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# Characterisation and phase behaviour of phospholipid bilayers adsorbed on spherical polysaccharidic nanoparticles

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**Abstract**

In this paper a new drug carrier, the Light-biovector, is described. These biovectors are composed of a neutral, anionic or cationic polysaccharidic core surrounded by phospholipids. They can be prepared with high yield and in a nearly pure form as determined by density analysis on sucrose gradients. These particles showed great stability with no sedimentation being observed after more than one year of storage. Physicochemical studies carried out with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol mixtures showed that in Light-biovectors, the lipids are organized in bilayer surrounding the polysaccharidic core. In presence of a neutral polysaccharidic core, the gel to liquid phase transition temperature $T_m$ of DPPC was only slightly affected as compared to liposomal dispersions of the lipid. In contrast, for cationic and anionic Light-biovectors, the $T_m$ of the lipids was affected by the electric charge born by the polysaccharidic core, indicating that electrostatic interactions contribute to the organization of the lipid bilayer in these systems. It was also found that the association of anionic membrane to anionic polysaccharidic cores and the association of cationic membrane to cationic polysaccharidic cores was possible. © 1997 Elsevier Science B.V.

*Keywords:* Fluorescence; Phase transition temperature; Lipid-sugar interaction; Biovector

## 1. Introduction

The therapeutic efficiency of numerous drugs is limited by their lack of specificity towards a given cellular target. The major portion of the drug remains unavailable for the intended therapeutic effect and increases the chances for undesirable effects. These shortcomings theoretically can be overcome by the design of a specific carrier for delivery to the desired site. Liposomes have been studied as drug carriers for the delivery in living organisms [1–3]. However, these systems are not very stable and their drug loading capacity depends on the drug and lipid chemical structures. Recently, supramolecular biovectors (SMBV), a new family of nanoparticulate drug carriers mimicking low density lipoproteins, have been studied [4–7]. These acylated SMBV are composed of a cross-linked natural polysaccharidic particle which is regioselectively acylated on its surface with fatty acids to give a peripheral hydrophobic surface onto which a phospholipid monolayer is adsorbed. Relatively stable particles and high drug loading ratio (unpublished results) can be obtained with these new biovectors. In these particles, when the polysaccharidic core was grafted with short chain fatty acids

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(C8, C12), the adsorbed phospholipid monolayer (dimyristoyl- or dipalmitoyl-phosphatidylcholine) displayed gel-to-liquid phase transition at a temperature identical to that of a liposomal dispersion of the lipid, thus suggesting an absence of energetic coupling between the phospholipid monolayer and the supporting fatty acid layer [4].

According to the work performed in our laboratories which showed that acylation of the polysaccharidic particle was not required for its association with lipids, we developed a new type of biovector made of non-acylated polysaccharidic particle and phospholipids. These new biovectors, which will be referred to as *Light-biovectors*, are currently studied as drug carriers and evaluated in developing vaccines. These particles were found to be very effective as protein carriers, against proteolytic hydrolysis and highly increase human cell TCD4 proliferation against a cytomegalovirus (CMV) antigen [8]. They were partly characterized using light scattering multiangle technique [9]. They exist in suspension as spheric microgel nanoparticles and the result indicates that the association of lipids to these nanoparticles decrease both inter and intraparticle interactions. In this paper, we describe the preparation and some of the structural properties of these novel nanoparticles which provide unique model systems for investigating polysaccharides/phospholipids interactions. Light-biovectors composed of neutral, cationic and anionic polysaccharidic particles were prepared, and the phase properties of the surrounding lipids were studied. These phase properties depend in particular on the charges of the polysaccharidic core, indicating that external charges can affect the molecular packing of lipids. The occurrence of clear-cut gel to liquid phase transitions and fluorescence quenching experiments with non-permeant ions indicate that phospholipids were organized as bilayers around the polysaccharidic particle.

## 2. Materials and methods

### 2.1. Chemicals

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from NOF corporation (Hyogo, Japan), 1,6-diphenyl-1,3,5-hexatriene (DPH), 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF), stearylamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and phosphate buffered saline (PBS) were obtained from Sigma (Saint Louis, Mo, USA). 1-Acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti polar lipids (Alabaster, AL, USA). 1-Chloro-2,3-epoxypropan (epichlorydrin) and glycidyltrimethylammonium chloride (hydroxycholine) were obtained from Fluka (Saint Quentin Fallavier, France). Phosphoryl chloride was obtained from Prolabo (Paris, France). Cobalt(II) chloride ($CoCl_2$) and other salts were of analytical grade.

### 2.2. Preparation of Light-biovectors

Polysaccharidic particles were prepared from U.S. Pharmacopoeia maltodextrin (Glucidex, Roquette, Lille, France) as described previously [5,6,8,10]. Briefly, 100 g of maltodextrin were dissolved in 2 N sodium hydroxide under magnetic stirring at room temperature. Addition to the crude mixture of 1-chloro-2,3-epoxypropan (epichlorydrin) (4.72 ml), or of a mixture of epichlorydrin (4.72 ml) and glycidyltrimethylammonium chloride (hydroxycholine) (31.18 g), or of phosphoryl chloride (28.46 ml) yielded neutral, cationic and anionic polysaccharidic gels, respectively. Then, the gels were neutralized with acetic acid and finally sheared under high pressure in a Minilab homogeniser (Rannie, APV Baker, Evreux, France). The neutral, cationic and anionic polysaccharidic nanoparticles obtained were ultrafiltrated on a SGI Hi-Flow system (30UFIB/1 S.6/40 kD) (Setric Génie Industriel, Toulouse, France) in order to remove low molecular reagents and salts.

Light-biovectors were prepared in a Minilab homogenizer by the mixing of polysaccharidic nanoparticles and phospholipids at a temperature above the gel-to-liquid phase transition temperature of the phospholipid [11]. Polysaccharide and phospholipid concentrations were 1.0 mg/ml and 0.2 mg/ml, respectively. Phospholipid concentration was determined using the method of Bartlett [12] for liposomes, non ionic Light-biovectors and cationic Light-biovectors. Additionally, an enzymatic colorimetric test PL/MRP2 from Boehrringer (Mannheim, Germany)

was used for anionic Light-biovectors. Polysaccharide concentration was determined using the method of Dubois [13].

### 2.3. Preparation of liposomes (small unilamellar vesicles)

Liposomes were prepared by the injection of an ethanolic solution of lipids in water as described in [14,15] and then homogenized [11]. All the samples prepared were stored in sterile tubes after filtration through a 0.2 μm filter.

### 2.4. Size determination of nanoparticles

The mean diameter of polysaccharidic particles, liposomes and Light-biovectors was determined by LASER light scattering with the N4MD Coulter nanoparticle analyzer (Coultronics, Margency, France). Measurements on dilute samples in 50 mM PBS were carried out in triplicate at a temperature of 25°C.

### 2.5. Fluorescence labelling of Light-biovectors

Labelling of the polysaccharidic core with fluorescein was achieved by adding 10 mg of a DTAF water solution (2 mg/ml) to 100 mg of polysaccharidic particles at pH 10, with magnetic stirring [16]. These labelled particles were washed and purified by ultrafiltration on a SGI HI-Flow system (30UFIB/1S.6/40KD) with 1 M NaCl and then subsequently with demineralized water until no fluorescein was detected in the filtrate. The fluorescein-labelled polysaccharidic particles (1 mg/ml) were stored in sterile tubes after filtration through a 0.2 μm filter.

The labelling of phospholipid bilayers with DPH was achieved by adding the probe ($10^{-3}$ M in tetrahydrofuran) to water suspension of Light-biovectors or liposomes. Temperature was kept at 60°C for 30 min [17]. The final DPH concentration with respect to the lipids was 0.5 mol%.

For double labelling of Light-biovectors with fluorescein and DPH, Light-biovectors were prepared with fluorescein-labelled polysaccharidic particles and the surrounding lipid bilayer was then labelled with DPH as described above.

All fluorescent Light-biovector and liposome preparations were kept in the dark.

### 2.6. Fluorescence spectroscopy

Fluorescence excitation and emission spectra were recorded with a SLM-Aminco Spectrofluorimeter, Model 500. In these experiments, and in the fluorescence polarization experiments described below, absorbance of Light-biovector and liposome suspensions (recorded with the Hewlett Packard 8452A Diode Array Spectrophotometer) was never greater than 0.1. For the fluorescence emission spectra, the excitation wavelength $\lambda_{ex}$ was 360 nm for DPH and 490 nm for fluorescein. For the fluorescence excitation spectra, the emission wavelength $\lambda_{em}$ was 426 nm for DPH and 522 nm for fluorescein.

### 2.7. Fluorescence polarization

Experiments were performed with a T-format automatic apparatus of our fabrication [4] connected to a microcomputer. Briefly, the detection system comprised two independent channels, one measuring the vertical component $I_V$ and the other measuring the horizontal component $I_H$ of the fluorescence emission simultaneously. The excitation channel consisted of a mercury light source, a shutter, a monochromator, a rotating polarizer (Glan prism) polarizing the incident light alternatively either vertically (fluorescence polarization measurement) or horizontally (determination of the relative sensitivity of the two detection systems). Cut-off and band-pass filters were interposed on the emission beam to eliminate the excitation light. Samples were placed in a closed, thermostated housing system and the temperature was monitored from 0°C to 60°C by a Peltier element. When using DPH, the fluorescence emission was measured over the wavelength range 400–500 nm. Polarization $p$ is calculated as $p = (I_V - I_H)/(I_V + I_H)$.

### 2.8. Fluorescence quenching experiments

Light-biovectors and liposomes were prepared with DPPC mixed with NBD-PC (2 mol%), and dispersed in 1.5 M NaCl. Sample preparation and analysis were performed extemporaneously to avoid any significant

and differential penetration of the cobalt quencher ions in the lipid bilayer with time. In a quartz cuvette, aliquots of Light-biovector or liposome suspensions were placed (phospholipid concentration: 27.2 μM) and fluorescence was measured to find the $F_0$ value. Then, $CoCl_2$ quenching solution (0.3 M in water) was added to obtain the desired quencher concentration. Reactants were mixed for 15 s and fluorescence was measured immediately to obtain the $F_1$ value. As many Light-biovectors or liposomes samples were used as the number of cobalt ion concentration tested. The quenching efficiency was calculated as $(1 - F_1/F_0) \times 100$. Excitation and emission wavelengths were 474 nm and 535 nm, respectively and temperature was 20°C.

### 2.9. Isopycnic separation on sucrose gradients

Sucrose gradients were prepared by thawing 9 ml of 20% (w/w) sucrose solution in 84 × 19 mm polyallomer Beckmann tubes, at room temperature. 1 ml of the desired sample (liposomes, or polysaccharidic particles, or crude Light-biovectors preparations) was deposited at the top of the tube and centrifugation (250 000 × $g$, 4 h, 8°C) was performed in a Beckmann ultracentrifuge (Model L5-65) equipped with a SW 41 rotor. After centrifugation, 0.5 ml fractions were collected from top to bottom with a Gilson automatic micropipet and analyzed by fluorescence spectroscopy.

## 3. Results

### 3.1. Association of polysaccharidic particles with phospholipids

The degree of association between polysaccharidic particles and phospholipids was analyzed by separating the various components on sucrose gradient. Fluorescein and DPH, which display distinct fluorescence spectra, were used to label polysaccharidic particles and the lipid bilayers, respectively. Control experiments showed that the excitation and emission spectra of both probes were not affected by sucrose. Four hours ultracentrifugation was the minimum time required for the sedimentation of lipid-free particles, liposomes and Light-biovectors to reach equilibrium. Note that measured fluorescence intensities were much larger for fluorescein than for DPH. For the sake of clarity, fluorescein and DPH fluorescence intensities shown in Fig. 1 were normalized with respect to their maximum values.

As can be seen in Fig. 1A, lipid-free polysaccharidic particles (1 mg of particles/ml) sedimented over a wide sucrose concentration range of 2–20% with a maximum around 7%, indicating a certain polydispersity in density of these particles. Liposomes (0.2 mg DPPC/ml) displayed greater density and narrower polydispersity than polysaccharidic particles with an isopycnic band around 18–20% sucrose.

As shown in Fig. 1B, Light-biovectors (0.2 mg DPPC for 1 mg cationic polysaccharidic particles/ml), labelled with fluorescein and DPH,

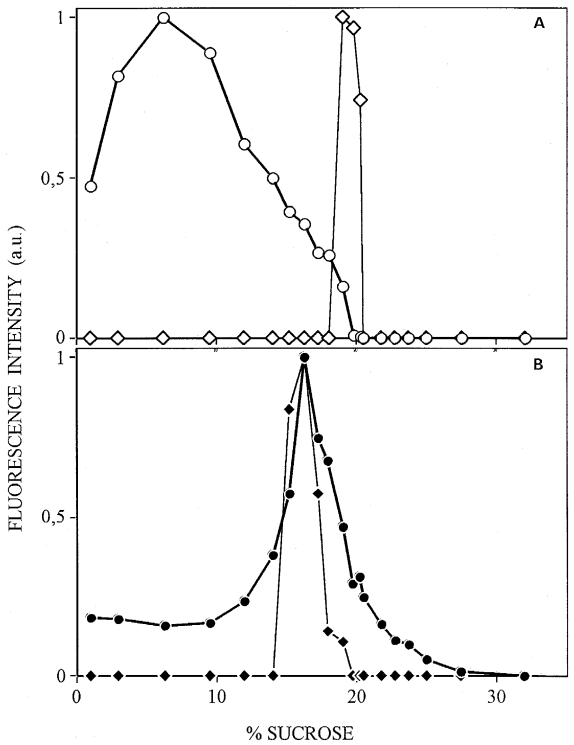


Fig. 1. Separation of polysaccharidic particles, liposomes and Light-biovectors on sucrose density gradients (0–35%). (A) Separation of fluorescein-labelled polysaccharidic particles (○) and DPH-labelled DPPC liposomes (◇). (B) Separation of Light-biovectors whose the polysaccharidic core was labelled with fluorescein (●) and the DPPC layer with DPH (♦). Fluorescein and DPH fluorescence intensities were normalized with respect to their maximum values.

were found at 14–19% sucrose concentration with the two probes, indicating that a large part of the polysaccharidic particles and lipids cosedimented. It is worth noting that the association between polysaccharidic cores and lipids resulted in increased density and a decreased polydispersity, with a final density for the Light-biovectors around 17% sucrose, close to that of liposomes. The fluorescence observed at low sucrose concentration (2–10%) probably corresponds to the presence of free fluorescein in the water phase and of a few light and lipid-free polysaccharidic particles. Traces of free fluorescein remained after purification of labelled polysaccharidic particles. Control experiments with a solution of free fluorescein showed that it spread over 0–7% sucrose. The small shoulder detected at 19% sucrose may correspond to the presence of denser Light-biovectors or of small amounts of liposomes. Control experiments showed that for up to 1 mg DPPC for 1 mg cationic polysaccharidic particles, homogeneous preparations of Light-biovectors were obtained, giving a single and well defined band on sucrose gradient, comparable to that shown in Fig. 1B. For higher DPPC/polysaccharidic particles ratio, Light-biovector preparations became non homogeneous with the appearance of a new band corresponding to the presence of free liposomes. Accordingly, mixtures of DPPC cationic Light-biovectors and DPPC liposomes, both labelled with DPH, yielded two well separated bands after centrifugation on sucrose gradient, indicating no overlap in density of the two entities (data not shown.)

In summary, this technique shows that Light-biovectors obtained were mainly composed of polysaccharidic particles associated with lipids.

### 3.2. Size determination of particles

Lipid-free polysaccharidic particles were relatively homogeneous in diameter A single particle population was detected in the nanosizer, around 35–42 nm for the neutral and anionic particles and around 75 nm for the cationic ones (Table 1).

Lipids alone gave relatively monodispersed liposomal suspensions with a mean diameter depending on lipidic composition: ~40 nm and ~20 nm for DPPC and DPPC/DPPG mixtures, respectively (Table 1).

In mixtures of cationic polysaccharidic particles (~75 nm in diameter) and DPPC liposomes (~40 nm in diameter) at concentrations equivalent to those found in Light-biovectors, the two populations were clearly differentiated by the nanosizer analysis. Surprisingly, simple mixtures of cationic polysaccharidic particles and anionic DPPC/DPPG (95/5 w/w) liposomes tended to precipitate, preventing size measurement of particles. In contrast, Light-biovector preparations exhibited a single population with a mean diameter similar to that of the corresponding lipid-free polysaccharidic particle. This strongly suggests that polysaccharidic core was coated with a thin phospholipid layer and not with a large multi-layered system or with adsorbed liposomes.

Table 1
Mean diameter of nanoparticles as measured by LASER light scattering

| Sample | Polysaccharidic core | Mean diameter (nm) |
|---|---|---|
| Polysaccharidic particles | Neutral | 42.3 ± 26 |
| | Cationic | 74.7 ± 29 |
| | Anionic | 34.6 ± 13 |
| DPPC liposomes | | 39.4 ± 13 |
| DPPC/DPPG liposomes | | 18.5 ± 9 |
| DPPC Light-biovectors | Neutral | 39.2 ± 9 |
| | Cationic | 82.9 ± 26 |
| | Anionic | 46.6 ± 18 |
| DPPC/DPPG Light-biovectors | Neutral | 40.7 ± 17 |
| | Cationic | 88.6 ± 12 |
| | Anionic | 45.4 ± 26 |

Phospholipid concentration was 0.2 mg/ml and polysaccharide concentration was 1 mg/ml. Samples were diluted in 50 mM PBS. Data represent means ± S.E. for 10 determinations.

### 3.3. Stability of Light-biovectors

We measured at various intervals the diameter of DPPC liposomal dispersions and Light-biovector preparations stored at 4°C or at room temperature. As can be seen in Table 2, no significant change in diameter was detected for cationic and anionic Light-biovectors stored at 4°C for up to one year. Similar results were obtained with Light-biovectors prepared with a 95/5 (w/w) DPPC/DPPG mixture (data not shown). In contrast, DPPC liposomes showed aggregation after 3 months at room temperature, or 6 months at 4°C.

Table 2
Effect of storage on the diameter of various nanoparticle preparations

| Sample | Mean diameter of particles (nm) | | | | | |
|---|---|---|---|---|---|---|
| | 24 h | 1 month | 3 months | 6 months | 9 months | 12 months |
| *DPPC liposomes* | | | | | | |
| 4°C | 39.4 ± 13 | 39.1 ± 15 | 40.6 ± 17 | 42.4 ± 9 | A | A |
| Room temperature | 39.4 ± 13 | 40.9 ± 11 | 38.8 ± 8 | A | A | A |
| *DPPC cationic Light-biovectors* | | | | | | |
| 4°C | 82.9 ± 26 | 86.2 ± 21 | 81.2 ± 19 | 88.7 ± 23 | 83.9 ± 14 | 87.4 ± 20 |
| Room temperature | 82.9 ± 26 | 79.5 ± 21 | 82.2 ± 18 | 86.1 ± 23 | A | A |
| *DPPC anionic Light-biovectors* | | | | | | |
| 4°C | 46.6 ± 18 | 40.3 ± 11 | 49.9 ± 23 | 45.1 ± 12 | 45.2 ± 19 | 47.0 ± 13 |
| Room temperature | 46.6 ± 18 | 48.0 ± 15 | 49.2 ± 16 | 48.1 ± 13 | 48.5 ± 17 | 49.4 ± 15 |

A: aggregated. Samples were stored at room temperature or at 4°C. Data represent means ± S.E. for 10 determinations.

### *3.4. Influence of the polysaccharidic core on the lipid shell.*

The phase behavior of the lipid shell surrounding the polysaccharidic cores was studied by measuring the gel to liquid phase transition temperature, $T_m$, of the lipid. In this study, the influence of charges born by the polysaccharidic core and the lipids for various ionic concentrations in the aqueous phase were examined. $T_m$ was determined by the fluorescence depolarization of the probe DPH inserted in the lipid phase [18].

Fig. 2A shows a typical $p$ versus temperature curve for DPPC liposomes, which accounts for a clear-cut gel to liquid phase transition with a $T_m$ value of 40.5°C, as expected for this lipid [18]. Well defined and cooperative gel to liquid phase transition was also observed for DPPC associated with Light-biovectors. As shown in Table 3, $T_m$ was found over the range 39–48°C, depending on the nature of the polysaccharidic core and of the lipids. Generally, in water and for particles coated with DPPC and DPPC/DPPG, a neutral polysaccharidic core only slightly affected $T_m$ as compared to liposomes. In contrast, $T_m$ slightly decreased in the presence of cationic polysaccharidic cores and markedly increased with anionic cores. For DPPC/stearylamine samples, $T_m$ increased in the presence of cationic and anionic cores.

Addition of PBS to Light-biovector suspensions decreased $T_m$, with a more pronounced effect on the anionic than on the cationic and neutral Light-biovectors.

All these observations clearly indicate that in Light-biovectors, the membrane is a well organized lipid bilayer whose phase properties depend partly on the sign of the charges born by the polysaccharidic core and the lipid and on the presence of salts in the water phase.

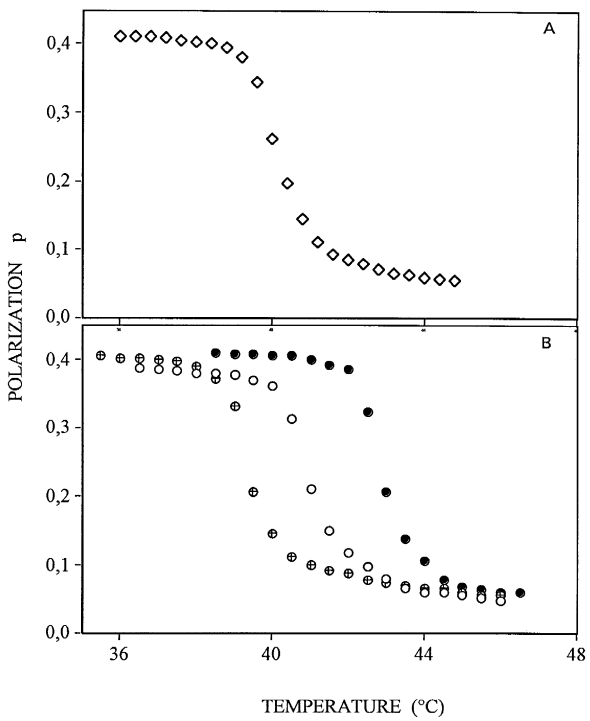


Fig. 2. Influence of temperature on the fluorescence polarization $p$ of DPH. (A) In DPPC liposomes (◇). (B) In DPPC associated with neutral polysaccharidic core (○), with cationic polysaccharidic core (⊕) or with anionic polysaccharidic core (●).

### 3.5. Number of lipid bilayers around the polysaccharidic core

Finally, it was important to determine whether the polysaccharidic core of Light-biovectors was surrounded by one or several lipid bilayers. Fluorescence quenching experiments were carried out using the probe NBD-PC in which the NBD group has been shown to be located in the glycerophosphate region of the lipid bilayer [19–21]. Cobalt ions were used as quenchers [21]. Because relatively high $Co^{2+}$ ion concentrations are required (up to 30 mM) to achieve a nearly complete fluorescence quenching of the NBD group, Light-biovectors were prepared in the presence of 1.5 M NaCl, in order to avoid important changes in ion concentrations upon addition of the quencher to the aqueous phase. Because $Co^{2+}$ ions are slightly permeant with time through lipid bilayers, experiments were carried out extemporaneously. To each sample, $Co^{2+}$ ions were added to the desired concentration and fluorescence was measured immediately. Superimposable fluorescence quenching curves were obtained for anionic and cationic Light-biovectors and unilamellar DPPC liposomes. For the sake of clarity, only the data obtained for unilamellar DPPC liposomes and DPPC anionic Light-biovectors is shown in Fig. 3. In both cases, changes in slope were detected with 50–55% quenching. Remembering that probe molecules located in the external lipid layer are affected first, a simple calculation using a bilayer thickness of 5.5 nm and a molecular area of 0.47 $nm^2$ for DPPC in the gel phase [22], indicates that for unilamellar vesicles of 40 nm and 80 nm in diameter, about 65% and 55% of the lipid molecules are present in the external layer, respectively. This and the great similarity between the two curves suggests that, like unilamellar DPPC liposomes, Light-biovectors were composed of a single lipid bilayer.

Table 3
Gel to liquid phase transition temperature $T_m$ of DPPC, DPPC/DPPG and DPPC/stearylamine (DPPC/SA) liposomes, or neutral, cationic and anionic Light-biovectors

| Lipid | Sample | $T_m$ (°C) | |
|---|---|---|---|
| | | Water | PBS |
| DPPC | Liposomes | 40.0 ± 0.2 | 39.2 ± 0.3 |
| | Neutral Light-biovector | 40.8 ± 0.2 | 39.8 ± 0.1 |
| | Cationic Light-biovector | 39.3 ± 0.3 | 38.4 ± 0.2 |
| | Anionic Light-biovector | 42.8 ± 0.2 | 39.0 ± 0.2 |
| DPPC/DPPG (95/5, w/w) | Liposomes | 41.1 ± 0.1 | 38.3 ± 0.2 |
| | Neutral Light-biovector | 41.0 ± 0.1 | 39.9 ± 0.5 |
| | Cationic Light-biovector | 39.3 ± 0.3 | 39.9 ± 0.2 |
| | Anionic Light-biovector | 44.8 ± 0.3 | 39.1 ± 0.2 |
| DPPC/SA (95/5, w/w) | Liposomes | 43.4 ± 0.1 | 42.8 ± 0.1 |
| | Neutral Light-biovector | nd | nd |
| | Cationic Light-biovector | 44.9 ± 0.2 | 43.4 ± 0.1 |
| | Anionic Light-biovector | 48.5 ± 0.1 | 44.3 ± 0.2 |

nd: not determined. Experiments were carried out in water or 100 mM PBS. Data represent means ± S.E. for six determinations.

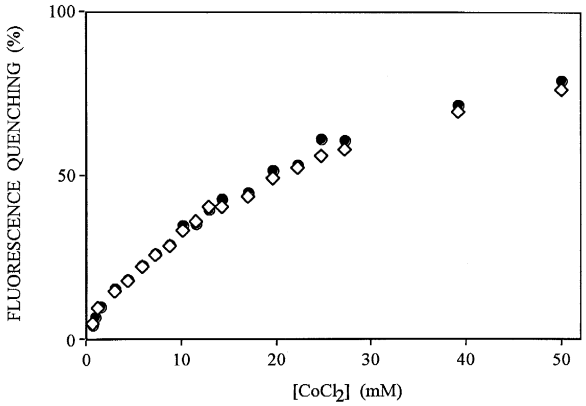


Fig. 3. Influence of Cobalt ions on the fluorescence of the probe NBD-PC inserted (2 mol%) in DPPC liposomes (◇) and DPPC Light-biovectors (●). Lipid concentration was 27.2 μM and temperature was 20°C.

## 4. Discussion

The above results clearly indicate that Light-biovectors are mainly composed of a polysaccharidic core surrounded by a single phospholipid bilayer. These nanoparticles are monodispersed and homogeneous in diameter population. They are reproducible in diameter and structure and are very stable. This presents a great advantage in the view for developing new drug carriers.

From the physicochemical point of view, one can assume that sugar–phospholipid interactions contribute to the ease of formation of these particles and their stability. Mono, oligo and polysaccharides have been described as structural and functionnal membrane-stabilizing factors [23–25]. Sugars and their

derivatives can be used as cryoprotectives to maintain the stability of liposomes [24–29] or the functional integrity and activity of natural membranes after desiccation or lyophilisation [30]. These molecules not only bind and entrap water molecules but they also interact with phospholipids [27–29,31–33]. These interactions depend on the nature of the sugar residues and phospholipid headgroups and they occur without the penetration of the sugar residues within the membrane [33]. More than likely, these interactions are stabilized by a complex network of hydrogen bonds between water, sugars and phospholipids [24,25,29]. In addition, these interactions are reinforced when the molecular weight of polysaccharides is increased [34]. In the present study, reticulation of maltodextrin resulted in a very high molecular weight polysaccharidic polymer. This may explain relatively stable profile of the Light-biovectors.

It is also noticeable that stable Light-biovectors could be obtained over a relatively wide phospholipid to polysaccharide concentration range (from 0.2 mg up to 1 mg DPPC for 1 mg cationic polysaccharidic particles). However in all cases, Light-biovectors appeared denser and therefore more compact than the lipid-free polysaccharidic particles. This strongly suggests a certain flexibility of the polysaccharidic chain enabling the formation of particles surrounded by a single lipid bilayer with a good adaptation in size of the polymeric core to the available number of phospholipid molecules.

Mono- and disaccharides have been reported to increase the gel to liquid phase transition temperature $T_m$ of phosphatidylcholines. Osmotic dehydration of the phosphocholine headgroups resulting in a decrease of the lipid molecular area and therefore an increase in the acyl chain order may account for this observation [24]. Our results clearly show that the phospholipid bilayer associated with the polysaccharidic core in the Light-biovectors displayed a degree of cooperativity and organization similar to that of liposomal structures. The $T_m$ of DPPC was also affected by its interactions with the polysaccharidic core, but now with a clear dependence on the charge born by the polymer. Thus, the $T_m$ of DPPC in neutral Light-biovectors was comparable to that of liposomes while it slightly decreased for cationic and increased for anionic Light-biovectors. In anionic Light-biovectors, incorporation of stearylamine in DPPC further increased $T_m$ up to the rather high value of $\sim 48°C$. A possible explanation is in the orientation of the phosphocholine dipole in response to the nature and location of charges born by the polysaccharidic core and the lipids. This dipole has been shown to behave like a 'sensor' of electric charges in membranes [35]. In phosphatidylcholine bilayers, the mean orientation of the phosphocholine moiety is approximately parallel to the membrane surface. Addition of a cationic amphiphile to the lipid has been shown to provoke a repulsion of the positively charged $N^+$ end of the choline residue which moves outward the membrane while an anionic amphiphile has the opposite effect, forcing the $N^+$ end toward the membrane interior [36].

In DPPC Light-biovectors, the electric charges are born by the polysaccharidic core and therefore are located outside the membrane. Consequently, opposite effects are expected, the negative charges being attractive for the $N^+$ end and the positive charges being repulsive. Tilting of the phosphocholine moiety outward or inward the membrane would lead to an increase or a decrease in the molecular packing of the lipids and thus an increase or a decrease in $T_m$ as was observed for anionic and cationic Light-biovectors, respectively. In anionic Light-biovectors, addition of stearylamine to DPPC would reinforce the influence of the polysaccharidic core, thus explaining the high $T_m$ value of 48°C found in this case. The fact that these differences in $T_m$ values between neutral, cationic and anionic Light-biovectors nearly disappeared when the ionic strength of the water phase was increased provides support to the idea that the electric charges born by the polysaccharidic core contribute to the organization of the surrounding lipid bilayer.

To conclude, Light-biovectors constitute a new class of nanoparticles in which the external lipid layer is stabilized by its interactions with the internal polysaccharidic core. The data presented reinforces the concept that sugars and their derivatives can contribute to the stabilization of membrane structures and show that these new biovectors provide an interesting system for investigating polysaccharide/phospholipid interactions. Incorporation of proteins in Light-biovectors is currently under investigation in our laboratories: either intrinsic proteins bound to lipids through hydrophobic interactions or soluble

proteins bound to the polysaccharidic core through electrostatic interactions.

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## References

[1] F. Puisieux, P. Couvreur, J. Delattre, J.P. Devissaguet (Eds.), Liposomes, New Systems and New Trends in their Applications, Editions de Santé, Paris, 1995.
[2] J. Senior, CRC Crit. Rev. Drug Carrier Syst. 32 (1987) 123–193.
[3] G. Gregoriadis (Ed.), Liposomes as Drug Carriers, Recent Trends and Progress, John Wiley and Sons, Chichester, 1988.
[4] M. Peyrot, A.M. Sautereau, J.M. Rabanel, F. Nguyen, J.F. Tocanne, D. Samain, Int. J. Pharm. 102 (1994) 25–33.
[5] D. Samain, I. De Miguel, J. Meniali, K. Ioualalen, L. Ding, M. Cervilla, V. Rieumajou, P. Delrieu, L. Imbertie, International Patent WO 92/21329, 1992.
[6] D. Betbeder, C. Davrinche, J.-L. Davignon, E. Prieur, International Patent WO 96/06638, 1996.
[7] I. De Miguel, K. Ioualalen, M. Bonnefous, M. Peyrot, F. Nguyen, M. Cervilla, N. Soulet, R. Dirson, V. Rieumajou, L. Imbertie, C. Solers, S. Cazes, G. Favre, D. Samain, Biochim. Biophys. Acta 1237 (1995) 49–58.
[8] E. Prieur, D. Betbeder, F. Niedergang, M. Major, A. Alcover, J.-L. Davignon, C. Davrinche, Vaccine 14 (6) (1996) 511–520.
[9] N.C. Santos, M.J.E. Prieto, A. Morna-Gomes, D. Betbeder, M.A.R.B. Castanho, Biopolymers (in press).
[10] N. Castignolles, D. Betbeder, K. Ioualalen, O. Merten, C. Leclerc, D. Samain, P. Perrin, Vaccine 12 (15) (1994) 1413–1418.
[11] H.C. Woodle, D. Papahadjopoulos, Methods Enzymol. 171 (1989) 193–217.
[12] G.R.J. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
[13] F. Dubois, Anal. Chem. 28 (1956) 350–356.
[14] S. Batzri, E.D. Korn, Biochim. Biophys. Acta 298 (1973) 1015–1019.
[15] J.M.H. Kremer, M.W. Esker, C. Pathmamanoharan, P.H. Wiersema, Biochemistry 16 (1977) 3932–3935.
[16] A.N. De Belter, K. Granath, Carbohydrate Res. 30 (1976) 375–378.
[17] B.R. Lentz, Chem. Phys. Lipids 64 (1993) 99–116.
[18] V. Borenstein, Y. Barenholz, Chem. Phys. Lipids 64 (1993) 117–127.
[19] S. Mazères, V. Schram, J.F. Tocanne, A. Lopez, Biophys. J. 71 (1996) 327–335.
[20] A. Chattopadhyay, E. London, Biochemistry 26 (1987) 39–45.
[21] A. Chattopadhyay, E. London, Biochim. Biophys. Acta 938 (1988) 24–34.
[22] W.J. Sun, R.M. Suter, C.R. Knewtson, C.R. Worthington, S. Tristam-Nagle, R. Zhang, J.F. Nagle, Phys. Rev., E. 49 (1994) 4665–4676.
[23] V. Rosilio, A. Baszkin, J. Sunamoto, in: F. Puisieux, P. Couvreur, J. Delattre, J.P. Devissaguet (Eds.), Liposomes, New Systems and New Trends in their Applications, Editions de Santé, Paris, 1995, pp. 43-71.
[24] L.M. Crowe, J.H. Crowe, Biochim. Biophys. Acta 1064 (1991) 267–274.
[25] G. Strauss, H. Hauser, Proc. Natl. Acad. Sci. USA 83 (1986) 2422–2426.
[26] C.H.P. Fabrie, B. De Kruijff, J. De Gier, Biochim. Biophys. Acta 1024 (1990) 380–384.
[27] D.E. Brooks, G.V. Seaman, Nature (London), New Biol. 238 (1972) 251–253.
[28] M. Minetti, P. Aducci, A. Teichner, Biochim. Biophys. Acta 437 (1976) 505–517.
[29] M. Minetti, P. Aducci, V. Viti, Biochemistry 18 (12) (1979) 2541–2548.
[30] J.H. Crowe, L.M. Crowe, S.A. Jackson, Arch. Biochem. Biophys. 220 (2) (1983) 477–484.
[31] J. Sunamoto, K. Iwamoto, H. Kondo, J. Biochem. 88 (1980) 1219–1226.
[32] M. Minetti, A. Teichner, P. Aducci, Biochem. Biophys. Res. Commun. 80 (1) (1978) 46–55.
[33] E.M. Arnett, N. Harvey, E.A. Johnson, Biochemistry 25 (1986) 5239–5242.
[34] K. Iwamoto, J. Sunamoto, J. Biochem. 91 (3) (1982) 975–979.
[35] J. Seelig, P.M. Macdonald, P.G. Scherer, Biochemistry 26 (1987) 7536–7541.
[36] P.G. Scherer, J. Seelig, Biochemistry 28 (1989) 7720–7728.