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Electrochemical and biological characterization of lyotropic liquid crystalline phases – retardation of drug release from hexagonal mesophases

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ABSTRACT

Self-assembled lipid liquid crystalline drug delivery systems of hexagonal (H2) and cubic (V2) structure were synthesized and characterized by means of small angle X-ray scattering, and electrochemical techniques. Biological analyses of the delivery potentials of the H2 phase were performed using cytotoxic assay and confocal microscopy and compared with the cubic phase. The study demonstrated a slow release rate from the H2 phase, which may serve as a novel matrix for sustained release. The data also indicate that empty H2 and V2 nanoparticles exhibit relatively low cytotoxicity, however when loaded with a chemotherapeutic drug (doxorubicin), cause a significant reduction in cell viability. It was confirmed that V2 nanoparticles likely present a more efficient and rapid drug release, while H2 nanoparticles exhibit a less prolonged drug discharge capability. We propose that H2 nanoparticles may be considered as valuable tools for sustained drug delivery systems in cancer therapy.

1. Introduction

Hydrated lipids can assemble into a variety of distinct material structures. This spectrum of lipid polymorphism includes: lamellar (Lα), inverted cubic (V2), inverted hexagonal (H2), and micellar phases [1–4]. Among them, V2 and H2 have received much attention due to their highly ordered internal structures, which offer the potential as a controlled release matrix for cargos of various sizes and polarities. The H2 phase consists of closed reverse micellar cylinders that are arranged in a hexagonal lattice. The structure of V2 mesophases comprises a curved bicontinuous lipid bilayer extending in three dimensions and two interpenetrating, but non-contacting, aqueous nanochannels. In the mesophase system that is based on monoacylglycerols three common bicontinuous cubic phases: the double-diamond (space group Pn3m), primitive (Im3m) and gyroid (Ia3d) phases may be formed [5]. Lipid-based liquid crystalline phases (LLCP) of hexagonal architecture offer the ability to provide sustained release for certain chemotherapeutics, where a prolonged delivery is preferable to immediate release. Sustained release delivery systems are advantageous since they offer prolonged therapeutic supply in time frame and may significantly reduce required drug dose and minimize its side effects.

Dispersing either the bulk H2 or V2 in excess of water and in the presence of stabilizer e.g. Pluronic F127, leads to the formation of H2 or V2 nanoparticles [6–9]. Dispersed hexagonal or cubic phases have much larger surface area and higher fluidity compared to bulk phases, and may exist as a solution of lower viscosity than the bulk phase [10–12]. Monoolein (glyceryl monooleate- GMO) is an example of a lipid exhibiting liquid crystalline behavior, which displays inverse types of architecture in the water environment. At room temperature the transition to the H2 phase may be obtained by addition of a third component that increases the negative curvature of the lipid layer leading to the formation of H2 mesophase at physiological conditions. Generally, the parameters that may affect the symmetry of LLCP include chain length, splay, and unsaturation within the chain and the size of the head-group [13]. Addition of lipophilic additive such as oleic acid (OA) to GMO induced phase transition of the V2 to H2 phase [14,15].

The amphiphilic character of the H2 mesophase enabled incorporation of drugs, peptides and proteins, improving their stability [16–18]. Phan et al. demonstrated that the state of the water compartments, whether open or closed, has a great influence on the rate of drug release from the distinct liquid crystalline materials [1]. The possibility of switching between the V2 (fast release) and H2 phase

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(slow release) by changing variables such as temperature and/or pH allows for designing a responsive drug delivery systems (DDS) [1,19]. Fong et al. used temperature 'switches' to change the nanostructure of bulk liquid crystalline matrices, which resulted in changes in drug release rate [20]. Changes in pH have been used to alter the delivery of phloroglucinol from monoolein doped with linoleic acid phases [21]. The system was able to reversibly switch from the V2 to H2 phase, when changing the pH from neutral to acidic. Release of the incorporated guest molecules from LLCP also depends on the composition and structure of the lipid bilayer and its interactions with guest molecules, and can be tuned by applying lipids with different acyl chains to change the charge delineating the aqueous channels [22,23].

Recently, we described the properties of doxorubicin (DOX) loaded in the monoolein cubic phase, where the liquid-crystalline cubic phase is considered as a potential drug delivery gel to the affected cells [24–26]. We showed that a neutral form of DOX (pH ~7), embedded in the lipidic bilayer, is less prone to being removed from the cubic phase to the solution, compared to the protonated form, which is mainly located in the aqueous channels. Here we have shown that due to low cytotoxicity and a more prolonged drug release capability, H2 nanoparticles are promising drug delivery vehicles. Cytotoxic assay showed a relatively small cytotoxic effect of non-doped H2 or V2 nanoparticles at the concentration of 100 μg mL^{-1} GMO in the nanoparticle formulation. Nevertheless, H2 and V2 nanocarriers loaded with DOX caused a significant reduction in the viability of HeLa cells in comparison to treatment with free doxorubicin.

2. Experimental

2.1. Chemicals and substrates

Monoolein (1-oleoyl-rac-glycerol) purity ≥99% (GMO), oleic acid (OA), 2-(N-morpholino) ethanesulfonic acid (MES), and doxorubicin hydrochloride (DOX), were purchased from Sigma-Aldrich (USA). All solutions were prepared with Milli-Q water (18.2 MΩ cm^{-1}; Millipore, USA). To prepare MES buffer, 0.1 M 2-(N-morpholino) ethanesulfonic acid was titrated with 0.1 M NaOH to obtain a pH of 5.5.

Human cervical carcinoma cells - HeLa (ATCC, USA) were cultured in RPMI 1640 Medium (Biological Industries, Israel), supplemented with 10% of fetal bovine serum (FBS; Biological Industries, USA) and 1% of Penicillin-Streptomycin Solution (Biological Industries) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Sample preparation

To prepare bulk non-doped hexagonal phase, GMO and OA were dissolved in chloroform at the ratio 8/2 w/w. The selected GMO/OA ratio has been shown to lead to the formation of a hexagonal phase gel [14]. Before hydration, chloroform was evaporated and the sample was left overnight to remove traces of chloroform. The final composition of the non-modified H2 phase was 50/12.5/37.5 wt% for the GMO/OA/MES buffer and 50/12.5/0.6/36.9 wt% for the MO/OA/DOX/MES buffer for the DOX loaded H2 phase. Non-doped bulk V2 phase was obtained by mixing melted GMO with buffer solution at the ratio chosen on the basis of the phase diagram as described previously [26]. V2 formulations with compositions of 62.5/37.5 and 62.5/0.6/36.9 wt % for the GMO/MES and GMO/DOX/MES, respectively were prepared. In order to compare the drug release rate from V2 and H2 phase lipid and DOX ratio were kept constant for all formulations. All samples before being used were stored at room temperature in darkness for a few days to equilibrate. To prepare DOX-loaded H2 and V2 phases, DOX was dissolved first in MES buffer before hydrating the lipids and prepared as described for non-doped systems. H2 and V2 nanoparticles were synthesized in the presence of Pluronic F127 solution, used as a stabilizer. The hexagonal or cubic phase were fragmented by a high-shear dispersing emulsifier (IKA T10 homogenizer). The total lipid content was kept at 94.7 wt%. To produce H2 particles lipid mixture of MO/OA at ratio 4:1 in MES buffer pH 5.5 was used. The final compositions of all formulations studied contained 0.1 wt% of doxorubicin. The selected F127/lipid ratio has been demonstrated to allow the formation of dispersed hexagonal phase particles.

2.3. Characterization of LLCP systems

SAXS has been used to characterize the phase behavior and structural parameters of liquid crystalline systems. Diffraction patterns were recorded using a Bruker Nanostar system working with CuKα radiation, equipped with Vantec 2000 area detector. To analyze, the 2D pattern was integrated into 1D scattering function I(q) where q($Å^{-1}$) is length of the scattering vector. The scattering vector q, was determined from the scattering angle using the relationship $q = (4\pi/\lambda)\sin\theta$, with 2θ being the scattering angle and λ being the wavelength of radiation. Before measurement, samples were loaded into 1.5 mm capillaries and left to equilibrate at room temperature for at least 12 h. Measurements were performed at 25 °C; scattered intensity was collected over 5 h for dispersed systems and 10 min for bulk mesophases. The lattice parameters (a) of the mesophases were calculated from the corresponding reciprocal spacings and used to determine structural parameters such as the lipid bilayer thickness and water channel diameter of formulations used. Structural parameters of mesophases were determined as described in S1 [27,28].

The average sizes and polydispersity of nanoparticles were determined through the dynamic light scattering (DLS, Zetasizer Nano ZS Malvern, UK) at 25 °C assuming a viscosity of pure water and presented as an average of three separate determinations. The refractive indexes used for lipid and water were 1.48 and 1.33, respectively. The encapsulation efficiency (EE%) was calculated using Eq. (1).

$$EE(\%) = \frac{C(DOX_{TOTAL}) - C(DOX_{FREE})}{C(DOX_{TOTAL})} \times 100\% \quad (1)$$

where $C(DOX_{TOTAL})$ is the concentration of DOX in nanoparticle, $C(DOX_{FREE})$ is the DOX concentration found in the ultrafiltrate. Free doxorubicin was separated from the nanoparticle formulation in centrifuge using Amicon tubes. The amount of free DOX was determined using absorption at 490 nm using UV/Vis spectrophotometer (Cary 60, Agilent, USA). Calibration curve of the DOX solutions was prepared and absorbance of the solutions was measured at 490 nm.

2.4. Electrochemistry

Electrochemical measurements were recorded using a CHI bipotentiostat with a standard three-electrode arrangement in buffered solution. Ag/AgCl was used as the reference electrode and a platinum foil as the counter electrode. The working electrode was glassy carbon electrode (GCE) modified with the H2 or V2 mesophase (the surface area of GCE was 7.01 mm^2). Before the experiments, the working electrode was polished on alumina of decreasing size (from 0.3 to 0.05 μm) on a polishing cloth. The electrodes were subsequently sonicated to remove adhered alumina particles, rinsed with ethanol and water and left to dry. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to study the influence of matrix on the DOX electrode reaction and to determine the DOX release from the matrix. To determine the release profile from bulk mesophases, electrode was modified with V2 or H2 phase. The mesophase was deposited on the electrode surface in the cylindric hole of a Teflon cap where the electrode surface was exposed to buffer. The thickness of the mesophase layer was kept constant (0.5 mm), so that the geometric volume of this layers remained the same during the experiments. The modified electrode was then immediately immersed in a deoxygenated supporting electrolyte solution. To obtain the release profile from dispersed systems, nanoparticles with DOX were placed in the dialysis membrane (MWCO 12–14 kDa) and submerged in 50 mL of MES buffer. Dialysis