European Journal of Pharmaceutics and Biopharmaceutics 88 (2014) 551–555

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Assessment of drug entrapment within liposomes using photophysical probes

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ARTICLE INFO

Article history:
Received 30 January 2014
Accepted in revised form 10 June 2014
Available online 26 June 2014

Keywords:
Cinacalcet
Drug carrier
Drug delivery
Fluorescence
Laser flash photolysis
Liposome
Naphthalene chromophore
Naproxen
Singlet excited states
Triplet excited states

ABSTRACT

The photophysical and photochemical behavior of (*R*)-cinacalcet (CIN) and (*S*)-naproxen (NPX) entrapped within liposomes has been studied. For this purpose, liposome encapsulated drugs have been prepared through thin layer evaporation and characterized by transmission electron microscopy, cryoscopy scanning electron microscopy and dynamic light scattering. Steady state and time-resolved fluorescence experiments showed similar spectra, emission quantum yields, singlet energies and lifetimes for the selected drugs, outside and inside liposomes. By contrast, laser flash photolysis experiments revealed an important enhancement of the triplet lifetimes for entrapped drugs inside liposomes, indicating the spatial confinement existing in the microenvironment prevailing in these biomimetic entities. Thus, this photophysical property shows potential as a non-invasive, direct and valuable tool to monitor encapsulation of photoactive drugs and to probe the intraliposome environment. In addition, it provides a new quantitative indicator of the capability of liposomes to act as drug carriers.

1. Introduction

Liposomes are the smallest artificial vesicles of spherical shape that can be produced from natural phospholipids and cholesterol. They are very versatile tools in biology, biochemistry and medicine [1–5] and have attracted attention as potential vehicles for drug delivery to selected cells or tissues *in vivo* and as carriers of drugs or other bioactive compounds including proteins, hormones and diagnostic agents [6,7]. Although the interest has been mainly focused on drug delivery, a variety of other applications have been found in cosmetics, cleansing and food technology [6,8].

Liposomes are simple biomimetic models for cell membranes and provide appropriate microenvironment for the study of photoinduced electron transfer [9] and lipid peroxidation [10,11]. Adoption of this matrix seems very promising in photodynamic therapy, based on the generation of reactive oxygen species that destroy the tissues or target cells by the use of photosensitizing compounds, which absorb photons and are excited to a triplet state, capable of generating superoxide anion or promoting molecular oxygen to its highly reactive singlet state. This mechanism seems responsible for cell death in tumor tissues or for stimulation of immune responses that cause cell death. Despite the progress made in the study of clinically available photosensitizing agents, there are still several drawbacks limiting their use. For example, their hydrophobic nature limits their efficiency and delivery in the body. To circumvent these problems, alternative formulations are required to enhance solubility and to achieve selective delivery to tumor sites. In this context, liposomes have the ability to encapsulate hydrophobic molecules. Inclusion of photosensitizers within this type of matrix has revealed a good correlation between the physicochemical features of formulations and their photosensitizing

Abbreviations: CIN, (*R*)-cinacalcet; CryoSEM, Cryoscopic Scanning Electronic Microscopy; DLS, Dynamic Light Scattering; EE, encapsulation efficiency; E_s, singlet energy; HPLC, high performance liquid chromatography; k_{Fq}, fluorescence quenching rate constant for oxygen; k_{Tq}, triplet quenching rate constant for oxygen; Lip, liposome; LFP, laser flash photolysis; LUV, large unilamellar liposomes; NPX, (*S*)-naproxen; PBS, phosphate buffered saline solution; PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; PTI, Photon Technology International; TEM, Transmission Electronic Microscopy; TLE, thin layer evaporation; τ_s, singlet lifetime; τ_T, triplet lifetime; UV–Vis, ultraviolet–visible.

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http://dx.doi.org/10.1016/j.ejpb.2014.06.013
0939-6411/

efficiency [12]. Moreover, interaction of light with photosensitive liposomes may cause membrane reorganization and offers an appropriate tool for selective drug release in the target site, with a significant impact on the therapeutic index of drugs. Additional applications of this effect can be found in imaging or sensing [13]. The study of molecular mechanisms that result in photo-destabilization of liposome membranes is essential in developing light-activable liposomes for photodynamic therapy with higher opportunities of success in the clinical practice [14].

Recently, naphthalene-like triplet excited states have been used as reporters for the interaction of drugs with transport proteins [15–17] and for the incorporation of photoactive compounds within cholic acid aggregates [18] and mixed micelles [19]. In the present work, two naphthalene-derived drugs, namely (*R*)-cinacalcet (CIN) and (*S*)-naproxen (NPX) have been entrapped within liposomes, and photophysical studies have been performed in order to establish how this microenvironment influences the excited state dynamics of both drugs. The chemical structures of CIN and NPX are reported in Chart 1. The former (CIN) is a recently marketed calcimimetic drug, mimicking the action of calcium on tissues, by allosteric activation of the calcium-sensing receptor [20,21]. This agent is used for the treatment of persistent hyperparathyroidism in renal transplanted patients, with chronic kidney disease and renal failure in dialysis [22–24]. It is also used for the treatment of hypercalcemia in patients with parathyroid carcinoma, in order to decrease the calcium level in blood [25–27]. As regards NPX it is widely used as a highly effective nonsteroidal anti-inflammatory drug. Although NPX is rather safe and tolerable, serious gastrointestinal side effects, and in some cases skin photosensitivity, may appear after oral administration [28–31]. Besides, it exhibits red blood cells photohaemolytic activity and causes photocleavage of DNA [28,29,32].

At the present stage, our goal is to assess the entrapment of both naphthalene-derived drugs within biomimetic liposomes by means of non-invasive and straightforward spectroscopic tools, specifically fluorescence and laser flash photolysis (LFP). Selection of CIN and NPX as probes seems appropriate, since they present a two-channel responsive chromophore and appear in principle suitable systems to evaluate their encapsulation inside liposomes by photophysical techniques.

2. Materials and methods

2.1. Materials

NPX and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC), with purity higher than 99%, were purchased from Sigma–Aldrich (Steinheim, Germany). The latter was stored as purchased at −20 °C. CIN was extracted from commercial Mimpara 60 mg (Amgen, Spain). The content of six pills was powdered in a mortar, suspended in NH_4OH 1 M (50 mL) and extracted with CH_2Cl_2 (4 × 50 mL). The combined organic layers were washed with brine (3 × 150 mL) and water (3 × 150 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography through silica gel 60 (dichloromethane:methanol:ammonium hydroxide 98:2:0.1 v/v/v) to give CIN as a colorless oil. The purity of isolated CIN was higher than 98% as indicated by ^1H and ^{13}C NMR spectroscopy. Chloroform and methanol were of HPLC grade from Scharlab (Sentmenat, Spain) and were used without further purification. Phosphate buffered saline solution (PBS) (pH = 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate amount of purified water (Milli-Q system, Millipore).

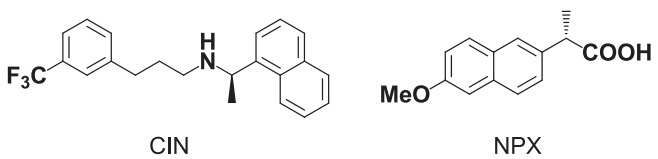

Chart 1. Chemical structures of CIN and NPX.

2.2. Preparation of liposomes

Liposomes including CIN and NPX were prepared by thin layer evaporation (TLE) method according to standard conditions [33,34]. Liposomes were obtained by dissolving 12.1 mg of PC in 10 mL of chloroform containing the drug (*ca.* 2×10^{-3} M) in a 50-mL round-bottomed flask. Drug-free liposomes were prepared as blank. The solutions were evaporated at 58 °C by using a vacuum rotary evaporator for 10 min, to remove the organic solvents. Then, the residual solvent was completely eliminated by drying under vacuum for 2 h. The obtained lipid film was hydrated with 2 mL of PBS, leading to a liposomal formulation. Large unilamellar liposomes (LUV) were produced starting from multilamellar ones by sonication in an ultrasonic bath for 30 min at 50 °C. In this way, more homogeneous size vesicles were obtained; they were kept under controlled temperature (4 °C) until their use within 3 days. Immediately before use, 0.5 mL of liposome emulsion was added to the same amount of PBS and purified by centrifugation at 14,000*g* during 10 min for 3 times at room temperature in a HERAUS Pico 21 centrifuge (Thermo electron corporation, Germany).

2.3. Size analysis

Liposomes were characterized by Transmission Electronic Microscopy (TEM), Cryoscopic Scanning Electronic Microscopy (CryoSEM) and Dynamic Light Scattering (DLS). TEM observation of liposomes was carried out by a JEOL2010-FEG TEM/STEM microscope, equipped with a field emission electron source and an objective lens with *Cs* value of 0.5 mm. The images were digitally recorded on a 1024 × 1024 CCd camera. Image analysis was performed using routines implemented in the software plugins of Digital Micrograph. For TEM measurements, 5 μL of purified liposomes were put on a holey-carbon coated 3-mm Cu-grid and dried at room temperature under a cabinet equipped with an aspiration system. After complete evaporation of the solvent, the sample was ready for direct observation. SEM images were obtained using a JEOL JSM-6300 microscope operating at 20 kV. For CryoSEM analysis, samples were frozen, and thin frozen sections were imaged without staining. For both microscopic analysis liposomes were rehydrated with Millipore water instead of PBS, in order to avoid the optical interference of saline crystals in the medium. DLS measurements were conducted using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom). The liposomes were diluted with deionized water and the experiments were performed at 25 °C and 173° scattering angle. The mean hydrodynamic diameter was determined by cumulant analysis.

2.4. Drug entrapment efficiency

The encapsulation efficiency (EE) of CIN and NPX was determined using Eq. (1) [35]; it is expressed as the percentage of the drug trapped in purified liposomes referred to the non-purified solutions.

$$\text{Encapsulation efficiency } (\%) = \frac{Ct - Co}{Ct} \times 100 \quad (1)$$

where *Co* is the concentration in the supernatant diluted with PBS and *Ct* refers to the liposome emulsion diluted with warm ethanol