Journal of Colloid and Interface Science 532 (2018) 782–789

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

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

Regular Article

Role of oligo(malic acid) on the formation of unilamellar vesicles

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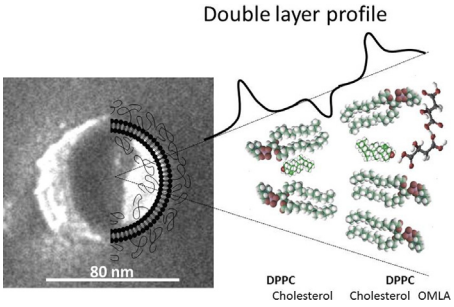
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 May 2018
Revised 4 August 2018
Accepted 7 August 2018
Available online 9 August 2018

Keywords:
Sterically stabilized vesicle
Oligo(malic acid)
Freeze fracture
Small-angle X-ray scattering (SAXS)

ABSTRACT

Stable unilamellar dipalmitoylphosphatidylcholine vesicles were produced by using oligo(malic acid) and cholesterol. Detailed physico-chemical characterization prove that by using oligo(malic acid) the substitution of PEGylated lipids for sterically stabilization comes possible. The polymer molecules cover the outer surface of spherical-shaped vesicles, and an asymmetrical composition occurs in the two leaflets of the phospholipid bilayer. The oligo(malic-acid) and cholesterol are enriched in the outer side assuring the stabilization of vesicles. Cholesterol plays an important role in the self-assembly of components as it makes the entering of oligomers possible deep into the polar head-region of lipids. The presence of oligo (malic acid) molecules does not induce degradation by hydrolysis of lipid molecules but the vesicle system turns into a sensitive form giving a possibility for pH sensitive targeting. Preliminary investigation on the investigated oligo(malic acid)-stabilized vesicles do not show any toxic effect promising their applicability in the field of liposomal drug delivery.

1. Introduction

The long-term circulation of sterically stabilized vesicles (SSV) in the vascular system is typically achieved by anchoring biocompatible polymer molecules onto their surfaces [1–3]. These small (≤100 nm), unilamellar vesicles are typical nanocarriers for therapeutic drugs and are already widely used in clinical practice [4]. Poly(ethylene-glycol), the commonly used biopolymer responsible for stabilization is covalently bonded to the head group of phospholipids. From these compounds the lipid part is embedded into the bilayer while the PEG chain covers the outer surface of the bilayer. These properties result in a biocompatible, "stealth" character of the whole object that hides them from the immune sys-

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https://doi.org/10.1016/j.jcis.2018.08.020
0021-9797/

tem. The first medical product based on SSVs was the liposomal doxorubicin, which was followed by many other formulations dominantly dedicated for cancer therapies [5]. Further modifications of SSVs provide broad possibilities in biological, pharmacological, cosmetic and medical applications [6,7]. The usage of PEGylated SSVs is widespread, although it can cause a complement activation-related pseudo-allergy (CARPA) in some patients, resulting in dramatic cardiovascular and clinical abnormalities [8]. Consequently, in order to achieve fully biocompatible SSVs without any side-effect, great scientific effort is invested in research worldwide. It has been found that biopolymers used as nanoconjugate delivery systems can form a compact shell on the vesicles. For example, Rinaudo et al. have studied the stabilization of liposomes by polyelectrolytes like chitosan and hyaluronan [9,10]. Without doubt, hyaluronan, as a fully biocompatible biopolymer and a constituent of the extracellular bio-matrix is an effective molecule to assure the long circulation time of vesicles [11–13].

The multifunctional, nontoxic and nonimmunogenic poly(L-glutamic acid), poly(L-aspartic acid) and poly(L-malic acid) (PMLA) are also prominent biopolymer constituents of nanoconjugates [14]. PMLA is a fully biocompatible polyester that is degradable by hydrolysis, leading to the production of the nontoxic malic acid, which also participates in the citrate-circle. A further advantage of PMLA is that it contains carboxylic groups where various bioactive ligands can be attached. Numerous synthesis routes are known to produce both racemic and optically active PMLAs [15–17]. In order to reveal the nature of the interactions between PMLA and the cell membrane surfaces, the changes in thermotropic and structural behavior of fully hydrated multilamellar dipalmitoylphosphatidylcholine (DPPC) system was studied in the presence of low molecular weight PMLA [18]. It was found that PMLA attaches to the headgroups of the phospholipids through hydrogen bonds between the free hydroxyl groups of PMLA and the phosphodiester groups of DPPC. The acidity of PMLA is not dominant and it does not causes hydrolysis in the lipid system [19,20]. Low molecular weight PMLA, however, induces a change on the electrostatic state of lipid headgroup region, even in the presence of buffer in the solvent, whereby uncorrelation between the lamellae can occur indicating the possible utilization of this charged polymer in stabilization of vesicles. Consequently we have constructed a new type of unilamellar vesicles with oligo(malic acid) for the purposes of drug delivery. Here we show the complex characterization – applying morphological, structural and theoretical calculation methods – of these vesicles and elucidate the role of each constituent in the colloidal stabilization of the system.

2. Materials and methods

Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, >99% purity) was purchased from the NOF Corporation (Japan). Phosphate buffered saline (PBS) and cholesterol (>99% purity) were obtained from Sigma-Aldrich. These materials were used without further purification. The low-molecular weight oligo(malic acid) (abbreviated further on as OMLA) was purchased from Nanochem Ltd. (Szeged, Hungary) synthetized by the method of Kajiyama et al. [21,22].

2.1. Preparation of vesicle systems

Concentrated hydrated samples (20 wt% lipid), forming multilamellar vesicles, were made to elaborate the proper preparation protocol. The weight ratio of OMLA to lipid was 0.1. The samples were prepared using two different routes: (i) the main components – lipid and OMLA – were mixed in solid powder state and hydrated in 10 mM PBS buffer (7.4 pH); (ii) the dry lipid was hydrated with a solution of OMLA prepared with 10 mM PBS buffer. The resulting viscous suspensions were homogenized by cooling and heating cycles (4°–50 °C) applied twenty times.

The unilamellar sterically stabilized vesicles (SSVs) containing also cholesterol were prepared by lipid film hydration and extrusion method [23] in small (2 w/w%) lipid concentration. Solid DPPC and cholesterol (3:1 M ratio) were dissolved in a chloroform: methanol (2:1 v/v ratio). The solvent was then evaporated at 40 °C and the dry film was kept in vacuum overnight to remove traces of the solvent. The lipid films were hydrated with OMLA solutions (10 mM PBS made of ultrapure water (18 MΩ cm) corresponding to 0.05 and 0.1 OMLA/DPPC weight ratios. Weight ratio was used to characterize the composition because of the uncertainty of OMLA molecular weight. Taking into consideration the LC-MS analysis, the 0.05 and 0.1 w/w ratios correspond to approx. 0.05 and 0.1 mol/mol ratios, respectively. Twenty thermal cycles (4°–50 °C) were applied in order to ensure proper hydration and homogenization. The samples were extruded (at 60 °C) eight times through polycarbonate filters (Nucleopore, Whatman Inc.), first with 200 nm then 80 nm pore sizes using a LIPEX extruder (Northern Lipids Inc. Canada).

2.2. Liquid chromatography-mass spectrometry (LC-MS)

The molecular weight distribution of OMLA dissolved in water was analysed by liquid chromatography-mass spectrometry. LC-MS measurements were performed on a Sciex API2000 tandem mass spectrometer equipped with electrospray source. A Perkin Elmer Series 200 HPLC pump was used for solvent delivery system. Sample was introduced in flow injection mode into a water: acetonitrile flow (1:1). Flow rate of the eluent was 200 μL/min. Ions were detected in negative ion detection mode. Spray voltage was −4800 V. Source temperature was 300 °C. The LC-MS spectra show the occurrence of oligomer forms from dimer up to eleven units. The most intensive peaks correspond to 6 and 8 times of units. Monomer, however, was also present in low amount.

2.3. Dynamic light scattering (DLS)

The size distribution profile of SSVs was determined by an AvidNano W130i dynamic light scattering apparatus (AvidNano, UK). 80 μL samples were used in a low-volume cuvette (UVette, Eppendorf Austria GmbH). The analysis of the measurement data was performed using the i-Size 3.0 software.

2.4. Transmission electron microscopy combined with freeze fracture (FF-TEM)

For freeze fracturing, an approximately 1 μL droplet of the samples was pipetted onto golden sample holders and rapidly frozen in liquid freon, then transferred into liquid nitrogen [24]. Fracturing was performed at −100 °C in a Balzers freeze fracture device (Balzers BAF 400D, Balzers AG, Liechtenstein). The surface of samples were etched for 30 s at −110 °C. The replicas, prepared by platinum-carbon shadowing, were cleaned with surfactant solution and washed by distilled water. From pure water the replicas were picked up on 200 mesh copper grids for examination in a Morgagni 268D (FEI, The Netherlands) transmission electron microscope.

2.5. Small angle X-ray scattering measurements (SAXS)

Small-angle X-ray scattering measurements were carried out at the ID02 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) [25]. The energy of the incoming beam was 12 keV and the two-dimensional scattering patterns were collected with a FReLoN 2000 CCD detector. The data were corrected