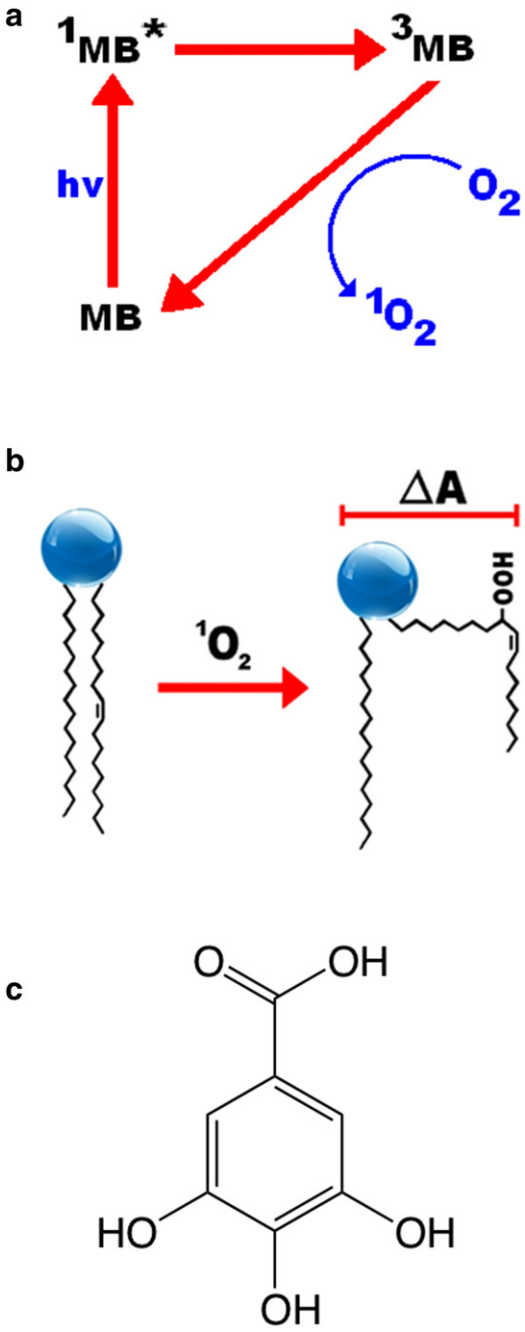

Scheme 1. Methylene blue (MB) absorbs light, is promoted to singlet state $^1MB^*$, by intersystem crossing to triplet state $^3MB^*$ where the absorbed energy is transferred to surrounding oxygen leading to $^1O_2$ that reacts with acyl chains of unsaturated lipids producing hydroperoxide with larger area per molecule (A). The molecule of gallic acid is shown on the right.

genomic DNA [15]. In this manner, we followed the procedure of [14] and went a step further in the photo protection development of biological membranes.

2. Materials and methods

2.1. Materials

Stock solutions of the phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) from Avanti Polar Lipids (Birmingham, AL) were prepared in chloroform (99.8%; Synth). Methylene blue (MB) was purchased from Sigma-Aldrich and all stock solutions were prepared in purified water. The MB molar concentration was checked using a spectrophotometer (Ocean Optics USB-2000; Dunedin, FL) by taking into account the molar extinction coefficient of 81,600 at $\lambda =$ 664 nm. Gallic acid (Sigma-Aldrich) solutions were prepared in MilliQ quality water. Chitosan was a gift from Primex (Germany), with 95% degree of deacetylation (DDA) and average molecular weigh 199 kDa (corresponding to 1223 repeat monomers per molecule). Fluorescent chitosan was obtained as previously described [16] introducing a fluorescent probe (fluorescein isothiocianate isomer I, FITC; 90%; Fluka BioChemika) on a few polymer monomers at a proportion of 1:100 (labeled:not labeled monomers) according to the procedure of Qaqish and Amiji [17]. Sucrose (99%; Sigma-Aldrich), glucose (99%; Sigma-Aldrich) and all other reagents were of analytical grade. All solutions were prepared using deionized water from MilliQ Millipore system with a total organic carbon value of less than 15 ppb and a resistivity of 18 MΩ cm.

2.2. Preparation of gallic acid labeled chitosan

Reaction was performed following Curcio et al. [14]. Briefly, 0.5 g of chitosan was dissolved in 50 mL of 2% acetic acid (v/v) by vigorous stirring during 2 h. Under slight stirring, 1 mL of 1.0 mM H_2O_2 containing 0.054 g of pre-dissolved ascorbic acid was added. The solution was kept under stirring during 30 min and then 0.264 g of gallic acid was carefully added under slight stirring. The solution was kept at rest during 24 h at room temperature. Afterwards, in order to eliminate unreacted components, the solution was transferred into dialysis tubes (MWCO 12,000–14,000 Da) and dipped into a 2 L glass vessel containing deionized water. The dialysis was performed under slight stirring at 20–22 °C during 48 h with eight changes of the glass vessel water. The labeled chitosan solution was further diluted to 1 mg/mL with the same acetate buffer (pH 4.48 ± 0.01) used for dissolving plain chitosan. This solution was used to prepare giant vesicles covered with labeled chitosan (see below).

The gallic acid amount, which was bonded on chitosan, was determined by the amount of total phenolic equivalents using Folin-Ciocalteu reagent procedure [14]. Labeled chitosan was precipitated from part of the solution obtained above by adding an equal volume of NaOH aqueous solution (0.1 M). The precipitate was filtered and vigorously washed until the pH of water was constant and later the solid was dried in a desiccator under vacuum during 2 h. GA-labeled chitosan (20 mg) was dissolved in 6 mL of water and 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich) was added under vigorous stirring. Next, 3 mL of a 2% Na_2CO_3 aqueous solution were mixed and the system was shaken continuously for 2 h. The absorbance of the solution was measured at 760 nm. An absorbance calibration curve was also obtained for GA solutions in the same conditions from six concentrations of GA ranging from 1 to 25 μg/mL. The total phenol content in chitosan was calculated using the standard curve equation obtained from the GA solution calibration, where y is absorbance at 760 nm and x is total phenolic content. The results amounted to a ratio of GA to chitosan monomers of 1:2, i.e., 50% of the monomers were grafted.

2.3. Preparation of giant unilamellar vesicles

Giant unilamellar vesicles of POPC were prepared using the traditional electroformation method [18]. Briefly, 10 μL of a 2 mM lipid solution in chloroform were spread on the surfaces of two conductive glasses (coated with Fluor Tin Oxide), which were then placed with their conductive sides facing each other separated by a 2 mm thick Teflon frame. This electro swelling chamber was filled with 0.2 M sucrose solution and branched to an alternating power generator (Minipa MFG-4201A; Korea) at 1.5 V and 10 Hz frequency during 2 h at room temperature (22–24 °C). Afterwards, the vesicle solution was carefully transferred to an Eppendorf vial and kept at rest at 4 °C before use. A typical observation experiment, using an inverted microscope