Colloids and Surfaces B: Biointerfaces 142 (2016) 214–222

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Delivery of fluorophores by calcium phosphate-coated nanoliposomes and interaction with *Staphylococcus aureus* biofilms

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ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form 22 February 2016
Accepted 1 March 2016
Available online 2 March 2016

Keywords:
Nanoliposomes
Calcium phosphate
Acridine orange
5, 10, 15, 20-Tetrakis(1-methyl-4-pyridinio)porphyrin
Staphylococcus aureus

ABSTRACT

The delivery capacity and mechanical stability of calcium phosphate (CaP) coated 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) liposomes free and adsorbed on bacterial surface was investigated introducing either acridine orange (AO) or 5,10,15,20-Tetrakis(1-methyl-4-pyridinio)porphyrin (TMP) in the aqueous core of the liposomes. The obtained nanomaterials were thoroughly characterized by electron and optical microscopy and by fluorescence techniques. Distribution of the AO and TMP molecules between the aqueous liposomes core and the outer solution was demonstrated by the band shifts and broadening of the excitation–emission matrices and the modified Stern-Volmer model for fluorescence quenching. In aqueous suspensions, c.a. 40% of AO was released to the outer solution while only a small percentage of TMP was observed to reach the outer liposome surface. The nanoliposomes adhesion capacity and the leaking of fluorophore molecules to *Staphylococcus aureus* (*S. aureus*) biofilms were further evaluated. A close interaction between liposomes and *S. aureus* biofilm was evidenced by TEM and SEM imaging. Epifluorescence experiments demonstrated that CaP-coated liposomes have good biofilm staining capability after two hours incubation of the biofilms with the liposomes, thus supporting an important release of the fluorophores when in contact with the biofilm. Altogether, the obtained results strongly suggest that CaP-coated liposomes are capable of activating drug release when in presence of *S. aureus* biofilms and smears. The studies herein presented, indicate that CaP-coated liposomes are potential vehicles for the selective delivery of drugs to *S. aureus* biofilms, as is the case of the singlet oxygen photosensitizer TMP, a well known photodynamic antibacterial agent.

1. Introduction

Liposomes carrying entrapped reporter molecules have been used in immunodiagnostics to provide signal amplification and to lower the detection limits of analytes [1]. Also, fluorescent dyes and quantum dots entrapped into hydrophilic lipid-based particles were developed as attractive targets for selective delivery of imaging agents to tumor cells [2,3]. The photophysical properties of encapsulated fluorophores are often regulated by the micellar structure improving the sensory capability of the dye [2,4]. Although liposomes promise the improvement of diverse abilities of the entrapped molecules [5], several drawbacks inherent to their structural assembling need being considered. Among the deficiencies of the lipid vesicles are the entrapped molecules leakage due to diffusion which results in a diminution of the efficiency [1], the liposome tendency to coalesce which turns their suspensions poorly stable, and the organic nature of their surface which converts them in labile structures [6]. In order to overcome these problems, coating with biocompatible and biodegradable substances such as polymers and calcium phosphate shells has been reported [7–12]. The calcium phosphate stoichiometry of the shell may be variable as the distinct phases are highly dependent on pH and the activity of ions in solution. As a consequence, they are denominated as CaP [9,13]. In particular, CaP-coated 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) liposomes have been reported as efficient nanoreactors for H_2O_2 sensing and drug delivery [9,14].

In the present study we assessed the potential ability of CaP-coated DOPA liposomes as vehicles for drug delivery in media inoculated with bacteria. To that purpose, the CaP-coated DOPA nanoliposomes were loaded with two extensively used dyes: acridine orange (AO) and 5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin (TMP), Fig. 1. Acridine orange is a versatile and cell-

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http://dx.doi.org/10.1016/j.colsurfb.2016.03.003
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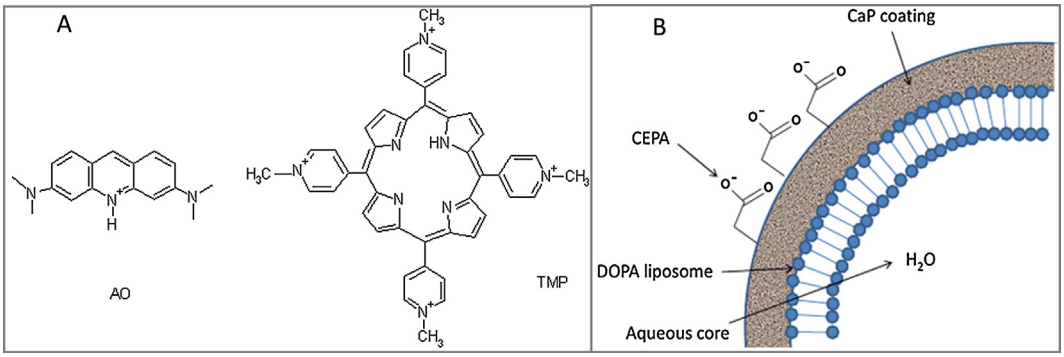

Fig. 1. (A) Acridine orange (left) and 5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin(right) molecular structures. (B) Schematic view of the CaPLi nanoliposomes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

permeable fluorescent dye which has been long and extensively used in fluorescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status, and in the microscopic examination of microorganisms [15,16]. AO is adequate to stain planktonic bacteria, sessile cells, and biofilms. On the other hand, the water-soluble TMP is an efficient sensitizer of singlet oxygen [17,18] and therefore an efficient antimicrobial photodynamic agent [19]. The larger molecular size of TMP than that of AO allows the investigation of the effect of size on the diffusion of the fluorophores out of the CaP-coated liposome core.

Since *Staphylococcus aureus* (*S. aureus*) is one of the major worldwide pathogen causing community-acquired and nosocomial infections and its biofilms have been associated with poorer postsurgical outcomes [20,21], it was selected as a model of opportunistic pathogen target to evaluate the capability of CaP-coated liposomes as vehicles for the delivery of the encapsulated antimicrobial and staining fluorophores.

2. Experimental

2.1. Chemicals

1,2-Dioleoyl-sn-glycero-3-phosphate (DOPA) lipid was obtained as a lyophilized powder and stored at −18 °C until used (Avanti Polar Lipids). 2-Carboxyethyl phosphonic acid (CEPA)(94%,Sigma-Aldrich), glutaraldehyde (25%, Sigma −Aldrich) $CaCl_2$ (analitycal grade, Anedra), H_3PO_4 (85%,Cicarelli), NaCl (ACS,Anedra), NaOH (ACS, JTBaker), acetone (HPLC grade, Merck), glacial acetic acid (pro analysis, Merck), acridine orange (AO,Sigma, St Louis, MO, USA) and 5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin (TMP,97%, Aldrich) were used without further purification. Regenerated cellulose dialysis tubing Spectra Por 1 with a MWCO of 6000–8000 Da was obtained from Spectrum Labs. A handheld extrusion apparatus with 100-nm polycarbonate (PC) filters (Millipore) was obtained from Avanti (Mini Extrusor 610000). Deionized water (>18 MΩ cm, <20 ppb of organic carbon) was obtained with a Millipore system.

2.2. Liposome synthesis

Liposomes were prepared by extrusion of 1 mL of 1 mg/mL DOPA lipid suspension in distilled water through a 100-nm PC filter at room temperature 11 times. The suspension contained alternatively AO (1×10^{-3} M) or TMP (1×10^{-4} M). After this procedure, the suspension became clear, indicating liposome assembly formation. In order to draw out the excess of AO or TMP, the solution was subsequently dialyzed against distilled water for 2 h and then overnight. DOPA liposomes are stable enough to act as a template for CaP nanoshells [10], as its negatively charged phosphatidyl groups assists the deposition of calcium ions around the vesicles [9].

2.3. Calcium phosphate (CaP) coating

CaP-coating of liposomes was performed following an adaptation of the method reported in the literature [9]. Freshly prepared 1 mL aqueous suspensions of liposomes and 100 μL of 0.1 M $CaCl_2$ were added to a 50 mL aqueous solution containing 10 μL of 1 M H_3PO_4 and 40 μL of 1 M NaOH (pH 10), while continuously stirring at 400 rpm. After 10, 30 or 120 min, 50 μL of 0.1 M CEPA was added to stop the CaP growth reaction. Subsequently, the suspension was stirred for additional 10 min, after which the samples turned opalescent. In order to eliminate the excess of AO and TMP, solutions were subsequently dialyzed against distilled water first for 2 h and then overnight. The resulting suspension was stored at 4 °C. The obtained samples were identified as: CaPLi, CaPLi-AO, and CaPLi-TMP, for CaP-coated DOPA liposomes with no added chromophore in the aqueous core, with added AO, and with added TMP, respectively.

Since the shell thickness was reported to depend on the elapsed reaction time of the synthesis mixture until CEPA addition [9], the thickness of the CaP shell capable of minimizing light scattering was optimized. To that purpose, the fluorescence intensity of CaPLi-AO particles as a function of the shell formation time was evaluated. Negligible emission was already observed after 30 min of CaP precipitation. Therefore, CEPA addition time was set to 10 min. During this period 40% of the initial emission is retained.

2.4. Bacterial suspensions and biofilm and smears formation

S. aureus (ATCC-25923) was inoculated in 150 mL of sterile liquid nutrient broth (Merck, Darmstadt, Germany) and grown for 24 h under gentle agitation at 37 °C until the optical density at 600 nm was greater than 1. Biofilms were prepared pouring 20 μL of bacterial suspension of OD = 1 over a glass slide which was incubated at 37 °C. After 2 h, the slides were washed with sterile water. 30 μL of CaP-Li AO suspension was poured over the biofilm. The liposomes were left to interact with the biofilm either for 15 min or 2 h at 37 °C. Then, slides were rinsed by immersion in a buffer solution. Alternatively, smears of planktonic culture were placed on a glass slide and 40 μL of 1:10 concentrated CaPLi-TMP were dripped over them and left to interact with the smears for 2 h at 37 °C prior to their observation.

2.5. Minimum inhibitory concentration determination

The Minimum Inhibitory Concentration (MIC) of AO, CaPLi-AO, TMP and CaPLi-TMP against S. aureus strain was determined by