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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.

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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

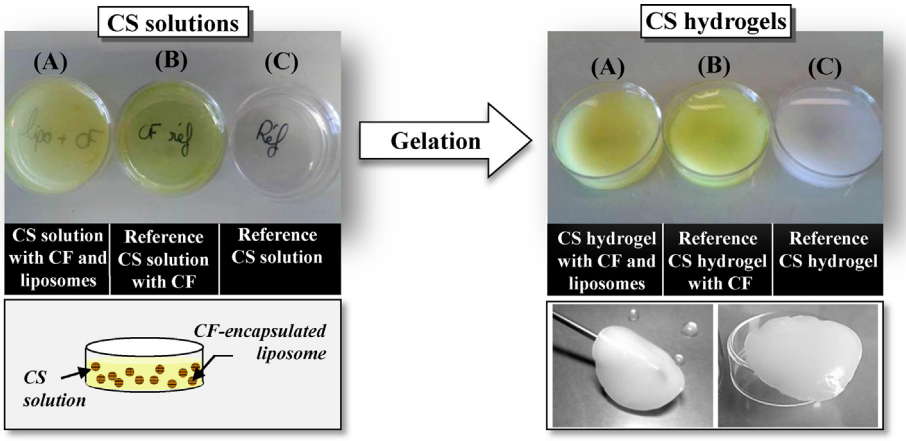

Fig. 1. Images of "chitosan (CS) solutions" before the gelation step (on the left), and "CS hydrogels" after gelation process under ammonia vapors before the washing step (on the right): (A) CS solution or hydrogel with liposomes encapsulating CF, (B) CS solution or hydrogel with free carboxyfluorescein (CF), and (C) "reference" CS solution or hydrogel, without liposome and without CF.

was then taken off the reactor, and washed with deionized water to eliminate the formed ammonium acetate as well as the excess of ammonia, until neutral pH. In this process, the ammonia bath concentration was optimized in order to limit the number of successive washings, while allowing the formation of the hydrogel. A protocol for the washing step was established: (i) the gel was successively immersed into 3 mL of deionized water during 15 min for the first five washings, (ii) then, during 30 min for the next four washings, (iii) and 45 min for the following five washings.

2.4. Fluorescent analyses

Fluorescent analyses of washing waters (200 μL per well) were carried out using a multi-mode Synergy Mx microplate reader (Bio-Tek Instruments) at 25 °C (with excitation wavelength at 492 nm, and emission wavelength at 518 nm). The fluorescence intensity of CF solutions prepared in a carbonate buffer at pH 8.6 (0.1 mol L^{-1}) was measured as a function of the CF concentration, in the range from 1×10^{-8} to 1×10^{-6} mol L^{-1}. The calibration curve (6 points) was obtained with a correlation coefficient above 0.9950.

2.5. Rheological measurements

A dynamic mode was used for rheology measurements which consisted to apply an oscillatory strain to the sample and then to measure the resulting stress calculated from the torque. The rheological studies were performed thanks to a rheometer ARES (TA Instruments) fitted with a plate-plate tool. The diameter of the plates was 25 mm. The normal force sensor of the rheometer was used to measure the first contact between the hydrogel slab and the upper plate. The temperature was 22 °C for all measurements. The applied strain was chosen as 1% from the linear viscoelastic region. The storage and loss moduli of gels were measured from a constant-strain frequency sweep over frequency ranges of 100–0.05 rad s^{-1}.

2.6. Fluorescence microscopy

Hydrogels were directly observed with an inverted wide-field microscope Axiovert100 M (Zeiss) at PLATIM (ENS Lyon, IFR 128/UMS3444). The fluorescence was excited with a band-pass filter 470/40 nm and observed with a band-pass filter 525/50 nm. This apparatus is equipped with a 63×/1.4 oil immersion objective. The measurements were achieved on CS physical hydrogels after washing, without (*i.e.*, the "reference" hydrogel) and with fluorescent liposomes (DPPC/NBD-PC 99/1 mol/mol.

2.7. Cryo-scanning electron microscopy (cryo-SEM)

Small chitosan hydrogel pieces of about 5 mm^2 were directly immersed in slush nitrogen (*ca.* −210 °C). In these freezing conditions, calefaction and formation of crystalline ice were limited. The frozen samples were introduced into the cryotransfer chamber (Gatan, Alto2500), fractured and transferred into the microscope chamber. In the cryo-transfer and microscope chambers, the temperature sample was maintained at −150 °C. The samples were observed under high vacuum on a FEI Quanta 250 scanning electron microscope with an accelerating voltage of 5 kV.

3. Results and discussion

3.1. Formation of the CS hydrogel/liposome biosystem

Chitosan physical hydrogels can be obtained by acting on the balance between hydrophobic and hydrophilic interactions within the polymeric aqueous solution without adding any cross-linking agent. This original gelation process was previously set up in our team, and described elsewhere (Montembault et al., 2005b). The first step consists in the dispersion of CS in water and the stoechiometric addition of acetic acid *versus* the polymer amine sites in order to dissolve the CS chains. The high charge density of CS *via* the presence of many protonated amine groups prevents the establishment of polymer interchain interactions. Then, the resulting CS solution is subjected to gaseous ammonia for gelation. Ammonia easily diffuses in the acidic CS solution leading to the neutralization of protonated amine functions, and to a consecutive decrease of the apparent charge density of polymer chains. This favors hydrophobic effects and hydrogen bonds between CS chains inducing the formation of a tridimensional network. As shown in Fig. 1, this gelation process occurs simultaneously with an opacificying phenomenon of CS solutions.

In this work, the incorporation of liposomes in CS hydrogels was carried out by the supplement of a pre-formed liposome suspension to the CS acidic solution just before the gelation process. The mixture (final CS concentration = 1.7% w/w, lipid concentration = 2 mM) was stirred for 20 min at room temperature. For the "reference" hydrogel (*i.e.*, without liposome), an equal volume of buffer was added instead of the liposome suspension. Then, the mixture was left for 4 h at room temperature for degassing, and put under ammonia vapors for gelation. In this way, the liposomes are expected to be entrapped in the meshes of the hydrogel matrix.

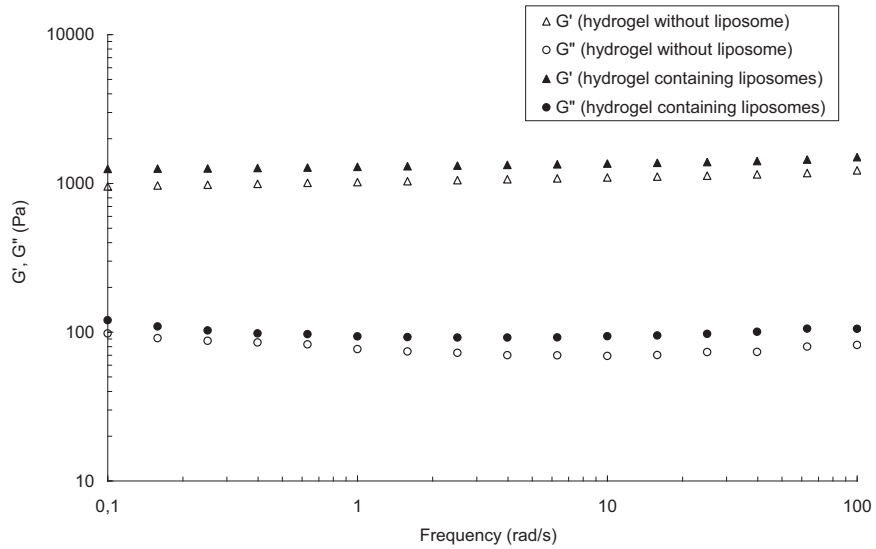

Fig. 2. Variation of G' (triangles) and G'' (circles) moduli *versus* frequency for two CS hydrogels with liposomes (open symbols) and without liposome (full symbols). Polymer and lipid concentrations in these hydrogels were 1.7% (w/w) and 2 mM, respectively.

It is then necessary to wash the hydrogel to eliminate ammonia salts (ammonium acetate, $CH_3CO_2NH_4$) as well as the excess of ammonia. To this end, several washing baths were achieved until neutrality of washing waters. The pH value of each washing water was measured to precisely follow the pH evolution during the washing step. The pH value of the first washing waters was about 10.3, whereas it was around 8.9 for the fourteenth one (data presented in the Supporting information).

This experimental process implied acidic (for CS dissolution) as well as basic conditions (for gelation), it is consequently important to evaluate if the structural organization of liposomes is not impacted by such pH extremes. In order to check it, a liposomal suspension was dispersed in an acid solution exactly in the same experimental conditions as for the CS dissolution (pH, concentration, duration), and another liposomal suspension was put in contact with ammonia vapors, also in the same conditions as for the hydrogel formation (pH, concentration, duration). After that, both liposomal suspensions were analyzed by QELS to compare the object sizes and size distributions, before and after the considered treatments. The measurements showed that there was no evolution, and confirmed no destruction of liposomes by the experimental conditions imposed by the synthesis process of CS physical hydrogels. The next step aims at demonstrating that the presence of liposomes does not hinder the formation of CS hydrogels.

3.2. Characterization of the CS hydrogel/liposome biosystem by rheology

The final hydrogels obtained after the washing step, loaded or not with liposomes, were characterized by rheology measurements. For each hydrogel, the moduli G' and G'' were measured over a frequency-range of 100–0.05 rad s^{-1}. The polymer concentration

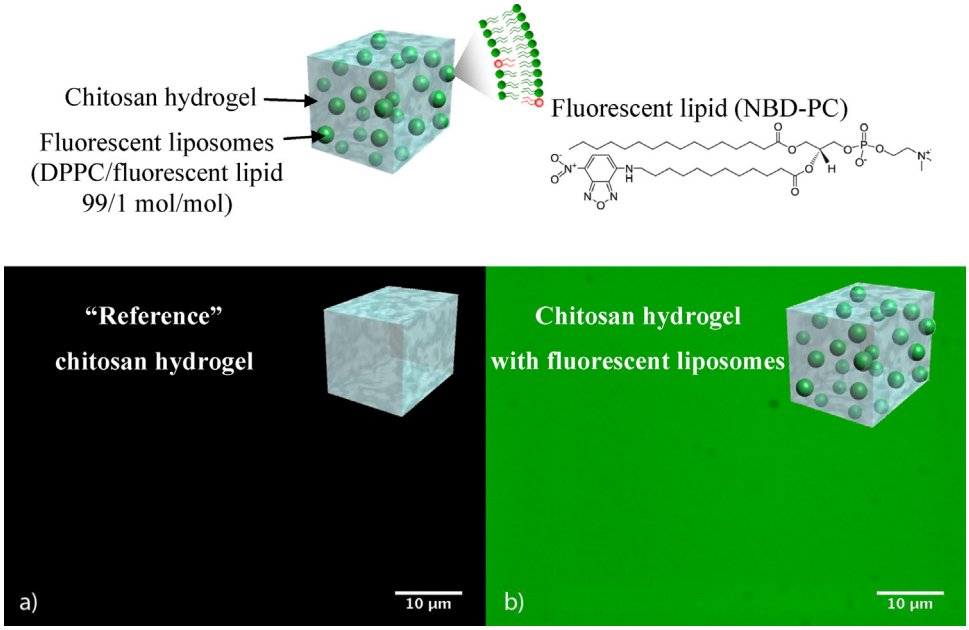

Fig. 3. Fluorescence microscopy of CS hydrogels: (a) "reference", without liposome, (b) with liposomes (lipid concentration = 2 mM, DPPC/fluorescent NBD-PC 99/1 mol/mol). The fluorescence was excited with a band-pass filter 470/40 nm and observed with a band-pass filter 525/50 nm.

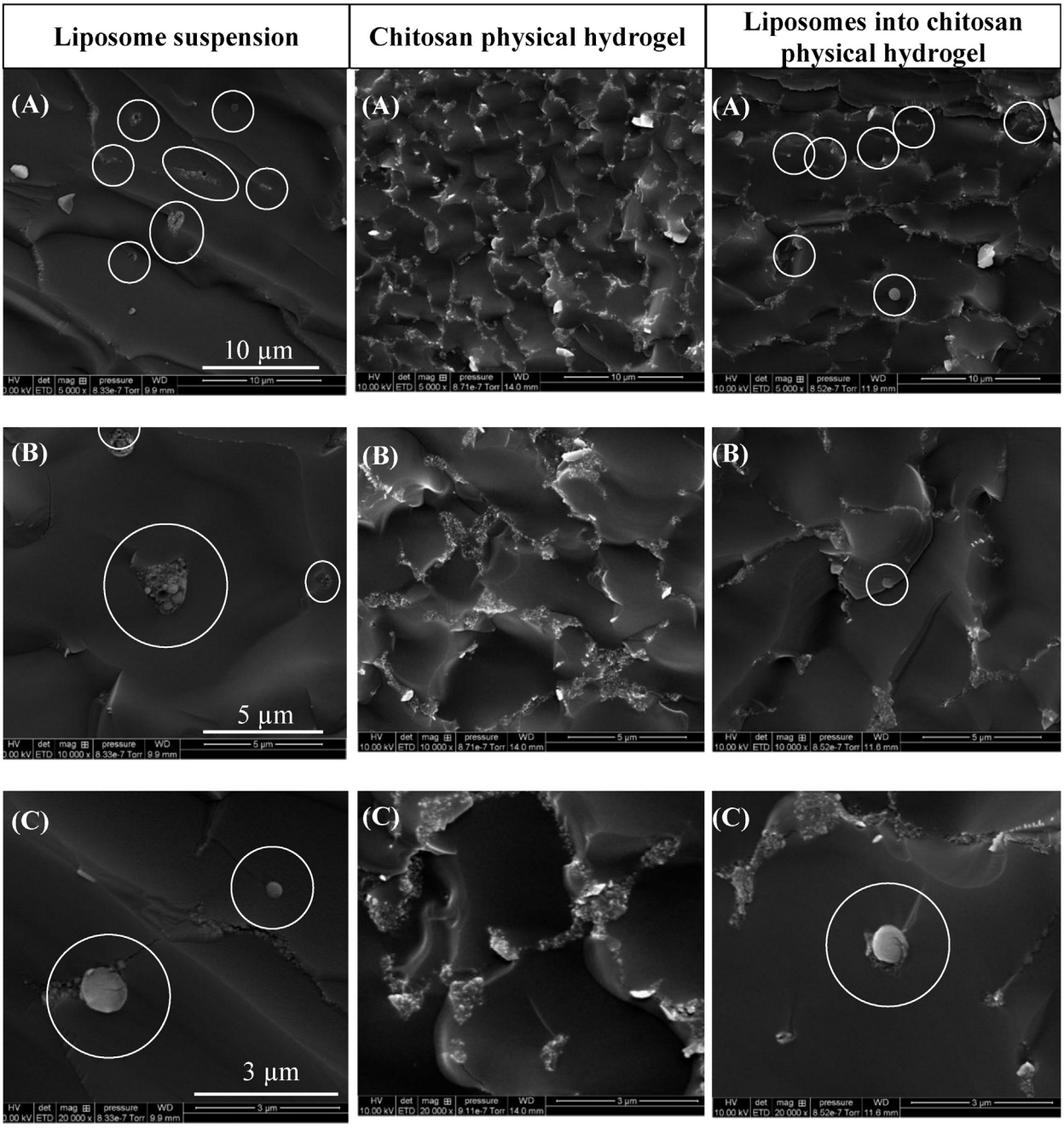

Fig. 4. Cryo-SEM observations of DPPC liposomal suspension at 10 mM (first column), hydrogel without liposome and with CF (second column), CF/liposome-loaded hydrogel (third column). Scale bars: (A) 10 µm, (B) 5 µm, (C) 3 µm. The white circles are to guide the eyes.

and liposomal amount in hydrogel were 1.7% (w/w) and 2 mM, respectively. Fig. 2 shows an example of the curves obtained for the systems with and without liposomes. The curves always presented the same trend: the moduli were nearly frequency-dependent, and G' was at least ten times higher than G'' (a condition that defines a gel) (Almdal, Dyre, Hvidt, & Kramer, 1993). Thus, these results proved that the presence of liposomes did not prevent the gelation process of the CS solution, as already demonstrated for other CS hydrogel kinds (Ruel-Gariepy et al., 2002; Mulik et al., 2009; Mourtas et al., 2007).

From these rheological curves, the equilibrium moduli G_{eq}' and G_{eq}'' (corresponding to the values of G' and G'' at the plateau for low frequencies) can be determined. The average values of G_{eq}' and G_{eq}'' for loaded hydrogels were 1400 ± 100 Pa and 150 ± 10 Pa, respectively. In all the cases, the values of moduli were found to be around 15–20% higher when hydrogels are loaded with liposomes. As a result, it would be interesting to increase the liposome concentration within the hydrogel in order to observe if this effect on the rheological moduli would be more accentuated.

3.3. Characterization of the CS hydrogel/liposome biosystem by microscopy

After these experimental verifications, the characterization of "hybrid" hydrogels was carried out by fluorescence microscopy and cryo-SEM in order to confirm the presence of liposomes inside the CS hydrogels.

3.3.1. Fluorescence microscopy

The first type of microscope considered in this investigation was a fluorescence microscope which involved the elaboration of fluorescent liposomes to incorporate them into the CS hydrogel matrix. They were prepared by adding 1% molar of NBD-PC to the 99% molar DPPC formulation. The resulting composite material was observed after the washing step by fluorescence microscopy (Fig. 3b), and the image was compared to the same CS hydrogel one, without liposome (Fig. 3a).

This comparison allowed us to prove the presence of NBD-PC lipids in the hydrogel. Indeed, the observation of the "reference" hydrogel did not reveal a fluorescent signal whereas a typical "green" signal was detected for the CS hydrogel loaded with fluorescent liposomes. Unfortunately, the optic resolution is not high enough to distinguish the liposomes (size ca80 nm), and only their fluorescent halo was detected. Furthermore, this technique is not quantitative and did not allow us to evaluate the amount of NBD-PC (and thus of liposomes) incorporated in the hydrogel.

3.3.2. Cryo-scanning electron microscopy

In order to distinguish the liposomes within hydrogels, the second type of microscope used was electron microscope which has a very high resolution (up to 2 nm in SEM) whereas a limit of 250 nm is theoretically expected for the light microscopes. Furthermore, low temperature SEM (cryo-SEM) is an established technique for capturing and observing hydrated samples after a rapid freezing. This is particularly important in the case of liposomes, and CS hydrogels composed of at least 95% of water (w/w).

Thus, hydrogels with and without liposomes were observed by cryo-SEM at different magnifications (Fig. 4A: 5000×, B: 10,000×, and C: 20,000×, and additional images are presented in SI). The first column of Fig. 4 corresponds to images of a liposome suspension which shows clusters of white (convex fracture) (Severs, 2007) and black (concave fracture) (Severs, 2007) vesicles at lower magnifications (Fig. 4A and B), as well as individualized spheres (3D-organization expected for lipid vesicles) at higher magnification (Fig. 4C).

The middle column corresponds to images of "reference" hydrogels, without liposome. Note that these images of fractured gels, obtained after surface ice sublimation, show a "compartmented" morphology which cannot be associated with the native-state structure of physical chitosan hydrogels. Indeed, the experimental conditions to observe this microstructure require additional and considerable investigations comprising the optimization of freezing step, sample preparation, and observation conditions.

Finally, in the last column, the observation of spheres similar as the ones detected in the liposomal suspension is another proof of the presence of liposomes in the CS hydrogels. These spherical objects are not observed in the "reference" hydrogels, without liposome (second column). After the validation of the "liposome-in-hydrogel" system, the next step of the study was to examine the concept of this biosystem as delayed-release device for a model molecule.

3.4. Characterization of CF release from the CS hydrogel/liposome biosystem

The main aim of the liposome incorporation in these composite hydrogels is to delay the release of drugs by taking advantage of the reservoir role of liposomes. In our case, this delayed release property was studied during the hydrogel washing step. The molecule chosen in this study to proof this concept was the carboxyfluorescein (CF) because of its fluorescence as well as its water-solubility (optimal from pH 6 to 12, and for low concentrations between 1×10^{-8} and 1×10^{-6} mM, data not shown). To this end, the CF molecules were encapsulated in the aqueous cavity of liposomes

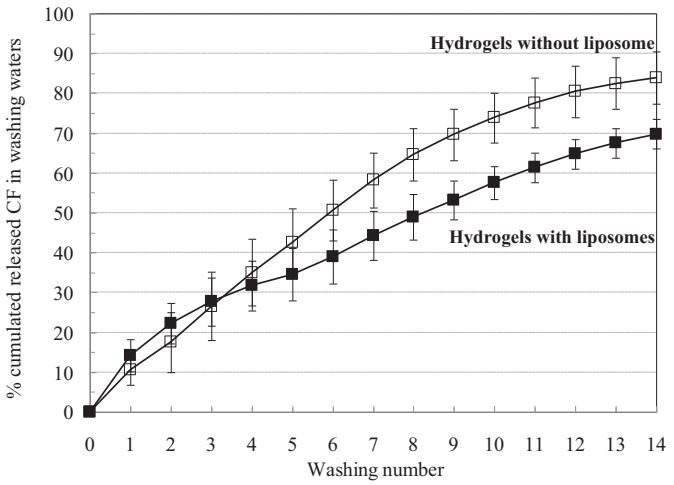

Fig. 5. Cumulated percentage of carboxyfluorescein released for each washing water for the hydrogels without liposomes (open symbols), and with liposomes (full symbols). One point equal to 1 washing. For each point, average and standard deviation on five series of different hydrogels.

for "hybrid" hydrogels, or directly introduced in the CS solution for "reference" hydrogel (*i.e.*, without liposome). This led to a yellow coloring of the CF-loaded hydrogels and "reference" ones, as observed in Fig. 1A and B. Note that this coloration was more opaque when hydrogels were loaded with liposomes containing CF (Fig. 1A, at left) in comparison with free CF-loaded gels (Fig. 1B, at left).

The CF release during the hydrogel washing step was monitored by fluorescence measurements of each washing water (see detailed protocol for the washing step in Experimental section). These fluorescence data, presented in Fig. 5, were compared between hydrogels comprising CF encapsulated in liposomes, and those comprising only free CF into polymer network (without liposomes). Note that for both hydrogel types, the initial total CF amount was strictly equal. These analyses required a previous verification on the fact that the fluorescence of CF molecules was not influenced by the pH value range of the different washing waters (from pH 7 to 10). Complementary fluorescence experiments were also carried out to check the stability of CF with time, and under particular process conditions due to the different steps of the liposome elaboration process and hydrogel formation (heating at 70 °C and sonication step for the liposome elaboration, acid medium and ammonia vapor atmosphere for the CS gelation process). The position of the CF fluorescence emission peak was not modified by these conditions and remained around 518 nm in every case (data not shown).

Fig. 5 shows the average cumulated percentage of CF released for each washing water (average carried out on 5 series of hydrogels). This study was achieved on "reference" CS hydrogels and CS hydrogels containing liposomes, and revealed a delayed CF release thanks to the liposomes entrapped in hydrogels since those without liposome (with only free CF molecules) presented an average CF release of ca $84 \pm 7\%$ at the end of 14 washings, while in the case of hydrogels containing CF-liposomes, the average CF release was about $70 \pm 5\%$ after the same number of washings. Furthermore, a satisfactory reproducibility of these data was attained (with and without liposome) for different hydrogels. The quantity of CF lost in a "simple" CS hydrogel by diffusion through the meshes of the polymer network during the washings is consequently higher than the one of a composite hydrogel. The role of reservoir for liposomes, as well as their consecutive interest in such "hybrid" systems were thus confirmed.

4. Conclusion

In this work, a novel system "liposome-in-hydrogel" was developed, entirely biocompatible, biodegradable, and with no external cross-linking agent. The results of this study demonstrated that the formation of physical CS hydrogels was not disturbed by the incorporation of liposomes. These liposome-loaded hydrogels present nearly the same rheological properties as "simple" CS hydrogels. Fluorescence microscopy and cryo-SEM techniques allowed us to confirm the presence of liposomes inside hydrogels. Intensive washings of hydrogels revealed a delayed release of carboxyfluorescein molecule encapsulated in liposomes for the "liposome-in-hydrogel" system (cumulated release of ca 70%) in comparison with the system where CF was free in hydrogels (cumulated release of ca 85%). These data proved the concept of "delayed release" of this system "liposome-in-hydrogel". The next step of this work will be to study the drug release in controlled environmental conditions (temperature, stirring, presence of biological fluids, etc). The influence of the liposome concentration and size on the release will also have to be explored. The weight-average molecular weight of CS chains should also probably play a role on the release property of the system, as well as the CS acetylation degree, which is a structural parameter impacting hydrophobic and hydrophilic domains within the tridimensional network, and thus impacting the potential interactions with liposomes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.120.

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