5

Liposil, a Promising Phospholipid/Silica Composite Material

Begu Sylvie, Aubert-Pouëssel Anne,
Lerner Dan A and Devoisselle Jean-Marie
Institute Charles Gerhardt Montpellier, Advanced Materials for Catalysis and Health
Montpellier
France

1. Introduction

Since their discovery by Bangham (Bangham, 1965), liposomes have become paradigms for biomembranes, models of self-assembling colloids as well as vehicle for pharmaceutical, diagnostic and cosmetic agents. The word 'liposome' was coined by Weissmann (Sessa, 1968) according to Bangham's expression (Bangham, 1995) and is derived from the Greek: 'lipo' referring to their fatty constitution and 'soma' referring to their structure. Liposomes are constituted by phospholid bilayer (s) surrounding an inner aqueous volume. Due to this constitution, they are used as drug-carrier for hydrophilic, lipophilic or amphiphilic molecules (Gregoriadis, 1976). Depending on the method of preparation (Vemuri, 1995), liposomes can vary widely in term of size (0.02–10 mm) and number of lamellae (uni or multi lamellae). Usually, liposomes are classified into three categories on the basis of their size and lamellarity (number of bilayers): small unilamellar vesicles (SUVs) or oligolamellar (OLVs), large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). Interest in liposomes results from this vesicular structure limited by one or more outer bilayer lipid and an inner water pool. The application of liposomes was first an artificial model for the biological membrane (Chapman, 1984). Forming lipid bilayers through hydrophobic interaction, liposomes are then considered as excellent platforms for the delivery of hydrophobic and hydrophilic drugs. These phospholipid dispersions in water solutions were able to trap and release solutes, to which they were selectively permeable. Then, active compounds have been loaded either in the aqueous volume, if they were water-soluble, or in the lipid membrane, if they were lipid soluble. Liposomes have been administered in a variety of ways but intravenous injection is the most practical route because liposomes present considerable persistence in the blood. This feature facilitates efficient drug delivery to target tissues. Lipids are characterized by different fatty acid chain lengths, different head groups, and different melting temperatures. Consequently, temperature (Jeong, 2009) or pH sensitive (Obata, 2010) liposomes can be assembled by manipulating the formulation. The effectiveness of 1-methylxanthine (1MTX) as a radiosensitizer and the in vivo efficiency of the temperature sensitive liposomal 1-methylxanthine (tslMTX) were evaluated when combined with regional hyperthermia and ionizing radiation (Jeong, 2009). Intraperitoneal injection of the tslMTX inhibited tumor growth in the mouse xenograft tumor model.

Moreover, the combination of tslMTX with regional hyperthermia and ionizing radiation clearly inhibited tumor growth. Most recently, to target leukemic cells, pH sensitive immunoliposomes including a terminally alkylated N-isopropylacrylamide (NIPAM) in the bilayer, were coupled with the anti-CD33 monoclonal antibody (Simard et al., 2009). The pH sensitive immunoliposomes exhibited high cytotoxicity against HL60 cells, suggesting that these immunoliposomes could be active in acute myeloid leukemia therapy. Commercial liposomes have already gained approval from US Food and Drug Administration (FDA). The typical examples are doxorubicin encapsulating liposomes (Doxil® ALZA Corporation), which has shown a strong antitumor activity against a wide range of cancers and amphotericin B encapsulating liposomes (AmBisome®, Nexstar Pharmaceuticals, Inc) for the treatment of fungal infections refractory to amphotericin B.

Despite all the work done, liposomes still have not attained their full potential as drug and gene delivery vehicles (Guo et al., 2003) and their use was mostly limited to pH close to neutrality and above, due to their destabilization in acidic media.

Recently, the self-assembly of organized nanoscopic structures has been the subject of a strong interest in both colloidal and materials science. Vesicle templating presents an unique opportunity to obtain hollow submicrometer particles (Hubert et al., 2000; Hentze et al., 2003). Templating agents used in the synthesis of organized materials range from lyotropic lamellar phases of non-ionic and ionic surfactants, micro-emulsions, phospholipid tubules to polymer particles. Among recent examples, one may cite novel vesicular particles described by Katagiri (Katagiri et al., 2003). The walls of these hybrid particles consist of a bilayer built from dialkyl amphiphiles modified by alkoxysilanes. Cha et al. used polyelectrolyte homopolymers to spontaneously obtain micron size vesicles using citrate stabilized quantum dots as a template (Cha et al., 2003). A careful balance between charge control and hydrogen bond leads to the self-assembly.

Other spherical particles made from various materials have been obtained, some being multilamellar (El Rassy et al., 2005). Some of them were used to encapsulate fluorescent dyes, enzymes, polymer particles (Schmidt & Ostafin, 2002). In a different approach, the polymerization of trialkoxysilane bearing a double alkyl chain lead directly to hybrid analogs of liposomes, named “cerasomes” (Katagiri et al., 2003). Liposomes were also trapped into sol–gel silica materials and loaded with a fluorescent probe to build a pH sensor (Besanger et al., 2002), with proteins (Li & Tip, 2005) or with a transmembrane peptide ion channel (gramicidin A) to control ion diffusion in sensors or in drug screening devices (Besanger & Brennan, 2003). The stability of supramolecular structures such as vesicles and microemulsions depends on pH, ionic strength and on the presence of organic solvents in the reaction mixture, and as such, they were often affected by the reaction conditions.

In our work, we have shown that nanoscale liposomes could be used as templates for the deposition of silica to create hollow silica nano-shell systems called “liposils” (Bégu et al., 2003) in which the trapped unilamellar liposomes maintained their fundamental properties. The conditions for the template directed assembly were fulfilled due to electrostatic charge density matching or hydrogen bonding between template and inorganic precursor. The silica walls being non-porous, the dried silica spheres retained the inner aqueous pool. The use of zwitterionic phospholipids (non toxic) as templates required a modified approach since the liposome structure was sensitive to low pH and high ionic strength and organic solvents were not allowed for pharmaceutical applications.

The non-porous and amorphous silica shell of liposils protected the trapped liposomes from pH and temperature variations and maintained the specific properties of the phospholipid bilayer. Furthermore, the synthesis of these hybrid nanoparticles allowed loading the liposomes with hydrophilic or lipophilic drug (Bégu et al., 2004).
The stability of drug-loaded liposils was tested at two different pH (1.2 and 7.4), in a flow cell according to the USP 28 norm. Characterizations of liposils were done at various steps of the kinetics. The stability observed for liposils make them good starting material for drug storage and release schemes. For instance, functionalization of their external surface should improve their capture by cells whereby drug release could then be induced by a pH variation after endocytosis. In these conditions, the stability observed made them potential candidates for various drug storage and release schemes. Another release process was developed using triggered release of the content by either low-frequency ultrasound or by microwave treatments (Steinberg et al., 2007). The bursting of a capsule by using an external signal has considerable potential applications, wherever remote activation of content-release is needed. This concept has been also demonstrated for polymeric capsules (Radziuk et al., 2007) and for soft liposomes (Schroeder et al., 2007) and micelles, (Husseini et al., 2000) leaving the idea of external triggered release from rigid ceramic capsules relatively unexplored.
The objective of the present chapter aimed at the study (i) of the morphological characterization of the hybrid material (ii) of the behaviour of the liposome phospholipids and (iii) of the ability of liposils to act as drug delivery system. The morphological characterization, which was expected to reveal the presence of the silica coating, was provided by different methods such as electron microscopy (TEM and SEM), FTIR measurements and nitrogen adsorption. The presence of the entrapped materials (phospholipids) was studied by FTIR, elemental analysis and fluorescence anisotropy measurements.
Keywords: Liposomes; Liposils; Silica nanospheres; Drug delivery

2. Methods and tools

1. Liposils synthesis

Liposils synthesis involved two steps. Large unilamellar liposomes were prepared first and then the silica spheres enclosing these liposomes were formed (Rapuano & Carmona-Ribero, 2000). Two liposome samples were prepared according to the method described by Bangham (Bangham et al., 1995). For the first one, a suitable amount of L-alpha-dipalmitoyl phosphatidylcholine, DPPC (phase transition temperature = 41°C), was dissolved in chloroform. Small amounts of cholesterol were added to DPPC for the second sample, leading to a liposome composition with a DPPC:cholesterol molar ratio of 7:3. After complete removal of the chloroform (at 40°C under reduced pressure), a 5,6-carboxyfluoresceine (CF) solution (100mM in PBS, 50 mM, pH 7.4) was added to both dry phospholipid films in order to obtain a 10 mg/ml lipid suspension in the form of large multilamellar vesicles loaded with the water soluble label in their aqueous core.
These suspensions of multilamellar vesicles were then extruded using an extruder (Lipex Biomembranes Inc.) equipped with polycarbonate membranes (mean size diameter: 400, 200 and finally 100 nm, Nucleopore) in order to reduce and homogenize the liposomes size distribution. The extrusion of the lipids was done above their respective transition temperature, 41°C for DPPC and 45°C for the DPPC:chol mixture (Hamilton, 1980).

After extrusion, the external aqueous phase still contained the fluorescent label. So the liposomes were dialyzed in cylindrical membranes (12,000 Da, SpectraPor) for 1 day with PBS (150 mM, pH 7.4) in order to eliminate any 5,6-CF present in the external aqueous phase. After the dialysis step, 5,6-CF was only present in the internal aqueous core of the liposomes.

To determine the phase transition temperature of both types of liposomes, the hydrophobic fluorescent probe diphenylhexatriene (DPH) was loaded in the phospholipid bilayer instead of 5,6-CF to prepare two new sets of liposomes (DPH added in tetrahydrofuran to obtain a 1:200 DPH:lipid ratio) (Bégu et al., 2004). The protocol was then the same as described above. For these experiments, and for each of the two samples, a small amount of DPH loaded liposomes suspension was diluted in PBS (150mM) and the resulting suspension was placed in a thermostated spectrofluorimeter cell. The cell temperature was slowly increased from 20°C to 60°C (1°C/min) while monitoring the fluorescence intensity (λex = 360 nm and λem = 430 nm).

The last step of the synthesis consisted in the silica shell formation. To that aim, the inorganic precursor tetraethoxysilane (TEOS, Fluka) was first hydrolyzed in the PBS buffer (150mM, pH 7.4) for 2 days at 40°C under stirring. The silicate solution was added drop wise to the liposome suspensions (DPPC or DPPC:chol, with a TEOS:DPPC molar ratio 8:1) at room temperature (20°C) under stirring. The final mixture was gently stirred for 1 day. Sodium fluoride (NaF, Sigma Aldrich) at a 4% molar ratio with respect to TEOS was then added to both samples, and the reaction medium was stirred at room temperature (48 h), protected from light.

The resulting samples were then controlled by dynamic light scattering (DLS), filtered (pore size 200 nm) and the residual solid was dried at 40°C for 24 h to give free flowing silica coated particles .

Two silica hybrid samples were prepared: the first referred to as sample 1 contains DPPC liposomes and the second, sample 2, contained DPPC:chol 7:3 liposomes. Both solid samples, loaded with 5,6-CF in the liposomes aqueous core, were washed twice with water and then with an isopropanol/acetic acid (0.1 M) solution. This washing was a precautionary step done in case some 5,6-CF and phospholipid were freed after disruption of some of the liposomes during the silica condensation step and could adsorb onto the external surface of the silica shell. The same experimental conditions, including the washing step, were followed to prepare the two sets of DPH loaded liposils used to determine the phase transition temperature of the trapped bilayer.

2. Characterization of the hybrid material
   a. Dynamic light scattering (DLS)

Liposomes and liposils size measurements were performed by DLS at a 90° angle, using a spectrogoniometer equipped with a He/Ne laser and a photon correlator (Sematech, SM633/RTG). In DLS, nanometric particles diffuse in the small volume of a sample hit by a laser beam and scatter the laser light. The movement of the particles across that volume is due to thermal activation (Brownian diffusion) which is such that the probability of a translation step of amplitude ΔR at time t ($\Delta R=R(0)-R(t)$) is described by a gaussian distribution. It results that the square of the average displacement ΔR^2 is given by the following (Eq. (1)):

$$\Delta R^2(t) = 6D_0t \quad (1)$$

D_0 is the diffusion coefficient that is used in turn to define a relaxation time for the diffusion. In short, DLS theory shows that the latter was quantified by an experimental autocorrelation function. So an analysis of the correlogram of scattered light will give access to relaxation times, hence to a diffusion coefficient, from which the hydrodynamic diameter R_h is deduced using the Stokes–Einstein relationship (Eq. (2)) :

$$D_0 = \kappa T / 6\pi\eta R_h \quad (2)$$

b. TEM and SEM

The morphological characterization was done on the samples by conventional transmission (JEOL 1200 EXII) and scanning electron microscopy (Hitachi S 4500) following standard preparation. The samples were first washed with an isopropanol acetic acid mixture and then dried.

c. TGA DSC

The experiments were done on as-synthesized and on isopropanol/acetic acid washed liposil (Perkin Elmer, DSC 111). The temperature was increased from 50 to 550°C at 5°C/min for washed and unwashed material.

d. DPPC determination (enzymatic).

After crushing the sample, the concentration of DPPC in the suspension was quantified by an enzymatic determination of phospholipids (PAP 150, Biomerieux, France). Phospholipids are hydrolized by phospholipase D and the liberated choline is measured by the Trinder reaction (Takayama et al. 1977).

e. Elemental analysis.

For the determination of the liposils composition, liposomes were prepared according to the method described above using NaCl 9% solution instead of the phosphate buffer solution and the C, P, N, Si and O analysis have been done.

f. Nitrogen adsorption

For liposil sample 1 and sample 2, the characterization of the material was carried out on the as-synthesized material immediately after synthesis, then after a drying step at 40°C, and finally after the stability test at the two different pH values after a drying step. The specific surface area of the samples was assessed by nitrogen adsorption measurements according to the Brunauer–Emmett–Teller standard method (Brunauer et al., 1938). Measurements were carried out at 35°C during 12 h under 10^{-2} Torr using an ASAP 2010 adsorption analyzer from Micromeritics.

g. Fluorescence anisotropy measurements

A fully equipped spectrofluorimeter (Shimadzu RF 5310), was used for the steady state fluorescence depolarization experiments. A sample of liposil loaded with DPH was specially prepared to that effect. The excitation and emission wavelength were set to 360 nm and 430 nm respectively and the relative intensities for the four combinations of vertically and horizontally polarized excitation and emission beams were recorded in the ratio mode in order to eliminate source intensity fluctuations. The steady state emission anisotropy was calculated as:

$$r = 2p/(3-p) \quad (1)$$

with

$$r = (I_{//} - I_{\perp}.G)/(I_{//} + 2I_{\perp}.G) \quad (2)$$

The transmission factor G was taken into account by the method of Azumi and McGlynn, as usual. This factor took care of all polarization effects due to the optical components in the excitation path, including those due to the grating (Azumi & McGlynn, 1962). The experiment was done after the treatment in the acidic medium. No contribution due to light scattering was present since a dilution of the vesicles labelled with DPH had no effect on the measured fluorescence anisotropy.

h. Dissolution kinetics

The tests were done in a flow-through dissolution cell (USP apparatus 4, Sotax; the norm chosen was USP 28-NF-23 (2005)) which was a dissolution testing unit for solid or powder dosage form, using the USP/EP flow-through method.

The cell was used in the experiments with a thermostated bath, and may be described as having 3 parts: an inverted cone at the bottom, the cylindrical portion of the cell in the middle and the filter holding head, on top (Fig.1).

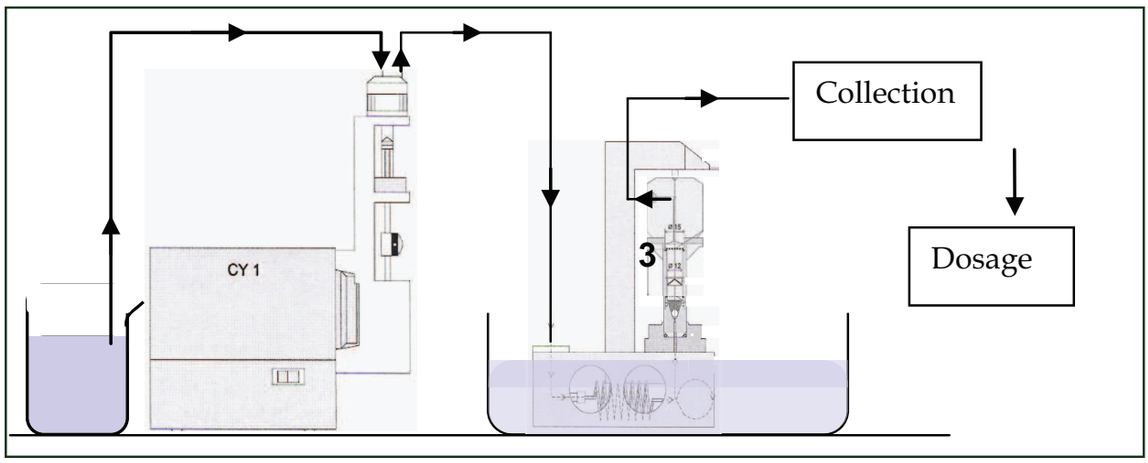


Fig. 1. Scheme of the flow cell apparatus used for kinetic dissolution tests

The dissolution medium entered the cone from underneath through a capillary bore and flowed upwards through the cell. The cone was separated from the cylindrical section by a 40-mesh screen holding a glass microfiber filter. The filter head on the top part of the cylinder also held a glass microfiber filter. At the bottom of the conical part, a single 6 mm diameter glass bead regulated the distribution of the solution jet entering the cell and the rest of the cone volume was filled with 1 mm diameter round glass beads. The role of the glass microfiber filter placed on the bottom part and on the top part (Whatman GF/B and GF/D) of the cylindrical section was to prevent some material particles from being drawn outside the cell. This detailed description was necessary since recent publications mentioned that the release was affected by the position of the glass beads (Bhattachar et al., 2002).

The sample to test was deposited in the flow-through cell, between the filters sitting at the bottom and top of the cylindrical part (Fig. 2). For each experiment, an amount of about 50 mg of the sample (the drug was featured here by the test molecule 5,6-CF) was needed. The flow rate of the medium, kept at 37 °C, was set at a value close to 4 ml/min. In a first experiment, the behaviour of liposil was tested in an acidic medium (pH 1.2) during 30 min. In a second independent experiment, a fresh sample was tested in a pH 7.4 medium for 1 h and a half. These experiments, done in triplicate, were carried out using a closed loop setup

connected in line to a UV–visible spectrophotometer in order to monitor the OD variation at λ=430 nm during the kinetics at pH 7.4.

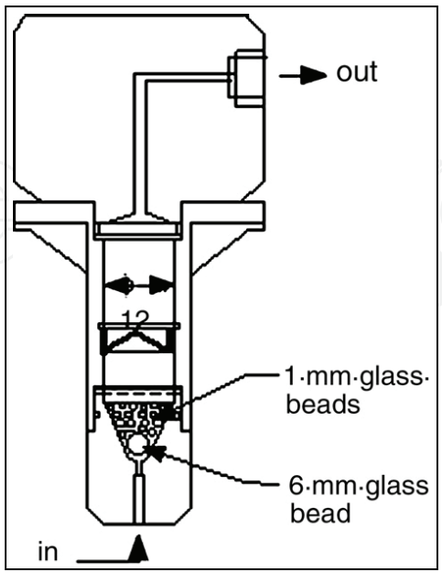


Fig. 2. Scheme of the flow-cell used in this study for the stability tests (the specific localization of the various glass beads are indicated by arrows)

3. Results

The liposomes used for the synthesis of the silica material were constituted by zwitterionic phospholipids: dipalmitoylphosphatidylcholine (Fig. 3a) presenting a 16 C long fatty acyl chain and a choline head group. The synthesized liposomes have a diameter of 80 nm (polydispersity: 0.181) for DPPC and 84 nm (polydispersity: 0.192) for DPPC:chol. They correspond to small unilamellar liposomes (Fig. 3b) with a narrow size distribution and a DPPC concentration of 9.5 mg/mL. The TEOS:DPPC molar ratio (8:1) was chosen to keep the amount of released ethanol under 4% (v/v) during the TEOS hydrolysis. The strong electrostatic interaction between the positively charged lipid head groups (quaternary ammonium) and the negatively charged silica monomers and oligomers was the driving force in the templating. The lateral mobility of the lipids in the outer leaflet of the bilayer (Fig. 3b) can explain efficient charge compensation. The resulting silica cladding is dense and non-porous.

The hybrid material was originally mostly obtained as clusters of monodispersed but aggregated spheres (Bégu et al., 2003). In the last synthesis process (Bégu et al., 2007), an improvement of the former, a pre-hydrolyzed silica precursor was added to the liposomes suspension. Improvements have also been done using PEG phospholipid (Avnir et al., 2007). From DLS measurements on freshly prepared samples of liposils in suspension (i.e. before the drying step), the size distribution of the liposil spheres was found to be centred at 90 nm with a low polydispersity index (0.20). DLS measurements done at each individual step of the synthesis showed that as long as the liposils remained in suspension in the buffer solution, there was no aggregation of the liposil particles. Interestingly, the liposils size polydispersity reflected the size distribution of the liposome template.

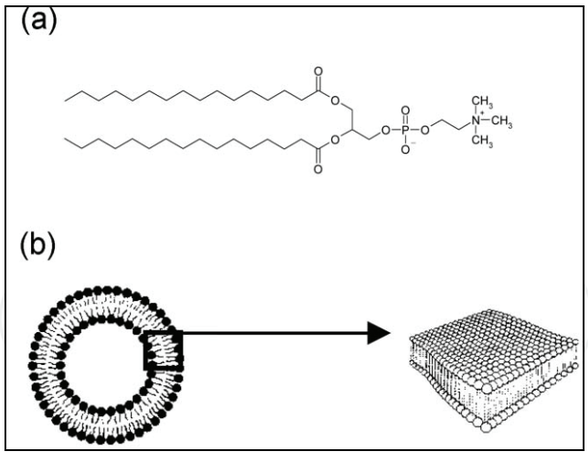


Fig. 3. a: Dipalmitoylphosphatidylcholine structure; b: Schematic representation of an unilamellar liposome and the organization of the phospholipid bilayer

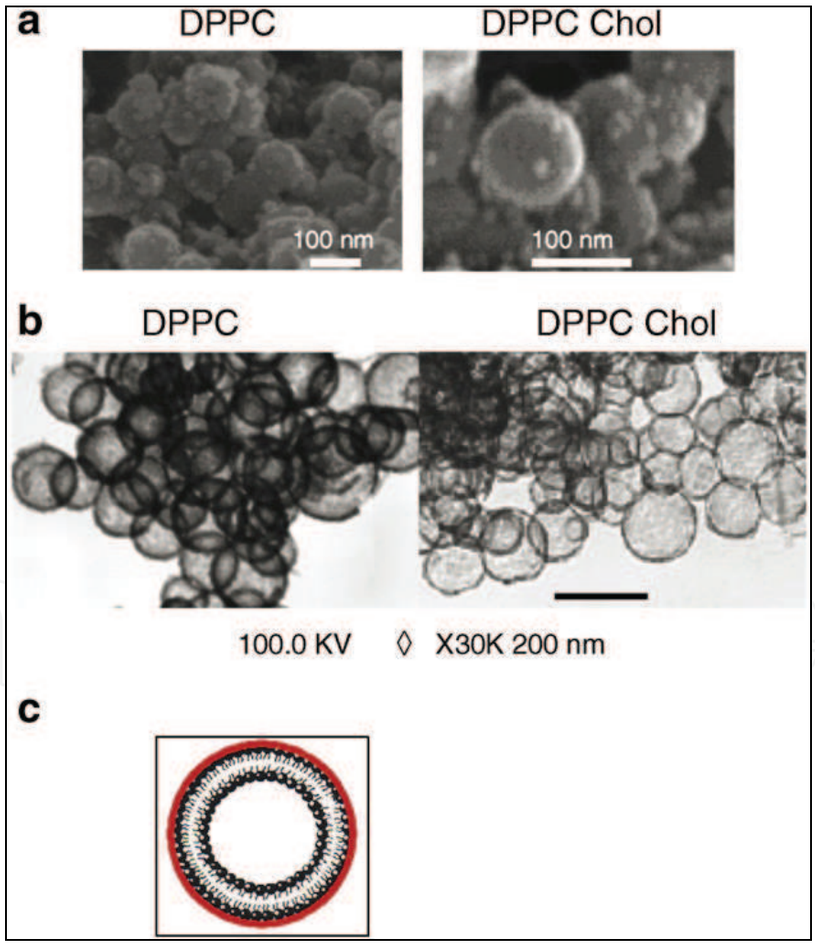


Fig. 4. SEM micrographs (a) and TEM micrographs (b) of dried DPPC and DPPC:chol liposils; schematic representation of a liposil (c) in which the silica shell is figured by the outermost thick line

After the drying step, these liposils still displayed the presence of spheres mostly grouped in clusters, with individual liposils retaining their perfectly spherical shape and wall integrity (Fig. 4a). SEM controls were done after the drying step. The reason was that before the latter, the presence of amorphous silica in excess of the amount needed for the coating process made the observation impossible. Aggregation occurred during the drying step.

TEM data clearly showed silica walls of even thickness of about 4 nm (Fig. 4b). The increase in particle size going from liposomes to liposils occurred during the condensation phase after addition of hydrolyzed TEOS, as a consequence of the formation of the silica shell. Aggregates formation resulted from interactions occurring between the OH groups of the silanols when the individual particles were brought into contact during the drying step (Park et al., 2002).

Here, aggregation generated microparticles which consisted of aggregated but well defined nanocapsules, each its own well delimited spherical shell. The improvement was seen first in the reduced size polydispersity, the thinner silica shell and a better homogeneity of the latter.

Furthermore, after the drying step, the particles from aggregates did not share a whole portion of their surface with their neighbours, but appeared to have only a small contact area, maintaining so the individuality of the silica shells. Many techniques, such as spray drying (Cheow et al., 2010) or freeze-drying, were tested in our laboratory to limit the aggregating effect of this drying step but they ended up with the breaking of a large number of the liposils, even if some of them appeared as free particles. SEM micrographs (Fig. 4a) showed particles with a size of one to several microns consisting in aggregated spherical nanoparticles. TEM results (Fig. 4b) were in agreement with the SEM observations and confirmed the presence of hollow nanocapsules with a mean diameter close to that of the unilamellar liposomes (80 nm).

The silica wall of these nanocapsules was thin enough (close to 4 nm) to be partially transparent to the electron beam. The material morphology was the same whatever the liposome composition used (1 or 2).

TGA-DSC studies show that between 25 and 150°C a very small amount of adsorbed water is desorbed from the surface of unwashed particles compared to the amount seen for washed particles. This means that the external surface of the former particles is almost totally coated by a single layer of lipid (as 70 percent of the total lipids are found inside the liposils), and that the washing step efficiently removes the latter. Between 160 and about 260°C, under an air flux, the DPPC coating the outer surface of the micro-spheres is burnt whereas the mass loss in this region is almost zero for the washed nanospheres (Fig. 5). For those, the inner bilayer is shielded from oxygen by the compact silica shell. Above 260°C, the particles explode under the pressure from vaporized water trapped in their core. Afterwards, the combustion of the now exposed lipid from the templating liposomes can be followed up to about 500°C.

The nanocapsules size was consistent with the liposome diameter augmented by twice the silica shell thickness. The synthesis conditions and specially the silicon alkoxide/phospholipid ratio (8:1) ensured a homogeneous deposition of the prehydrolyzed silica on the vesicle surfaces that were fully covered (refer to the SEM micrograph). During the latter process, the phospholipid bilayer integrity as well as the shape of the liposomes were maintained. This stability resulted from the choice of a phospholipid with a high phase transition temperature (Bégu et al., 2004; Bégu et al., 2007).

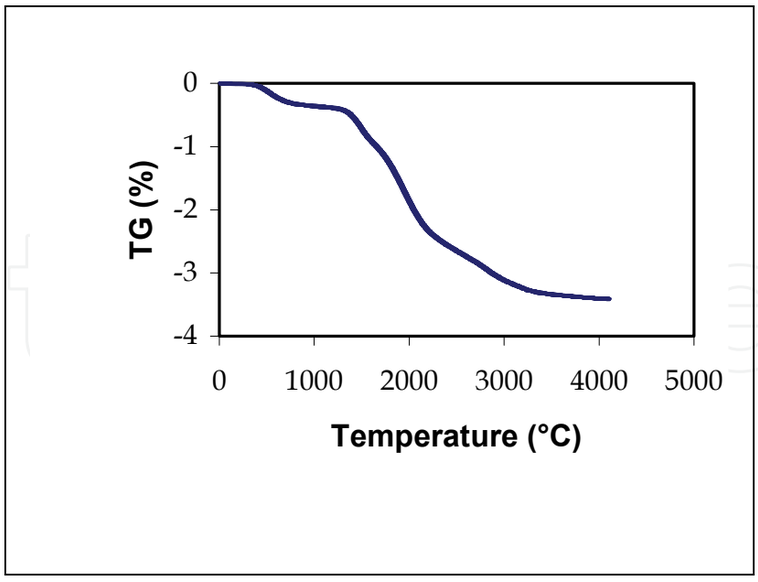


Fig. 5. TGA/DSC scans of liposils

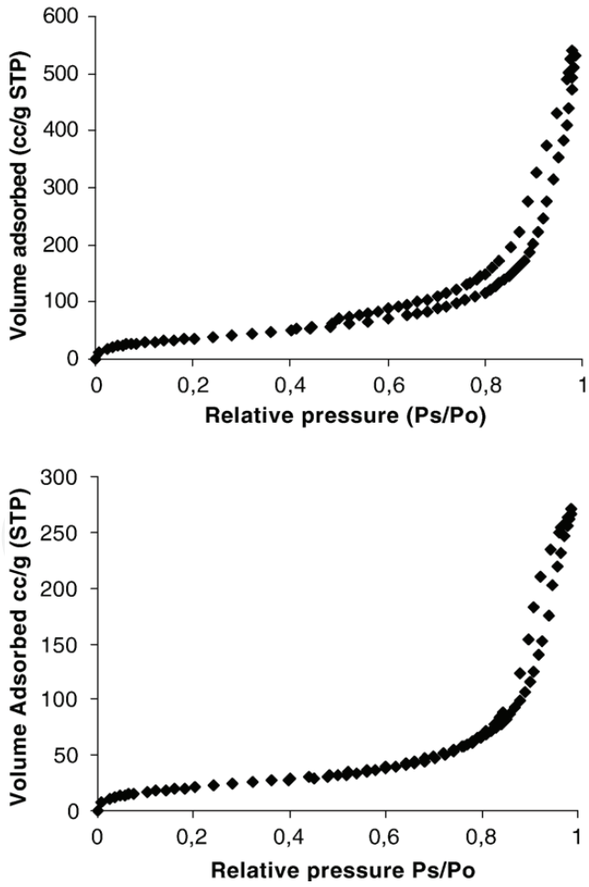


Fig. 6. Nitrogen adsorption isotherms for dried nanoparticles: a) top graph, as synthesized material; b) bottom graph, after the kinetics in acidic fluid

Elemental analysis carried out on unwashed particles at 200 and 350°C reveals a content of carbon of 11.2% and 1.6% respectively. This is in perfect agreement with the previous data concerning the early removal of the external lipid layer and the combustion of the inner bilayer after rupture of the vesicles.

Nitrogen adsorption isotherms (Fig. 6, top) showed that the as-synthesized materials had neither micro nor mesoporosity (type III isotherm). Further, measurements of the surface area of these hybrid solids lead to a small computed BET surface of 100 m^2/g (the porosity observed was mostly interparticular). These data confirmed the non-porous nature of the silica coating of the liposomes.

It is interesting to compare experimental data and results from a purely geometrical analysis. In a typical preparation, 15.53 mg DPPC are encapsulated in the silica liposil material. The total calcination of the latter leaves 22 mg of pure SiO_2. With an outer diameter of about 110 nm and an estimated shell thickness of 10 nm, the volume of the non-porous silica shell amounts to 4 10^{-16} cm^3 and its mass to 8.8 10^{-16} g based on a value of 2.2 $g.cm^{-3}$ for the density of amorphous silica. The total surface for the two layers of the included liposome amounts to 6.3 10^{-14} m^2.

Considering an accepted area of 0.60 nm^2 for the polar head group of the lipid in the film (Pidgeon & Hunt, 1981) the number of DPPC molecules and their expected mass (for a single particle) should be close to 1.1 10^5 and 1.3 10^{16} g respectively. Using the above values, 22 mg of silica and 15.5 mg of DPPC would correspond to 2.5 10^{13} and 12 10^{13} particles respectively. The difference between these computed values reflects a number of uncertainties, the largest being certainly the shell thickness that results from a visual estimation of TEM data. Taking the average of the two values as a reasonable guess, and the computed surface of a particle as 3.8 $10^{-16}m^2$, one ends up with a computed surface area of about 73 m^2/g of the core shell liposil. This is close to the 55 m^2 per gram found experimentally. The difference cannot be totally attributed to the aggregation of the shells, since the shell thickness and amount of aggregation is not precisely known. However these results point to the coherence of all the data.

Slight modifications of the material were observed after the kinetics in acidic fluid. The surface area increased from 100 to 140 m^2/g as a result of the partial destruction of the aggregates and breaking of a small fraction of the liposils induced by the dynamics of the flow in the cell (Fig. 3, bottom). This result was in agreement with the SEM and TEM micrographs which confirmed the integrity of most of the 1 and 2 liposil nanoparticles after the kinetics in acidic medium.

Liposils may be schematically represented as seen in Figure 4c. However, no particle was left intact after the complete kinetics at pH 7.4, and amorphous silica chunks only were seen, originating from the broken and partly hydrolyzed silica shells.

The behaviour of the liposil particles at two different pH values (1.2 and 7.4) was also tested using the hydrophilic probe 5,6-CF (Weinstein et al., 1983) expected to diffuse away from the aqueous core across the phospholipid bilayer. The integrity of the bilayer of encapsulated liposomes was also tested as described in the original synthesis (Bégu et al., 2004), using the emission polarization of the linear fluorescent probe DPH. This probe was only soluble in the inner region of the bilayer and helped monitor the rigidity of the lipids alkyl chains. When the temperature of the samples was varied across a region encompassing the transition temperature from the solid gel state to the liquid gel state, a typical decrease in the polarization of the emission was observed.

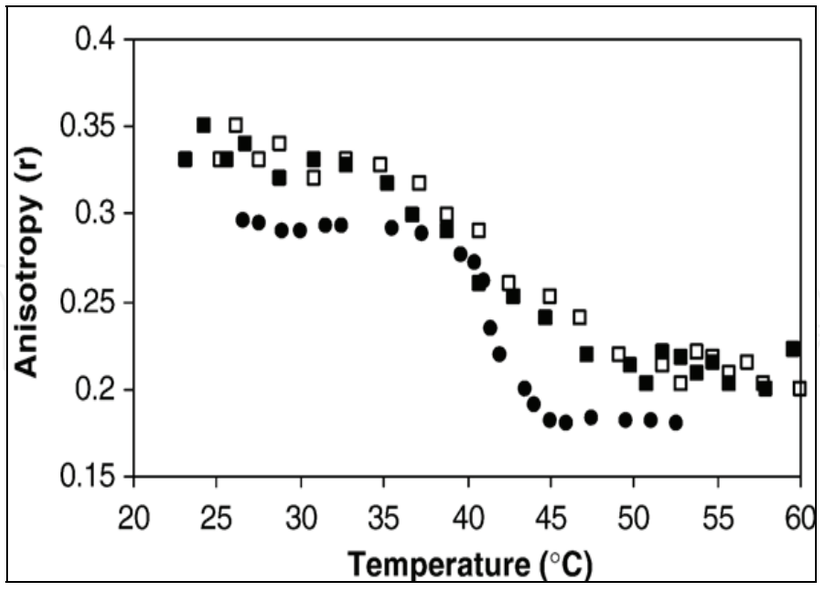


Fig. 7. Liposomes transition temperature from DPH fluorescence polarization: a) DPPC liposomes (•); b) as-synthesized DPPC liposils (▪); c) DPPC liposils after the kinetics in acidic fluid (□)

This effect is clearly seen for free DPPC liposomes for which the transition temperature was 41°C (Fig. 7a) [14]. In as-synthesized liposils, that transition temperature was shifted towards a higher value as the external leaflet of the bilayer interacted with the silica wall in a way that increased its cohesion (Fig. 7b). Measurement of the fluorescence anisotropy was also carried out on DPH loaded liposil after the 30 min stability step at pH 1.2 and it could be seen that the temperature of the phase transition of the template phospholipid bilayer was not affected by that treatment (Fig. 7c).

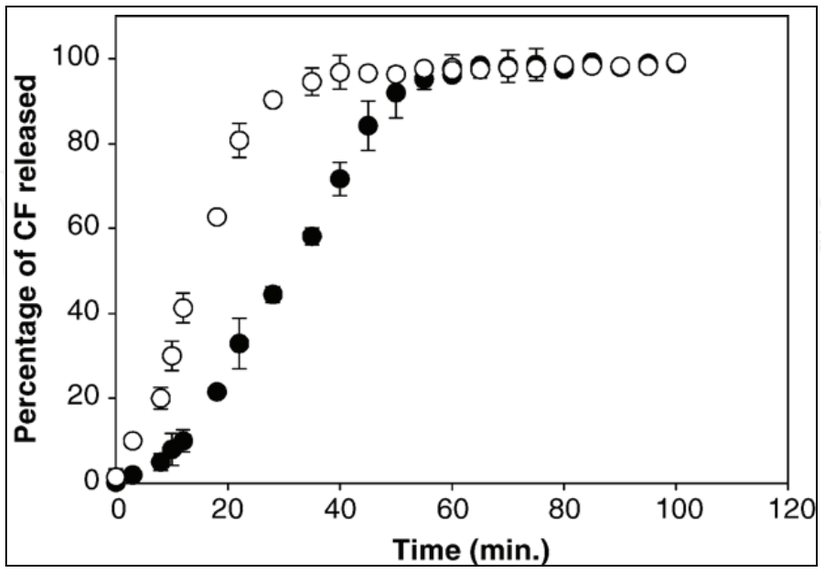


Fig. 8. Kinetics profiles for the release of 5,6-CF from the composite silica nanoparticles in suspension at pH 7.4 for 90 min: a) DPPC liposils (–**o**–); b) DPPC:chol liposils (–•–)

Samples were left for 30 min in contact with the acidic dissolution medium (pH 1.2), in order to test the stability of the silica shell in these conditions. At the end of this first step, the amount of released 5,6-CF in the medium was measured. That determination was done after raising the pH to 7.4 since 5,6-CF, in the form of a lactone at pH 1.2, would have been insoluble at that pH. It revealed that at most 5% of the total amount of the fluorescent probe associated to the hybrid solid was solubilized. The origin of that 5,6-CF could be due to residual adsorbed molecules that resisted the washing step. But it was most probably due to some probe molecules being trapped in the few closed inter-grain spaces formed as a result of the aggregation of the liposils, that were released in a breakage induced by the shearing stress. This was confirmed by the fact that further immersion of the treated particles in solution did not release 5,6-CF any more, and by the almost unchanged overall fluorescence intensity of the particles. These observations were identical for particles 1 and 2, and confirm the non-porous nature of the silica shell. This conclusion was also in agreement with the TEM and SEM micrographs results, which revealed at most minor changes in the silica shell of the treated liposils nanoparticles.

In the second part of the test, a kinetics run was done at pH 7.4 (Fig. 8) with another aliquot of the same liposil samples. In these conditions, a release of the probe occurs, due to leakage from the liposil core towards the pH 7.4 outer medium. To improve the signal value, the experiment was done with a closed loop setting; this was possible since any freed 5,6-CF was shown not to be readsorbed onto the silica surface. In the 37 °C bath, if the 5,6-CF probe could exit the liposomes at pH 7.4, that is, if a disruption of the liposomes had occurred concomitantly with the rupture of the silica shell, a burst should have been seen. This phenomenon was not observed whether or not the liposomes bilayer included cholesterol. A consequence of this observation was that the dissolution of the silica shell should initially free intact liposomes. This interesting hypothesis was also supported by the different rates of release of 5,6-CF observed for the two types of liposils, as the kinetics corresponding to the DPPC:chol templated material is the slowest. This reflects the known relative stability of the two different types of liposomes used as templates, the ones with cholesterol being more stable. Of course, due to the hydrodynamic shear forces in the flow cell, the liposomes were finally destroyed and released their content. The latter event defined the observed release kinetics. In physiological conditions, it was shown that liposomes were stable at pH 7.4 over a time stretch much longer than the 2h used for the kinetics of the release during which only a very limited release of 5,6-CF occurs (Agarwall et al., 1986; Semple et al., 1996). In any case, these tests cannot be assimilated to test for the standard release from liposomes. The shell was viewed as a protection towards acidic media, temperature and the presence of enzymes, all factors susceptible to destabilize bare liposomes. The test of the release of 5,6-CF in an acidic medium was only a control of the integrity of that silica shell. Later these liposil particles will be used for drug release in slightly improved form (functionalized external surface of the shell, etc.).

4. Conclusion

The stability of liposils, silica-based composites obtained via liposome templating was tested at pH 1.2 and pH 7.4 at the constant temperature of 37 °C. Two samples, one with DPPC and the second with DPPC and cholesterol, were carefully studied. The non-porous amorphous silica cladding of liposils protects the trapped liposomes which retain the fundamental properties of their bilayer. Carboxyfluorescein, 5,6-CF, simulating a typical hydrophilic drug

was loaded in the liposomes aqueous phase before the templating process. The stability tests were done using a flow cell, according to the USP 28 norm. As expected, at the acidic pH 1.2, the non-porous silica coating was stable and prevented the rapid degradation generally observed for free liposomes in these conditions. At a pH value of 7.4, the silica shell was hydrolyzed, and at first the intact liposomes were freed. This interesting feature was revealed by the release kinetics and was observed independently of the fact that the bilayer contained cholesterol or not. In turn, at that pH, the bilayers are destabilized in these conditions. The stability observed for liposils makes them good candidates for drug storage and release schemes. Their size and shape should facilitate their capture by cells and the presence of the water pool in the trapped liposome should allow for an externally triggered release using ultrasonic waves or microwaves. This would provide a fast release, complementing the slow release associated to erosion of the liposil particle wall. Further work is in progress to prevent the individual liposil nanospheres from aggregating when going to the dry state. Optimizing the latter factors will of course lead to a better control of drug release.

5. References

Agarwall, K.; Bali, A. & Gupta, C.M. (1986) Influence of the phospholipid structure on the stability of liposomes in serum, *Biochim. Biophys. Acta.* 856, 36–40.

Azumi, T. & McGlynn, S.P. (1962) Polarization of the luminescence of phenanthrene, *J. Chem. Phys.* 37, 2413–2420.

Bangham, A.D. (1965) Diffusion of univalent ions across unilamellar of swollen phospholipids. *J. Mol. Biol.*, 13, 238–252.

Bangham, A.D. (1995) Surrogate cells or Trojan horses. The discovery of liposomes. *Bioassays*. 17, 1081-1088.

Bhattachar, S.N.; Wesley, J.A.; Fioritto, A.; Martin, P.J. & Babu, S.R. (2002) Dissolution testing of a poorly soluble compound using the flow-through cell dissolution apparatus, *Int. J. Pharm.* 236, 135–143.

Bégu, S.; Durand, R.; Lerner, D.A.; Charnay, C.; Tourne-Peteilh, C. & Devoisselle, J.-M. (2003) Preparation and characterization of siliceous material using liposomes as template. *Chem. Commun.* 640–641.

Bégu, S.; Charnay, C.; Tourné-Peteilh, C.; Lerner, D.A. & Devoisselle, J.M. (2004) Characterisation of a phospholipid bilayer entrapped into non-porous silica nanospheres, *J. Mater. Chem.* 14, 1316–1320.

Bégu, S.; Aubert-Pouëssel, A.; Lerner, D.A. ; Tourne-Péteilh, C. & Devoisselle, J.M. (2007) Liposil, a promising composite material for drug storage and release. *Journal of Controlled Release*, 118, 1-6.

Besanger, T.R.; Zang, Y. & Brennan, J.D. (2002) Characterization of fluorescent phospholipid liposomes entrapped in sol–gel derived silica. *J. Phys. Chem.* 106, 10535–10542.

Besanger, T.R. & Brennan, J.D. (2003) Ion sensing and inhibition studies using the transmembrane ion channel peptide gramicidin A entrapped in sol–gel derived silica. *Anal. Chem.* 75, 1094–1101.

Brunauer, S.; Emmett, P.H. & Teller, E. (1938) Adsorption of gases in multimolecular layers, *J. Am. Chem. Soc.* 60, 309–319.

Cha, J.; Bartl, M.H.; Wong, M.S.; Popitsch, A.; Deming, T.J. & Stucky G.D. (2003) Microcavity lasing from block peptide hierarchically assembled quantum dot spherical resonators *Nano Letters*, 3, 7, 907–911.

Cheow, W. S.; Li S. & Hadinoto K. (2010) Spray drying formulation of hollow spherical aggregates of silica nanoparticles by experimental design. *Chemical Engineering Research and Design,* 88, 673–685.

Chapman D. (1984) Physicochemical properties oh phospholipids and lipid-water systems. In : Gregoriadis G, editor. *Liposome technology*. Boca Raton, FL : CRC press. 1-18.

El Rassy, H.; Belami, E.; Livage, J. & Coradin, T. (2005) Onion phases as biomimetic confined media for silica nanoparticle growth. *Langmuir* 21, 19, 8584–8587.

Guo, X.; Francis, C. & Szoka, J.R. (2003) Chemical approaches to triggerable lipid vesicles for drug and gene delivery. *Acc. Chem. Res.* 36, 5, 335–341.

Gregoriadis, G. (1976) The carrier potential of liposomes in biology and medicine. *New England J Med.,* 295, 765-770.

Hamilton, R.L. (1980) Unilamellar liposomes made with the French pressure cell: a simple preparative method, *J. Lipid Res.* 21, 981–992.

Hentze, H.P.; Raghavan, S.R.; McKelvey, C.A. &Kaler, E.W. (2003) Silica hollow spheres by templating of catanionic vesicles, *Langmuir*, *19*, 4, 1069–1074.

Hubert, D.H.W.; Jung, M. & German, A.L. (2000) Vesicle templating, *Adv.Mater.* 12, 17, 1291–1294.

Husseini, G. A.; Myrup, G. D.; Pitt, W. G.; Christensen, D. A. & Rapoport, N. Y. *(*2000) Combined cancer therapy by micellar-encapsulated drug and ultrasound. *J. Control. Release,* 69, 43-52.

Jeong, S.Y.; Yi, S.L. & Lim, S.K. (2009) Enhancement of radiotherapeutic effectiveness by temperature sensitive liposomal 1m ethylxanthine *Int J Pharm*, 372, 1-2, 132-139.

Katagiri, K.; Hamasaki, R.; Ariga, K. & Kikuchi, J.-I. (2003) Preparation and surface modification of novel vesicular nano-particle "Cerasome" with liposomal bilayer and silicate surface, *J. Sol-Gel Sci. Technol.* 26, 393–396.

Li, Y. & Yip, W.T. (2005) Liposomes as protective capsules for active silica sol–gel biocomposite synthesis, *J. Am. Chem. Soc.,* 127, 37, 12756–12757.

Obata, Y.; Tajima, S. & Takeoka, S. (2010) Evaluation of pH responsive liposomes containing amino acid based zwitterionic lipids for improving intracellular drug delivery in vitro and in vivo. *J Control. Release*, 142 2, 267-276.

Park, K.; Kim, K.D. & Kim, H.T. (2002) Preparation of silica nanoparticles: determination of the optimal synthesis conditions for small and uniform particles, *Colloids Surf., A Physicochem. Eng. Asp.* 197, 7–17.

Pidgeon, C. & Hunt, C.A. (1981) Calculating number and surface area of liposomes in any suspension, *J. Pharm. Sci.*, 70, 2, 173–176.

Radziuk, D.; Shchukin, D. G.; Skirtach, A.; Mohwald, H. & Sukhorukov, G. (2007) Synthesis of silver nanoparticles for remote opening of polyelectrolyte microcapsules. *Langmuir, 23*, 4612-4617.

Rapuano, R. & Carmona-Ribeiro, A.M. (2000) Supported bilayers on silica. *J. Colloid Interface Sci.*, 226, 299-307.

Schmidt, H.T. & Ostafin, A.E. (2002) Liposomes directed growth of calcium phosphate nanoshells. *Adv. Mater.,* 14, 7, 532–535.

Schroeder, A.; Avnir, Y.; Weisman, S.; Tzemach, D.; Najajreh, Y.; Gabizon, A.; Talmon, Y.; Kost, J. & Barenholz, Y. (2007) Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility. *Langmuir*, *23*, 4019-4025.

Semple, S.C.; Chon, A. & Cullis, P.R.; (1996) Influence of cholesterol on the association of plasma proteins with liposomes, *Biochim. Biophys. Acta,* 35, 2521–2525.

Sessa,G. & Weissmann, G. (1968) Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid. Res.,* 9, 310-318.

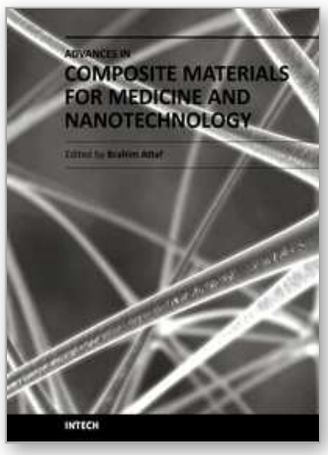
Simard, P. & Leroux, J.C. (2009) pH sensitive immunoliposomes specific to the CD33 cell surface antigen of leukemic cells. *Int. J. Pharm.,* 381, 2, 86-96.

Steinberg, Y.; Schroeder, A.; Talmon, Y.; Schmidt, J.; Khalfin, R.L.; Cohen, Y.; Devoisselle, J.M.; Begu, S. & Avnir, D. (2007) Triggered release of aqueous content from liposome-derived sol-gel nanocapsules, *Langmuir*, 23, 12024-12031.

Takayama, M.; Itoh, S.; Nagasaki, T.; & Tanimuzi, I. (1977) A new enzymatic method for determination of serum choline-containing phospholipid. *Clin. Chim. Acta*, 79, 93-98.

Vemuri, S. & Rhodes, C.T. (1995) Preparation and characterization of liposomes : a review. *Current Sci.* 68, 715-724.

Weinstein, J.N.; Ralston, E.; Leserman, L.D.; Klausner, R.D.; Dragsten, P.; Henkart, P.; & Blumenthal, R. (1983) Self quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome cell interaction, *Liposome Technol.* 13, 183–204.



Advances in Composite Materials for Medicine and Nanotechnology

Edited by Dr. Brahim Attaf

ISBN 978-953-307-235-7
Hard cover, 648 pages
Publisher InTech
Published online 01, April, 2011
Published in print edition April, 2011

Due to their good mechanical characteristics in terms of stiffness and strength coupled with mass-saving advantage and other attractive physico-chemical properties, composite materials are successfully used in medicine and nanotechnology fields. To this end, the chapters composing the book have been divided into the following sections: medicine, dental and pharmaceutical applications; nanocomposites for energy efficiency; characterization and fabrication, all of which provide an invaluable overview of this fascinating subject area. The book presents, in addition, some studies carried out in orthopedic and stomatological applications and others aiming to design and produce new devices using the latest advances in nanotechnology. This wide variety of theoretical, numerical and experimental results can help specialists involved in these disciplines to enhance competitiveness and innovation.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen