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Lipid vesicles loading aluminum phthalocyanine chloride: Formulation properties and disaggregation upon intracellular delivery

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ABSTRACT

Aluminum phthalocyanine chloride (AlClPc) is a second-generation photodynamic therapy (PDT) photosensitizer characterized for its high hydrophobicity and self-aggregation tendency in aqueous media, which hamper its potential application. Aiming at AlClPc solubilization we proposed here the use of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) at different proportions to form mixed lipid vesicles (LVs) as a drug delivery system. LVs were prepared by ethanol injection method and formed nano-sized vesicles (about 100 nm) with suitable polydispersity index, negative zeta potential, and stable in aqueous medium for at least 50 days. AlClPc strongly interacts with LV (high binding constant values), especially due to aluminum–phosphate specific interactions, which gives a surface localization to AlClPc molecules as demonstrated by fluorescence quenching data. Anisotropy, static and time-resolved fluorescence measurements corroborated with these results and demonstrated that AlClPc self-aggregation occurred even in the liposomes. However, formulation uptake by oral squamous cell carcinoma (OSCC) the AlClPc was distributed in cellular organelles and suffered a disaggregation process demonstrated by fluorescence life-time imaging microscopy. This amazing behavior is new and increases the scientific knowledge about the intracellular mechanism of action of PDT photosensitizers. In addition, these results open a new perspective to the potential use of AlClPc-LV formulations for photodynamic treatment.

1. Introduction

The use of well-conjugated molecules as active compounds in many photoprocess protocols in basic research and clinical trials and their use in the treatment of diseases associated with abnormal tissue growth have been described in the literature over the last three decades. Among these pathological diseases, cancer is one of the most extensively studied [1–3]. In addition, studies on antibacterial [4] and fungal disease activities [5,6], wound healing processes [6,7], and photooxidation processes focusing on current medical application have also been performed [8]. In recent decades, photodynamic therapy (PDT), photobiomodulation and photooxidative waste processes have also received attention from many researchers [9–11].

PDT comprises the administration of a photosensitizing agent (PS) to the specific target and the subsequent activation with visible light of appropriate wavelength. After excitation, the first excited state ($^1PS^*$) of the dye may undergo a reversal of spin to reach the excited triplet state ($^3PS^*$), which is able to react directly with biological substrates to form radical species and other reactive oxygen species (ROS). The energy transfer from $^3PS^*$ to molecular oxygen creates the high-reactive specie singlet oxygen ($^1O_2$) species of high reactivity [12]. All of these species in situ may cause structural damage at the cellular level, induce a cascade of biological events and, consequently, induce necrosis and/or apoptosis of the cancerous tissue [13,14], bacteria or fungus inactivation.

PS molecules need to exhibit some specific features: strong absorption of visible light at the wavelength used for activation; high quantum yield of singlet oxygen or ROS species production; low yield of photodegradation reaction avoiding loss of its photoactivity during the excitation process; favorable pharmacokinetics and pharmacodynamics; no prolonged photosensitivity after use, with a high clearance level from healthy or treated tissue; high stability; and low dark toxicity [15]. This set of conditions could be well designed for the specific proposed use, but generally, some of them are common to the studied photoprocesses.

Among the compounds that meet many of the above requirements are the metal-based conjugated phthalocyanines [16,17], a group of second generation molecules that present some of the behaviors expected for this class of molecules.

In particular, aluminum phthalocyanine chloride (AlClPc), a second-generation drug, has been widely used in our research group where numerous studies support the use of AlClPc as a PS for cancer, bacterial infections, and in the wound healing process [18–20]. However, AlClPc has a high tendency to self-aggregate in aqueous medium, which is an

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inconvenient process that drastically reduces AlClPc light absorption and reactive species generation, limiting its clinical application.

To overcome such issues, the engineering of nano-structured drug delivery systems (DDSs) to the efficient encapsulation of this type of compound has received widespread attention in recent years by influencing many of the functions of these molecules [21].

One nanomedicine-based technology for the delivery of PS as AlClPc is the liposomes (or lipid vesicles, LVs[1]) [22]. LVs may be the most effective for medical applications, given their excellent biocompatibility and targeting properties to the diseased tissue [14,23]. LV may be defined as colloidal particles with spherical internal aqueous compartments that exhibit one or more phospholipid bilayers. In addition, recent researchers have been demonstrating the potential use of this DDS for the formulation hydrophobic photosensitizer molecules [24].

The main objective of this work was the development and characterization of mixed lipid vesicles of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol and their use as a new DDS to deliver AlClPc for oral squamous cell carcinoma. The balance monomer/aggregate of AlClPc could be modulated by the fluidity of lipid vesicles using different ratios of DOPC/DSPC, which is very important to PDT. In addition, the influence of biological membrane, the primary barrier to DDS-PS systems, on form (monomer/aggregate) of AlClPc was also investigated. These studies will address most of the problems associated with the design of any types of DDS, for photoactive compounds, including the loss of the activity due to physico-chemical interactions or partitions that are not well controlled during the preparation of the DDS plus the active photosensitizer.

2. Materials and Methods

AlClPc (85% purity) was purchased from Sigma-Aldrich and used without further purification. Ultrapure water (Milli-Q) was used in all of the experiments, and analytical grade anhydrous ethanol was purchased from J.T. Becker®. The electronic absorption spectra were obtained using a UV/Visible Ultrospec 2100 pro-spectrophotometer from 300 to 800 nm. For the fluorescence measurements, an Amersham Ultrospec 2100 pro-spectrofluorometer with a fixed excitation wavelength of 610 nm and emission from 650 to 800 nm was used. The electronic absorption and fluorescence measurements were conducted with a 1-cm path length quartz cell. The average hydrodynamic diameter (z-average) and polydispersity index (PdI) were determined based on dynamic light scattering (DLS) with an angle of 173° using the Zetasizer Nano ZS (Malvern®). Zeta potential (ζ) and electrophoretic mobility were determined by using the same Zetasizer Nano ZS (Malvern®). The fluorescence lifetimes were measured using a Microtime 200 (PicoQuant) based on the Time-Correlated Single Photon Counting (TCSPC) method in the unique Time-Tagged Time Resolved (TTTr) measurement mode. Fluorescence Life-time Imaging Microscopy (FLIM) was performed using a Microtime 200 (PicoQuant) with an inverted microscope (Olympus IX-71) with a water immersion objective (UPlanSApo 60×, NA 1.2) and scanning with a monodirectional movement of the lens over an area of $80 \times 80\ \mu m^2$ with 1 nm position accuracy. A picosecond diode laser with an excitation wavelength of 640 nm, a pulse width of less than 70 ps and a repetition rate of 80 MHz was used as the excitation source. The experimental decay fit was performed using SymPhoTime® software. All experiments were performed at 25 (±1) °C. Fluorescence anisotropy measurements were also performed at 37 and 50 (±1) °C. The pH and ionic strength (μ) were 7.4 and 0.1396 mol L^{-1}, respectively, and were controlled by the presence of phosphate buffer solution (PBS) with a NaCl (0.1369×10^{-3} mol L^{-1}) and KCl (2.7×10^{-3} mol L^{-1}) mixture, except for the dynamic light scattering, zeta potential and polydispersity index, where μ was 1.396×10^{-3} mol L^{-1}.

2.1. Development of Mixed Lipid Vesicles

The encapsulation of AlClPc was conducted in the mixed lipid vesicles (LVs) containing DSPC, DOPC and cholesterol (chol). The LVs were prepared in the following proportions of DSPC, DOPC and chol, respectively: 69/00/31 (LV_I); 55/14/31 (LV_{II}); 34.5/34.5/31 (LV_{III}); and 21/48/31 (LV_{IV}) (mol%). Ethanolic solution was prepared by combining the compounds as follows by the dissolution in anhydrous ethanol (178.4 μL): 1.47 mg of DSPC and 0.32 mg of chol for LV_I; 1.18 mg of DSPC, 0.29 mg of DOPC and 0.32 mg of chol for LV_{II}; 0.73 mg DSPC, 0.73 mg of DOPC and 0.32 mg of chol for LV_{III} and 0.44 mg of DSPC, 1.02 mg of DOPC and 0.32 mg of chol for LV_{IV}. To the solution, 5.1 μL of AlClPc stock (1.79×10^{-3} mol L^{-1}) in ethanol was added. The final solution was injected using a peristaltic pump under controlled velocity (1 μL/s) against 3.00 mL of PBS at pH 7.4 contained in a window at 57 °C. Empty LVs were injected in the absence of AlClPc. The injection was performed under magnetic stirring in the dark with a speed injection of 1 μL/s.

2.2. Particle Size and Polydispersity Index Analysis

The mean hydrodynamic diameter (z average) and the polydispersity index (PdI) of the LV were determined using dynamic light scattering (DLS) at 25 °C and the scattering angle of 173° (Zetasizer® Nano ZS, Malvern PCS Instruments, UK). Samples were obtained by diluting 20 μL LV with 2 mL of ultrapure water. The reported values are the mean ± SD of three different batches of each colloidal dispersion.

2.3. Zeta Potential Measurement

The ζ potential of the AlClPc-loaded LV was measured using electrophoretic mobility with a Zetasizer® Nano ZS apparatus (Malvern PCS Instruments, UK). The analyses were conducted at 25 °C, and the samples were appropriately diluted (1/100) with ultra-purified water. Values reported are the mean of three different batches of each colloidal dispersion.

2.4. Fluorescence Anisotropy

To estimate the relative fluidity of the LV, the fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH, 0.5×10^{-6} mol L^{-1}) at several LV values ([lipid] = 1.75×10^{-3} mol L^{-1}) was studied. The wavelengths used for the excitation and emission were 327 and 430 nm, respectively. Values of r and G factor were calculated according to Eqs. (1) and (2), respectively.

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2.G.I_{VH}} \quad (1)$$

where,

$$G = \frac{I_{HV}}{I_{VH}} \quad (2)$$

G is an instrumental correction factor for the rate of polarized light [25].

2.5. Binding Constant of AlClPc in Mixed Lipid Vesicles

The binding constant (K_b) of AlClPc in several LVs was estimated using fluorescence spectroscopy. LV solutions at concentrations ranging from 0.0 mol L^{-1} to 1.2×10^{-3} mol L^{-1} were prepared in PBS (pH 7.4, μ = 1.396 mol L^{-1}) followed by the addition of AlClPc stock (0.12×10^{-3} mol L^{-1}) to obtain a final concentration of 0.3×10^{-6} mol L^{-1}. The fluorescence spectra of AlClPc into LV at different concentrations

[1] LV = lipid vesicles.