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Liposome-loaded chitosan physical hydrogel: Toward a promising delayed-release biosystem

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ABSTRACT

This work deals with the elaboration of an original biosystem in view of its application as drug delayed-release device in biomedical area. This innovative "hybrid" system is composed of phosphatidylcholine liposomes entrapped within a chitosan physical hydrogel (only constituted of polymer and water). To this end, pre-formed liposomes were suspended into chitosan solutions, and the polymer gelation process was subsequently carried out following particular experimental conditions. This liposome incorporation did absolutely not prevent the gel formation as shown by rheological properties of the resulting tridimensional matrix. The presence of liposomes within the hydrogel was confirmed by fluorescence and cryo-scanning electron microscopies. Then, the expected concept of delayed-release of this "hybrid" system was proved using a model water soluble molecule (carboxyfluorescein, CF) encapsulated in liposomes, themselves incorporated into the chitosan hydrogel. The CF release was assayed after repeated and intensive washings of hydrogels, and was found to be higher in the CF-in-hydrogel systems in comparison with the CF-in-liposomes-in-hydrogel ones, demonstrating a CF delayed-release thanks to lipid vesicles.

1. Introduction

There is a huge amount of research being undertaken worldwide to exploit hydrogels as scaffolds to restore, replace or regenerate defective tissues. Among the biocompatible gels developed, chitosan-based ones have been extensively studied due to remarkable biological properties of this biopolymer (non-toxicity, Thanou, Verhoef, & Jungiger, 2001; Illum, 1998; biodegradability, Guo et al., 2006; Hirano, Seino, Akiyama, & Nonaka, 1988; mucoadhesivity, Bhattarai, Gunn, & Zhang, 2010; Dash, Chiellini, Ottenbrite, & Chiellini, 2011; bacteriostaticity, Jumaa, Furkert, & Muller, 2002; improvement in transport across biological barriers, Mooren, Berthol, Domschke, & Kreuter, 1998). The chitosan (CS) hydrogels have been mainly developed for cartilage tissue engineering (Montembault et al., 2006; Ladet, Tahiri, Montembault, Domard, & Corvol, 2011; Hao et al., 2010), wound-healing (Boucard et al., 2007; Obara et al., 2003), as well as biomedical molecule delivery applications (Bhattarai et al., 2010) (such as anti-cancer drugs, Azab et al., 2007; Azab et al., 2006; growth factors, Mattioli-Belmonte et al., 1999; Suh & Matthew, 2000). In the latter application, the CS hydrogels are indeed able to provide local delivery of a variety of therapeutic agents incorporated but the diffusion of these agents outside hydrogels can be rapid and not easily time-controllable. On the contrary, systems such as liposomal formulations can enable the delivery of drugs (water-soluble or not) in a predictable and sustained manner (Chonn & Cullis, 1995). Indeed, since the first description of liposomes in 1965, numerous clinical trials have been achieved in the delivery of anti-cancer, anti-fungal, antibiotic drugs, gene medicines, anesthetics and anti-inflammatory drugs. These lipidic objects were the first nanomedicine delivery systems to make the transition from concept to clinical application (Allen & Cullis, 2013). The success of liposomes as drug carriers has been demonstrated in various liposome-based formulations, which are commercially available or are currently undergoing clinical trials (Goyal, Goyal, Kumar, Katare, & Mishra, 2005).

In this context, "hybrid" systems involving liposomes incorporated into CS hydrogels present a very promising potential in tissue engineering and regenerative medicine. With such assemblies, the liposomes can be maintained at the delivery site thanks to the hydrogel (avoiding rapid clearance of liposomes), and the release

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of drugs from liposomes incorporated in the hydrogels is expected to be controlled by the long term destabilization/degradation of the lipid bilayer (Ruel-Gariepy, Leclair, Hildgen, Gupta, & Leroux, 2002). For example, Hosny (2009) showed that similar systems could enhance the transcorneal permeation of ofloxacin sevenfold more than the corresponding aqueous solution, improved the ocular bioavailability, minimized the need for frequent administration, and decreased the ocular side effects of ofloxacin. Another study described the use of analogous systems as highly immunogenic vaccine formulations that combined the benefits of a sustained vaccine release, provided by an injectable system, with particulate antigen and adjuvant delivery (Gordon et al., 2012). Other authors (Ruel-Gariepy et al., 2002) demonstrated that the *in vitro* release of a low molecular weight molecule in CS hydrogels was completed within 24 h whereas the gels containing liposomes released only 26% of this molecule after only 2 weeks. Finally, gels constituted of cytarabine-loaded liposomes were studied *in vitro*, and the results displayed that they could sustain release of encapsulated drug for more than 60 h compared with drug-loaded liposomal suspension (up to 48 h) (Mulik, Kulkarni, & Murthy, 2009). Pharmacokinetic studies of these systems resulted in higher $t(1/2)$ (28.86 h) and AUC (area under the curve of plasma concentration of drug, 2526.88 $\mu g\,h\,mL^{-1}$) in rats compared with cytarabine-loaded liposomal suspension or chitosan hydrogels containing free cytarabine. It is worthy of note that all the CS hydrogels implied in these different previous works are formed thanks to the adding of glycerophosphate as gelling agent (Ruel-Gariepy et al., 2002; Hosny, 2009; Gordon et al., 2012; Mulik et al., 2009; Wang, Zhang, Shan, Gao, & Liang, 2013).

Hydrogels can be divided into two classes: (i) chemical hydrogels constituted of irreversible covalent links, (ii) and physical hydrogels formed by reversible cross-links. Chemical hydrogels generally present better mechanical behaviors but the use of cross-linker additives can be harmful to biological applications. This is the reason why the synthesis of physical hydrogels was selected in our work. The physical gelation process set up in our team occurs from a simple aqueous solution of chitosan (Montembault, Viton, & Domard, 2005a). It offers the great advantage to get physical hydrogels only composed of chitosan and water, without any other additive.

The aim of the study presented herein is to develop "intelligent" drug delivery devices combining liposomes and chitosan physical hydrogels obtained from aqueous biopolymer solutions as previously described (Montembault et al., 2005a). Such liposome/hydrogel systems are completely innovative. In this paper, the formation process of these systems, and the way to incorporate liposomes into the hydrogels are studied. A rheological and microscopic characterization of the resulting "hybrid" hydrogels is then presented. Finally, an investigation about the release of a model molecule (*i.e.*, carboxyfluorescein) encapsulated in liposomes, themselves incorporated in hydrogels, is described and discussed.

2. Experimental

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocoline (DPPC), chloroform, acetic acid (99.8%), ammonium hydroxide 28%, and carboxyfluorescein (CF) were purchased from Sigma Aldrich. 1-palmitoyl-2-(12-[(7-nitro-2-1,3-benzoxadiazol-4yl)amino]dodecanoyl)-*sn*-glycero-3-phosphocholine) (NBD-PC) fluorescent lipid and chitosan (CS) polymer were purchased from Avanti Polar and Mahtani Chitosan Pvt. Ltd (India), respectively.

Purification and characterization of chitosan. To obtain a high-purity material, chitosan was dissolved at 0.5% (w/v) in an aqueous acetic acid solution, by the addition of the necessary amount of acid to achieve the stoechiometric protonation of the $-NH_2$ sites. After complete dissolution, the chitosan solution was successively filtered through 3, 1.2, 0.8, and 0.45 μm Millipore membranes. Then, dilute ammonia was added to the filtered chitosan solution to fully precipitate the polymer. Finally, the precipitate was repeatedly rinsed with deionized water until a neutral pH was achieved. Then, it was centrifuged and lyophilized. The weight-average molecular weight of chitosan ($Mw = 550{,}000 \pm 50{,}000\,g\,mol^{-1}$), and molecular weight distribution ($Đ = 1.7 \pm 0.3$) were determined by size exclusion chromatography (SEC) coupled on line with a differential refractometer (RI, Optilab T-rEX from Wyatt Technology), and a multiangle laser-light scattering detector (MALLS, Dawn EOS from Wyatt Technology) equipped with a laser operating at 690 nm. SEC was performed by means of TSKgel G2500PW and G6000PW columns. A degassed and filtered (on 0.1 μm) 0.15 M ammonium acetate/0.20 M acetic acid buffer (pH = 4.5) was used as eluent at a flow rate of 0.5 $mL\,min^{-1}$. The degree of acetylation, DA, calculated from the ^1H NMR spectrum (Montembault, Viton, & Domard, 2005b), was close to 4%.

2.2. Liposome elaboration

Lipids were dissolved in chloroform, and the solvent was then removed by rotary evaporation under reduced pressure yielding a homogeneous and thin lipid film. Large multilamellar vesicles (LMV) were obtained by adding distilled sterile water or CF solution (to get a final lipid concentration of 10 $mmol\,L^{-1}$), and by stirring this mixture in a water bath at 70 °C. This temperature was chosen above the main phase transition temperature of lipid (Tm DPPC = 41.4 °C) (Thevenot, Troutier, Putaux, Delair, & Ladavière, 2008). Smaller vesicles with a lower number of bilayers were prepared by disruption of a LMV suspension, using a bath sonicator thermostated at 70 °C (Branson 3510, Branson Ultrasonics Co., Danbury, CT). The concentration of the CF solution added to the lipid film was 0.1 $mmol\,L^{-1}$ in a carbonate buffer at pH 8.6, 0.1 $mol\,L^{-1}$. The resulting liposomes encapsulating CF were not purified from free CF in order to reason with the same CF amount for the comparison between the hydrogels containing free CF and those incorporating liposomes (enclosing CF, inside and outside vesicles). The mean hydrodynamic diameters (*ca.* 80 nm) and mean size distributions (PDI ca 0.4) of liposomes were determined at 25 °C by quasi-elastic light scattering (QELS) at an angle of 173°, using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK).

2.3. Preparation of physical chitosan hydrogels and process of liposome incorporation

In this study, physical hydrogels were prepared from aqueous chitosan solutions, without any organic solvent or cross-linking additive, as previously described (Montembault et al., 2005a). First, aqueous chitosan solutions concentrated at 2% (w/w) were prepared by dissolving the purified chitosan in an aqueous acetic acid solution. Thus, the polymer was dispersed in water, and acetic acid was added so as to achieve the stoichiometric protonation of the $-NH_2$ sites. After complete dissolution, 800 μL of liposomes (encapsulating CF molecules) or a CF solution (with an equal CF concentration, 0.1 $mmol\,L^{-1}$) was added and shirred (1000 rpm) for 20 min. The mixture was transferred in a Petri dish (diameter = 35 mm) and let to stand for degassing. This Petri dish was then put in contact with gaseous ammonia. It was thus placed in a glass reactor and displayed over 100 mL of an aqueous solution of ammonia. The concentration of the ammonia bath was 2 $mol\,L^{-1}$. The sample was let for 15 h in the reactor. The formed hydrogel