Pharmaceutical Research, Vol. 17, No. 7, 2000 *Research Paper*

Proofs of the Structure of Lipid Coated Nanoparticles (SMBV™) Used as Drug Carriers

Ignacio De Miguel,[1,2] Laurent Imbertie,[1] Valerie Rieumajou,[1] Michel Major,[1] Roger Kravtzoff,[1] and Didier Betbeder[1]

Received March 27, 2000; accepted April 11, 2000

Purpose. Supramolecular Biovectors (SMBV™) consist of cross-linked cationic nanoparticles surrounded by a lipid membrane. The purpose was to study the structure of the lipid membrane and to characterise its interaction with the nanoparticles in order to differentiate SMBV™ from other polymer/lipid associations.
Methods. The interaction of lipids with the nanoparticle surface was studied using zeta potential, Fluorescence Energy Transfer (FET) and Fluorescence Microscopy. SMBV™ were compared to liposomes and mixtures nanoparticles/liposomes. Finally the structure of SMBV™ was visualised by Electron Microscopy.
Results. Zeta potential measurements showed that lipids on SMBV™ had a pronounced shielding effect on the surface charge. This was not the case for mixtures of nanoparticles and liposomes. FET experiments confirmed these results indicating that, for SMBV™, the lipids are much closer to the nanoparticle surface. SMBV™ Fluorescence microscopy on model microparticles showed a lipid crown on SMBV™ that was confirmed by electron microscopy on SMBV™ nanoparticles.
Conclusions. Results show that in case of SMBV™ lipids are strongly adsorbed on the polysaccharide core surface probably due to ionic/hydrophobic interactions. The resulting supramolecular structure is a spherical cationic polysaccharide particle surrounded by a phospholipid/cholesterol layer.

KEY WORDS: supramolecular biovector; liposome; characterisation; zeta potential; fluorescence; microscopy.

I. INTRODUCTION

A Supramolecular Biovector (SMBV™) is a drug delivery nanocarrier which consists of a discretely sized, ionically charged, cross-linked polysaccharide core (PSC) surrounded by a lipid membrane (1). In case of cationic SMBV™, the PSC is made of epichlorohydrin cross-linked polysaccharides modified with quaternary ammonium functions. The diameter is around 60 nanometers, with an ionic capacity 2.0 ± 0.2 mmol/g of ammonium groups. SMBV™ are prepared by the addition of a lipid layer, composed of DPPC and Cholesterol. Due to their bi-compartimental structure SMBV™ are able to load a variety of molecules. The PSC acts as an ion-exchange matrix, capable of including ionic compounds, and the external layer can adsorb amphiphilic or hydrophobic compounds. This layer can also act as a cell like membrane and insert membrane proteins. SMBV™ have numerous potential applications for different therapeutic domains, for example, entrapping antisense oligonucleotides and improving their stability and antisense efficacy (2,3,4), stabilising peptides or proteins and improving bioactivity (5) or enhancing the immunogenicity of antigens (6). In case of cationic SMBV™, the main characteristic is its strong mucoadhesivity and nasal residence times (7). When combined with antigens, SMBV™ protects them from proteolysis and enhances their immunogenicity after nasal administration (8,9,10). Their size and structure enable SMBV™ to entrap internal viral proteins into the hydrophilic core and transmembrane proteins into the phospholipid layer (9), resulting in a virus-like synthetic nanoparticle able to elicit, not only immune humoral responses, but to activate cell mediated responses (11).

The structure of SMBV™ was firstly examined by different methods as light scattering, electron microscopy (12,13), fluorescence polarisation and quenching (1), suggesting that the structure was a mono-bilayer of phospholipids adsorbed onto polysaccharide nanoparticles. In this work further characterisation of the interaction PSC/lipids was performed by the characterisation of the surface properties of the resulting SMBV™ compared to PSC/liposome mixtures. Further microscopic visualisation of the interaction was performed using Sephadex microparticles (microscopic model) and electron microscopy. The results seem to confirm the proposed structure in which the PSC are covered by a phospholipid membrane. The results also indicate that the SMBV™ structure is the result of multiple hydrophobic and electric interactions which clearly differentiates SMBV™ from hydrogel solid core liposomes or other lipid covered particles (14,15).

II. EXPERIMENTAL

II.A. Reagents

Dipalmitoyl phosphatidyl choline (DPPC) was purchased from Lipoid KG (Mannheim, Germany). Cholesterol and Rhodamine B isothiocyanate were obtained from Sigma (St. Louis, USA). Dipalmitoyl phosphatidyl ethanolamine derived with 7-nitrobenz-2-oxa-1,3-diazole (NBD-DPPE) and 5-hexadecanoyl amino eosin (H-Eosin) were obtained from Molecular Probes (Leiden, The Netherlands).

Cationic PSC of 60 ± 20 nanometers having a charge density of 2 mmol/g were used from a mother aqueous solution of PSC at 20 mg/mL.

Supramolecular Biovectors (SMBV™) were composed of PSC (77% w) and a mixture (23% w) of DPPC and cholesterol (70:30 w/w).

Sephadex QAE A-50 was obtained from Pharmacia (Uppsala, Sweden).

II.B. Preparation of PSC and SMBV™

PSC were prepared according to (8). Briefly, 100 g of maltodextrins (Glucidex, Roquette Freres, France) were suspended in 200 mL of NaOH 2M at room temperature. The maltodextrins were then cross-linked by addition of 4.7 mL of

[1] Biovector Therapeutics S.A., Chemin du Chene Vert, 31676 Labege Cedex, France.
[2] To whom all correspondence should be addressed. (e-mail: ignacio.demiguel@biovector.com)

ABBREVIATIONS: SMBV™, Supramolecular Biovector; PSC, Polysaccharide core; FET, Fluorescence Energy Transfer; DPPC, Dipalmitoyl phosphatidyl choline; NBD-DPPE, phosphatidyl ethanolamine 7-nitrobenz-2-oxa-1,3-diazole; DMF, dimethyl formamide; TEM, transmission electron microscopy.

0724-8741/00/0700-0817$18.00/0 © 2000 Plenum Publishing Corporation

2,3 epoxy chloropropane (epichlorohydrin) and functionalised with 31.2 g of glycidyl trimethyl ammonium. The resulting gel was homogenised at high pressure to give a suspension of 60 nm cationic nanoparticles that were further purified by ultrafiltration (System Minisette, 30 kD membrane, Filtron, France) and microfiltration (Spiralcap, 0.2 μm, Gelman, USA). It resulted in a suspension of 60 nm cationic nanoparticles at 20 mg/mL. Elementary analysis of nitrogen contents showed an ionic charge of 2 mEq/g.

SMBV™ were prepared as described in (8). Briefly, a suspension of PSC was maintained at 80°C and mixed with an ethanol solution of DPPC/Cholesterol (70:30). The ratio PSC to lipids was 77% of PSC and 23% of lipids (w/w). The suspension was homogenised at high pressure to obtain a clear solution of SMBV™ of around 60 nm. Ethanol was eliminated by ultrafiltration ((System Minisette, 30 kD membrane, Filtron, France) and SMBV™ were further purified by microfiltration (Spiralcap, 0.2 μm, Gelman, USA). It resulted in a clear suspension of 60 nm cationic SMBV™ at 25 mg/mL.

II.C. Preparation of Fluorescent PSC and SMBV™

10 mL of the mother solution of PSC at 20 mg/ml (200 mg of PSC) were placed in a round bottom flask and buffered with 25mg of Na_2CO_3. 2 mg of rhodamine isothiocyanate in 20 μL of DMF were added. The reaction was incubated at room temperature for 15 h. Unbound rhodamine was eliminated on an Amicon 8050 system equipped with a 10 kd cellulose acetate membrane (YM10) by diafiltration against a sodium chloride solution (0.15 mM). The solution was then diafiltered against water until the conductivity in the filtrate was lower than 10 μsiemens.

The concentration of rhodamine labelled PSC (Rho-PSC) was then adjusted at 20 mg/mL. Double labelled fluorescent SMBV™ composed of Rho-PSC (77%) and a mixture (23%) of DPPC, cholesterol and NBD-DPPE (70:30:0.25 w/w) were prepared as described in II.B. The double labelled SMBV™ were washed and concentrated to 25 mg/mL by ultrafiltration on an Amicon 8050 system equipped with a 10 kd cellulose acetate membrane (YM10).

II.D. Preparation of Liposomes

Liposomes consisting of DPPC/Cholesterol (70/30 w/w) were prepared by dissolving 210 mg of DPPC and 90 mg of cholesterol in 10 ml of ethanol and injecting them in 100 ml of water at 80°C. The liposome suspension was placed in an ultrafiltration system (Amicon model 8200, 30 Kd cellulose acetate membrane (Amicon YM 30), diafiltered against water then filtered through 0.2 μm filters (Acrodisc 25 mm, 0.2 μm, Gelman, France), conditioned in sterile containers and kept at 4°C. The resulting liposomes (3 mg/mL) showed a mean diameter of around 60 nm.

Alternatively, fluorescent liposomes were prepared by adding 0.25% w/w of NBD-DPPE to the lipid mixture.

II.E. Size and Z-Potential Measurements

Size measurements and the recording of ISL were performed for SMBV™, PSC, liposomes and mixtures of PSC/liposomes at 25°C in 5 mM phosphate buffer pH 7. All samples were prepared at the desired concentration using concentrate particles and buffer solutions and then filtered at 0.45 μm prior to use (Acrodisc 25 mm, 0.45 μm, Gelman, France). Measurements were performed in a Coulter Model N4 SD Submicron Particle Analyser (Coultronics, USA). Zeta Potential measurements were performed for SMBV™, PSC, Liposomes and mixtures thereof. Samples at different concentrations, pH and ionic strength conditions were prepared from concentrate particles and buffer solutions and then filtered at 0.45 μm prior to use. Measurements were performed at 25°C in a Malvern Model 3000 Zetasizer (Malvern Instruments Ltd, UK).

For mixtures of PSC/Liposomes, the concentrations were adjusted to obtain the same proportions as used for SMBV™.

II.F. Fluorescence Energy Transfer Experiences

Fluorescence measurements were performed in a Perkin Elmer Luminescence Spectrometer Model LS 50 B (Perkin Elmer, USA).

Three different suspensions were prepared: a suspension of SMBV™ (1 mg/ml) composed of Rho-PSC (0.7 mg/mL) and the lipid mixture of DPPC: cholesterol: NBD-DPPE (70:30:0.25, 0.3 mg/mL), a second suspension of liposomes of DPPC: cholesterol: NBD-DPPE (70:30:0.25, 0.3 mg/mL) and a third suspension of Rho-PSC (0.7 mg/mL) mixed with the described liposomes (0.3 mg/mL). Samples were excited at 470 nm and the fluorescence emission of NBD at 530 nm was measured. The energy transfer efficacy (E (%)) for SMBV™ and mixtures of PSC and liposomes was calculated by donor emission quenching using the equation:

$$E\,(\%) = (1 - F/F0) * 100$$

Where F is the measured fluorescence emission and F0 is the fluorescence emission of the liposome suspension of DPPC/cholesterol/NBD-DPPE.

II.G. Fluorescence Microscopy Experiments

Sephadex QAE A-50 beads (50 mg) were initially hydrated in 5 mL of water at 95°C for 3 hours.

DPPC (175 mg) and Cholesterol (75 mg) were dissolved in 9 mL of ethanol. A solution of Hexadecyl-eosin (H-eosin) in ethanol (1 mL, 1 mM) was then added to the lipid solution. The molar ratio of H-eosin to lipids was 1/300.

A liposome suspension was obtained by injecting 3 mL of the ethanol solution of lipids in 30 mL of water at 80°C. The resulting liposomes were 70 nm in diameter and were at a concentration of 2.5 mg/mL. In the first experiment, the tube containing 50 mg of Sephadex A-50 in 5 mL of water was placed in a thermostatic bath at 80°C. The ethanol solution of lipids was then injected (0.4 mL, 10 mg of lipids) under convenient agitation and the resulting suspension was maintained at 80°C for 1h. In a second experiment a tube containing 50 mg of Sephadex in 3 mL of water was mixed with the aqueous liposomes suspension (4 mL, 10 mg of lipids) and maintained at room temperature for a period of 1 h. In both cases the ratio of lipids to Sephadex A-50 beads was 20% (w/w). Sephadex beads were collected and placed in a cover glass for immediate microscopic observation. Fluorescence microscopy was performed on a Nikon Inverted Fluorescence Microscope model Elipse TE300 equipped with a DM505 fluorescence filter and a Nikon AF F601 photographic camera. Photographs were obtained for each preparation.

II.H. Electron Microscopy Experiments

Electron microscopy of PSC by cryofracture was performed using a JEOL 1200 EX apparatus. Samples were frozen at −200°C on liquid nitrogen and cryofracture was performed on a Reichert-Jung Cryofract at −150°C. Plating was performed with platinum and carbon and the prints were mounted on nickel grids for TEM observation.

SMBV™ were observed by negative stain transmission electron Microscopy. Formvar plastic coated grids were placed in a pair of anticapillary forceps and treated with a drop of SMBV™ suspension. After 15–30 minutes of incubation the excess water was carefully absorbed with a piece of filter paper. The grid was then treated with a drop of negative stain solution (phosphotungstic acid solution at 3% pH 6.8–7.2) for 1–5 minutes and the excess solution was then carefully removed by absorption with filter paper. The grid was left to dry at room temperature for a period of 1 h and then observed by TEM.

SMBV™ samples were also observed by cryo-electron microscopy. Holey type grids were treated by glow discharge to obtain a hydrophilic surface. An aqueous solution of SMBV™ (5 μL) was placed in the grid and incubated 1 min. The grid was immersed in liquid ethane and maintained at −180°C with liquid nitrogen. Cryo-electron microscopy was then performed using a Philips CM12 Cryomicroscope at minimal irradiation conditions.

III. RESULTS AND DISCUSSION

SMBV™ were initially described as multi-compartimental nanoparticles (16,17) composed of an internal cross-linked polysaccharide core on which an external crown of fatty acids was covalently grafted. The role of this external layer was to ensure the anchoring of phospholipids by hydrophobic interaction in order to form an external phospholipide monolayer. The internal polysaccharide particles were functionalised with a variety of ionic ligands (phosphate, quaternary ammonium) in order to provide the core with ion exchange properties. Further developments indicated that the fatty acid crown was not necessary and that phospholipids could be adsorbed around the polysaccharide particles (1). This new type of SMBV™ was characterised by light scattering techniques (12,13). Analysis of the Light Scattering data indicated that the PSC behave as spherical, microgel nanoparticles which are slightly more dense at the centre of the particle. The particle hydrodynamic radius was affected by the ionic strength, which is consistent with the concept of a ionically charged hydrogel nanoparticle. The lipid layer around the particle had little effect on particle shape and dimension but a more pronounced effect on particle density, indicating a strong interaction between the lipids and the PSC. Confirmatory experiments by TEM showed multilayer structures, which could not well be explained as a multilayer structure should have a more pronounced effect on the particle diameter.

Further characterisation (1) by separation on a sucrose density gradient showed that the association of phospholipids and PSC was nearly quantitative. The authors observed that lipids in SMBV™ presented a transition temperature, indicating that bilayer structures were present. The transition temperature of lipids on SMBV™ was modified with respect to free liposomes of the same composition and this modification was dependent on the type of PSC and ionic strength. The authors suggested that modification of the transition temperature was due to the lipid polar head polarisation because of its interaction with the strongly charged PSC. Finally, fluorescence quenching experiments, using Co2+ as quencher, showed that quenching kinetics were equivalents to those obtained with single bilayer liposomes, and they suggested that SMBV™ were statistically composed of PSC surrounded by a single lipid bilayer.

Table I. Z Potential (mV), Size (nm), and ISL (Counts per Second) Measurements of PSC (3 mg/mL), SMBV™ (4 mg/mL), Liposomes (1 mg/mL), and Mixtures of PSC and Liposomes (3 and 1 mg/mL) in 5 mM Phosphate Buffer at pH 7

Sample type	Size (nm)	ISL (cps)	Pot Z (mV)
PSC	56 ± 19	1.4 10^5	18.9 ± 6.1
SMBV™	57 ± 20	5.1 10^5	2.9 ± 6.3
Liposomes	60 ± 17	6.9 10^5	−4.9 ± 5.7
PSC + Liposomes	62 ± 20	7.4 10^5	15.4 ± 6.1

Note: Plus and minus indicates standard deviation.

The zeta potential is an interesting tool in the study of surface modifications (18) and we sought to examine the influence of the lipid layer of SMBV™ on the electrophoretic mobility of PSC. This study was performed by comparing the electrophoretic behaviour of PSC, SMBV™ and mixtures of PSC/liposomes with the same size and composition as SMBV™.

The zeta potential gives information about the charge of a solid particle surface at the plane of shear. This is classically true for solid particles, but is more complex in case of highly hydrated ionic hydrogel nanoparticles. For those, the ionic strength may modify not only the surface characteristics but also size and swelling properties. Moreover, the ionic charge and counter-ions are distributed throughout the particle and zeta potential changes cannot be easily rationalised.

This work deals with zeta potential changes due to shielding effects and with electrophoretic mobility changes due to particle-particle interactions. In this context, the relationship between electrophoretic mobility and zeta potential cannot always be interpreted in the same way. Results are then discussed as electrophoretic mobility modifications rather than zeta potential changes, even if all results are presented as measured zeta potential.

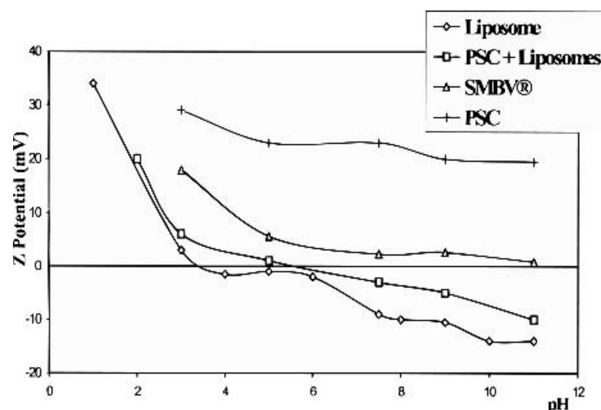

Fig. 1. Z Potential analysis as a function of pH for PSC (3 mg/mL), SMBV™ (4 mg/ml), Liposomes (1 mg/mL) and mixtures PSC/Liposomes at the corresponding concentrations. Measurements were performed in phosphate buffer 15 mM.

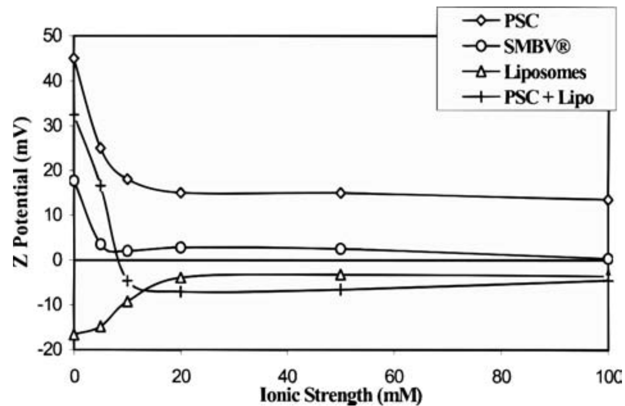

Fig. 2. Z Potential analysis as a function of ionic strength for PSC (3 mg/mL), SMBV™ (4 mg/ml), Liposomes (1 mg/mL), and mixtures PSC/Liposomes at the corresponding concentrations. Measurements were performed in phosphate buffer pH 7.5-8.

Table II. Z Potential Measurements of Different Batches of PSC (3 mg/mL), SMBV™ (4 mg/mL), Liposomes (1 mg/mL) and Mixtures of PSC and Liposomes (3 and 1 mg/mL) in 20 mM Phosphate Buffer at pH 8.5

Type	Batch 1 (mV)	Batch 2 (mV)	Batch 3 (mV)	Average
PSC	17.7	16.8	20.3	18.3
SMBV™	1.9	2.5	1.0	1.8
Liposome	−6.0	−8.8	−6.6	−7.1
PSC + Liposome	−6.5	−6.7	−5.1	−6.1

The first measurements of the zeta potential and size characteristics were performed at pH 7 in phosphate buffer 5 mM. The results are shown in Table I. Cationic polysaccharide cores are clearly positive and liposomes are negatively charged on their surface at this pH. The measured zeta Potential for SMBV™ is almost neutral whilst the zeta potential of mixtures of liposomes and PSC is largely positive with values close to those of PSC. It seems from these results that, in case of SMBV™, the lipids act as a shield for the PSC surface charge whilst for mixtures of PSC/liposomes this effect is less pronounced.

Despite their theoretical neutrality, the negative zeta potential of DPPC liposomes is not surprising. Makino *et al.* (19) showed that the zeta potential of neutral phospholipids depends on the polarisation (or position) of the phospholipid polar head and this may vary with the ionic strength and temperature.

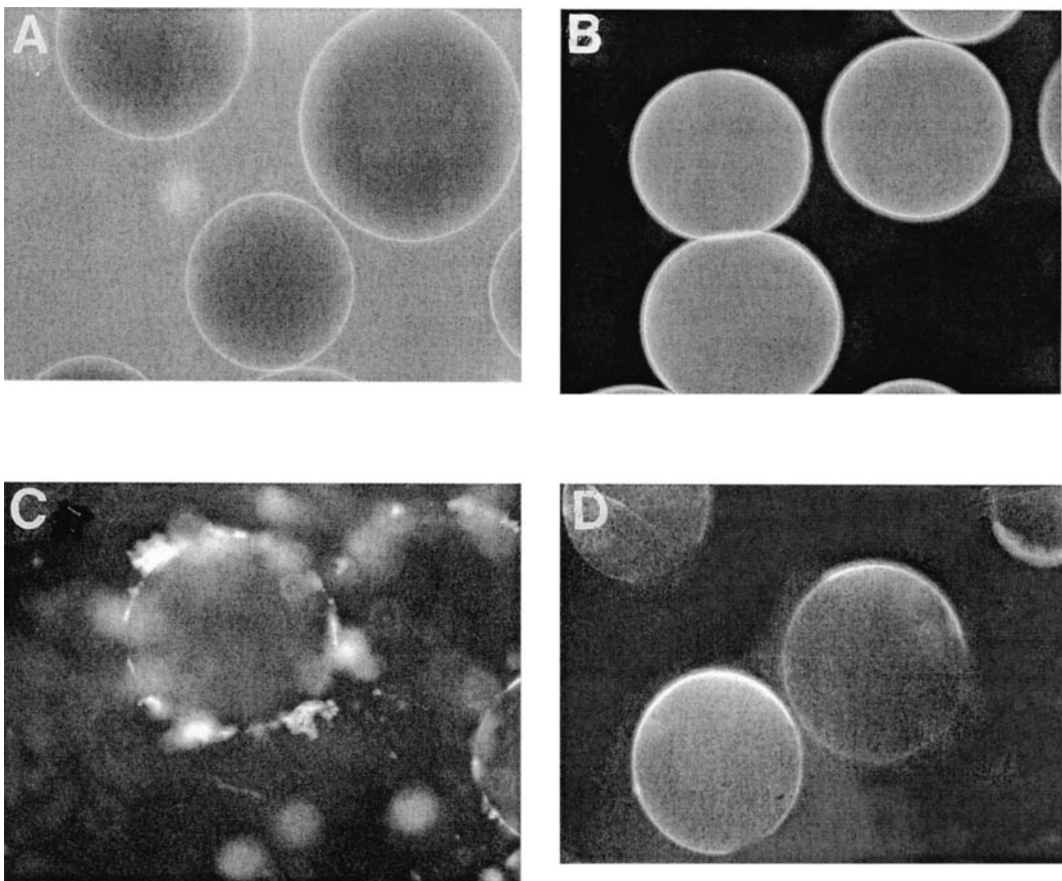

Fig. 3. Fluorescence miscroscopy (× 400) of Sephadex A-50 beads treated with a lipid mixture of DPPC/Cholesterol (70:30 w/w) containing H-Eosin (1/300 mol/mol). The ratio of lipids to Sephadex beads was 20% (w/w). (A) SMBV™ type process; (B) Mixture of beads with liposomes at room T°C; (C) is A after washing with PBS; (D) is B after washing with PBS.

They clearly showed that the electrophoretic mobility of DPPC liposomes was mainly negative at room temperature and over a wide range of ionic strength values.

Analysis of size and light scattering characteristics of each sample (Table I) showed that the size of all systems was distributed around 60 nm in diameter. These results also showed that the intensity of scattered light (ISL) was much higher for liposomes than for PSC at their respective concentrations. It seems that PSC are almost transparent to photons and the quantity of scattered light is quite low. Although these data do not allow any direct conclusion about sample structure, they have an important impact on the interpretation of zeta potential measurements for SMBV™ or mixtures of PSC and liposomes. In that case, the ISL is much higher for the lipid portion and, as zeta potential measurements are based on light scattering, the polysaccharide contribution may be masked by the lipids. The results should then be interpreted in terms of lipid mobility and, in this context, the positive zeta potential of a mixture of liposomes and PSC could be explained by a spontaneous surface interaction between the negatively charged liposomes and the positively charged PSC. This could lead to the formation of a complex PSC/liposome having a net positive charge due to the strongly cationic PSC. The liposomes are then carried along by the PSC. This type of surface interactions between charged polymers and lipids are well know (20,21).

In order to better understand these results, a systematic study of the zeta potential of PSC, liposomes, SMBV™ and mixtures as a function of the pH and ionic strength was performed. Figure 1 shows the results obtained as a function of the pH in 15 mM phosphate buffer solutions. Again the zeta potentials measured for liposomes could lead to a discussion as it should be independent of pH over a large range (4–10). However, in view of Makino *et al.* (19) and Jones *et al.* (22) these results could be accepted as changes in the polar region position on the phospholipids.

PSC remain clearly positive at all pH values due to their quaternary ammonium charge. The shielding effect of lipids is observed on SMBV™ over the entire range of pH tested.

For mixtures of PSC/liposomes, the zeta potential tends to follow the same curve as liposomes, being negative for a pH above 5.5 as if a single solution of liposomes was measured. This seems in contradiction with the above results in which the zeta potential of mixtures was positive at pH 7 in 5 mM buffer.

An explanation is given in Fig. 2 wherein the results of the zeta potential measurements as a function of the ionic strength are presented. Under these conditions (pH 7.5–8) PSC

Fig. 4. Cryofracture Transmission Electron Microscopy (× 150,000) of PSC in water.

and liposomes were, respectively, positively and negatively charged. An ionic strength shielding effect is observed in both cases from 5 to 20 mM.

SMBV™ remain nearly neutral at all ionic strengths, confirming the shielding effect of lipids and showing a good stability of the complex in saline medium. In contrast to SMBV™, the behaviour of mixtures of PSC/liposomes changes dramatically when increasing the ionic strength. At a low ionic strength the mixture behaves as a positively charged entity, confirming the results presented in Table I, but it appears that the interaction between liposomes and PSC is lost from a certain ionic strength (around 10 mM), thus inducing free liposomes with a negative zeta potential. Assuming that the negative charge of liposomes comes from the polarisation of a theoretically neutral zwitterionic head, this could be explained as a weak interaction between liposomes and PSC. Finally, the zeta potential measurements of three different preparations of PSC, SMBV™, liposomes and mixtures in 20 mM pH 8.5 phosphate buffer solution were performed to test the reproducibility of results (Table II).

Taking into account earlier characterisations (1,12,13) these results could further show that SMBV™ behave as a lipid coated polysaccharide nanoparticle. The layer of lipids acts as a shield to the surface charge of the cationic PSC and seems stable in saline conditions.

The different arrangement of lipids at the PSC surface in SMBV™ or mixtures of PSC and liposomes was also confirmed by two simple Fluorescence Energy Transfer (FET) experiments using Rhodamine labelled PSC (Rho-PSC) and NBD labelled phospholipids. FET is a widely used method to monitor molecular proximity, particularly in cellular biology, to study membrane fusion and in lipid physics to study vesicle-vesicle

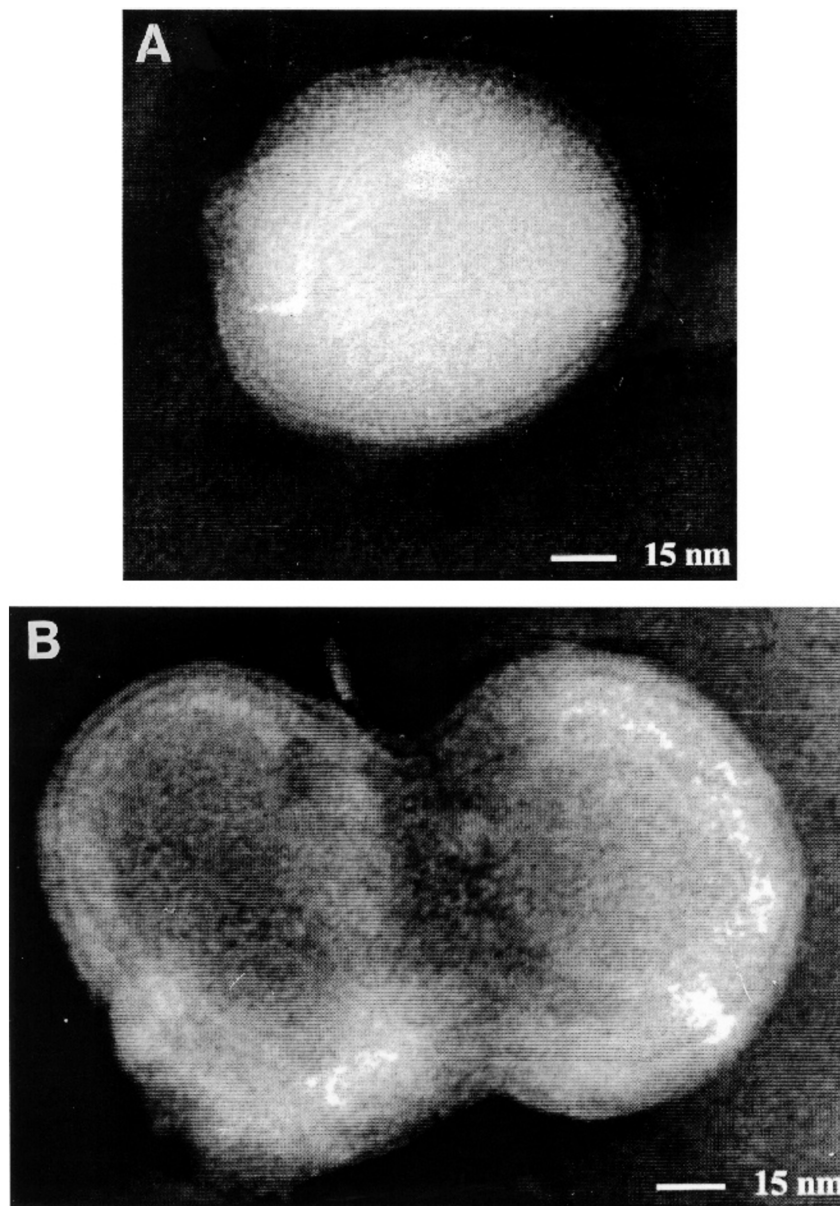

Fig. 5. Negative Staining Transmission Electron Microscopy (× 250,000) of SMBV™ in water. Negative stain was performed with phosphotungstic acid.

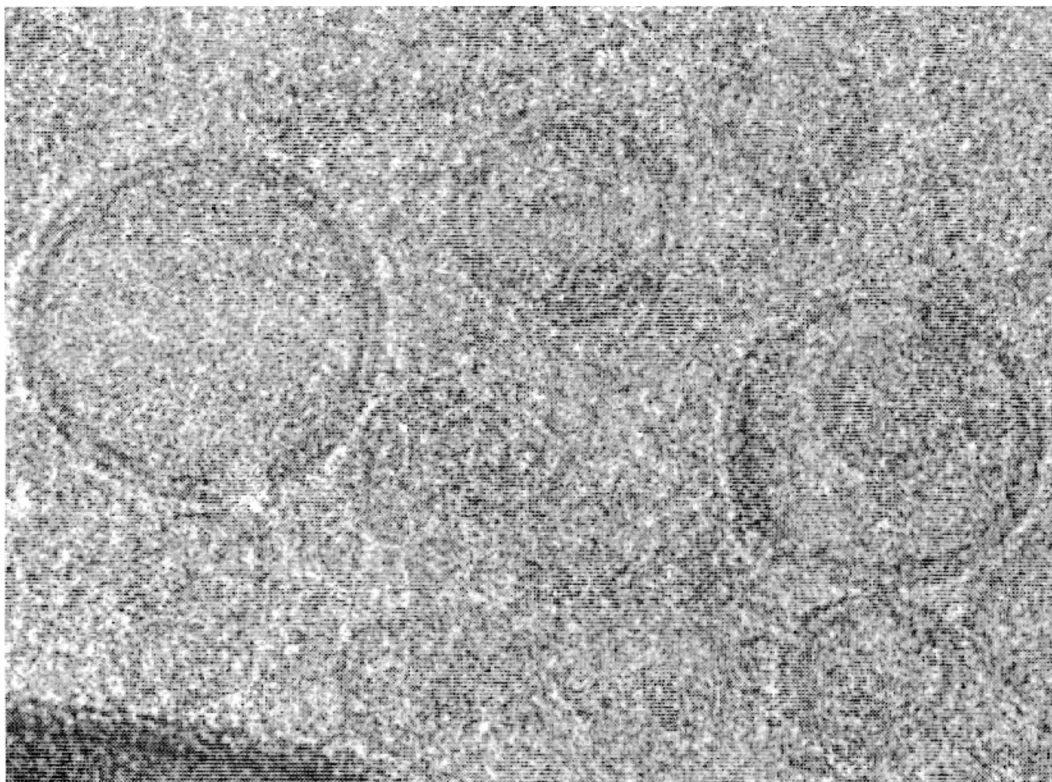

Fig. 6. Cryo-Electron Microscopy of SMBV™ in water (× 196,000).

interactions (23,24). The λ excitation and λ emission for NBD are, respectively, 470 nm and 530 nm. For Rhodamine the values are 555 nm and 580 nm, respectively. When the NBD is excited at 470 nm, its emission at 530 nm will be absorbed by the Rhodamine probe, resulting in fluorescence emission at 580 nm and a quenching of the NBD emission at 530 nm. The efficacy of energy transfer (E%) is calculated by the donor emission quenching as described in the experimental section. Classically, the distance between different pairs of probes required to obtain an efficient energy transfer is less than 10 nm and more often in the range of 2–7 nm (25). We have then compared the E% between lipids and PSC on SMBV™ and on mixtures of PSC and liposomes. The results obtained indicated that in case of SMBV™ the major part of the lipids are in close contact with the PSC (E% = 92%) whilst a much lower transfer efficiency was obtained for mixtures of PSC and liposomes in the same proportion (E% = 40%).

In order to confirm these interpretations we performed studies using Sephadex A-50 as model support. Sephadex A-50 is composed of cross-linked polysaccharide particles containing quaternary ammonium functions and have a diameter of around 100 μm. In this regard, they can be considered as a macroscopic model for nanometric cationic PSC. The Sephadex particles were treated with a mixture of lipids corresponding to the composition of SMBV™ and containing a lipophilic fluorescent probe (Hexadecyl eosin). One sample was treated using a "SMBV™ like" process and the second one by mixing Sephadex with DPPC/Cholesterol liposomes in the same proportion. The hexadecyl eosin probe is only fluorescent when solubilised in the lipid phase and no fluorescence is observed if the probe is mixed with pure PSC. Figure 3A clearly confirms that when the SMBV™ preparation process is used, a crown of lipids can be seen around the polysaccharide particles. The mixing of liposomes with the particles (Fig. 3B) does not produce the same result.

A spontaneous interaction between liposomes and Sephadex beads is clearly observable which creates some lipid aggregation at the particle surface. Due to their size (100 μm) the specific surface of Sephadex beads is quite low and lipids are in excess to the beads surface. The excess of lipids is in the form of a diffuse fluorescence in the background corresponding to free liposomes. After washing, the fluorescent crown around the Sephadex remained for particles treated by the SMBV™ process (Fig. 3C). For Sephadex/liposomes mixtures most of the fluorescence was eliminated as can be seen by the loss of contrast between the particles and the background (Fig. 3D). Some lipids remained adsorbed to the surface of the particles. These results are qualitative, but they confirm the zeta potential data interpretation and indicate that in the case of SMBV™ a stable crown of lipids is established around the polysaccharide cores.

Further confirmation was obtained by electron microscopy performed on PSC and SMBV™. First, experiments were performed on PSC by cryofracture electron microscopy. Figure 4 shows a representative micrograph of PSC on which 50–100 nm pseudo-spherical structures can be seen. Attempts to perform cryofracture on SMBV™ were unsuccessful; structures resembling PSC were poorly resolved in terms of the lipid membrane.

Negative stain transmission electron microscopy of SMBV™ shown in Fig. 5 A and B show a nearly spherical structure surrounded by a thin layer of lipids with a diameter of approximately 60 nanometers. In order to distinguish the structure of the lipid layer, an additional microscopy experiment was performed using the cryo-electron microscopy technique (CEM). The PSC

do not contain any contrast agent and are almost freely permeable to electrons as a highly hydrated hydrogel. Figure 6 shows a liposome like structure, composed of a 5 nm thickness phospholipid mono-bilayer having the same diameter as SMBV™. This suggests that the structure of SMBV is a polysaccharide nanoparticle surrounded by a mono-bilayer membrane.

IV. CONCLUSION

In this study the characterisation of polysaccharide cationic nanoparticles coated with a lipid membrane (SMBV™) was performed using various techniques.

Studies involving Fluorescence Energy Transfer and zeta potential data collection showed that the lipids are close to the polysaccharide and that they have a marked shielding effect on the surface charge. The SMBV™ structure cannot be obtained by simple adsorption of liposomes to the surface of nanoparticles.

The results showed that liposomes and cationic nanoparticles interact spontaneously but this interaction is dependent on the ionic strength whilst the SMBV™ structure remains stable under the same conditions.

Using Sephadex A-50 microparticles as a model, the results of zeta potential were confirmed and visualised by fluorescence microscopy. A crown of lipids around the particles was clearly observable using the SMBV™ preparation process.

Finally, electron microscopy showed that the SMBV™ seem to be composed of nearly spherical nanoparticles surrounded by a lipid layer. All results together indicate that this layer is a single phospholipid/cholesterol bilayer.

ACKNOWLEDGMENTS

The authors thank M. Schmutz (IGBMC, Strasbourg, France) and A. Thierry (Biovector Therapeutics, Labege, France) for performing cryo-electron Microscopy, S. Lenstch (Biovector Therapeutics, Labege, France) for her help on Fluorescence Energy Transfer experiences and M.A. Dupont (CNRS, IBCG, Toulouse, France) for performing negative stain transmission electron microscopy.

REFERENCES

1. M. Major, E. Prieur, J. F. Tocanne, D. Betbeder, and A. M. Sautereau. Characterisation and phase behaviour of phospholipide bilayers adsorbed on spherical polysaccharide nanoparticles. *Biochim. Biophys. Acta* **1327**:32–40 (1997).
2. C. Allal, S. Sixou, R. Kravtzoff, N. Soulet, G. Soula, and G. Favre. Supramolecular Biovectors (SMBV™) improve antisense inhibition of erbB-2 expression. *Br. J. Cancer* **77**:1448–1453 (1998).
3. M. Berton, S. Sixou, R. Kravtzoff, C. Dartigues, L. Imbertie, C. Allal, and G. Favre. Improved oligonucleotide uptake and stability by a new drug carrier, the Supramolecular Biovector (SMBV™). *Biochim. Biophys. Acta* **1355**:7–19 (1997).
4. D. Betbeder, R. Kravtzoff, I. De Miguel, S. Sixou, P. Pavco, and T. Jarvis. Conjugates of a particle vector and oligonucleotides, process for their preparation and pharmaceutical compositions containing them. *PCT WO 98/29557 (1998), Chem. Abstr. N° 129 (9) 105220 g*.
5. N. Castignolles, D. Betbeder, K. Ioualalem, O. Merten, C. Leclerc, D. Samain, and P. Perrin. Stabilization and enhancement of interleukine-2 in vitro bioactivity by new carriers: Supramolecular Biovectors. *Vaccine* **12**:1413–1418 (1994).
6. N. Castignolles, S. Morgeaux, C. Gontier-Jallet, D. Samain, D. Betbeder, and P. Perrin A new family of carriers (Biovectors) enhances the immunogenicity of rabies antigens. *Vaccine* **14**:1353–1360 (1996).
7. R. Kravtzoff, A. Fisher, I. de Miguel, A. Perkins, M. Major, D. Betbeder, and A. Etienne. Nasal residence time evaluation of cationic Biovector in human volunteers. *Proc. Int'. Symp. Control Rel. Bioact. Mater.* **25**:818–819 (1998).
8. D. Betbeder, A. Etienne, I. De Miguel, R. Kravtzoff, and M. Major. Mucosal administration of substances to mammals. *PCT WO 98/29099 (1998), Chem. Abstr. N° 129 (9) 113546 h.*
9. E. Prieur, D. Betbeder, F. Niedergang, M. Major, A. Alcover, J. L. Davignon, and C. Davrinche. Combination of human cytomegalovirus recombinant immediate-early protein (IE1) with 80 nm cationic Biovectors: protection from proteolysis and potentiation of presentation to CD4+ T-Cell clones in vitro. *Vaccine* **14**:511–520 (1996).
10. M. Major, R. Kravtzoff, I. De Miguel, T. Mabrouk, M. Laliberte, A. Etienne, and D. Betbeder. Biovector loaded with a trivalent split influenza vaccine administered intranasally induce a strong mucosal and seric response in mice. *Proc. Int'. Symp. Control Rel. Bioact. Mater.* **25**:820–821 (1998).
11. D. Betbeder, C. Davrinche, J. L. Davignon, and E. Prieur. Method for enhancing immunogenicity, product obtained and pharmaceutical composition. *PCT WO 96/06638, Chem. Abstr. N° 125 (1) 8470 y.*
12. N. C. Santos, M. J. E. Prieto, A. Morna-Gomes, D. Betbeder, and M. A. R. B. Castanho. Structural characterisation (shape and dimensions) and stability of polysaccharide/lipid nanoparticles. *Biopolymers* **41**:511–520 (1997).
13. N. C. Santos, A. M. A. Sousa, D. Betbeder, M. Prieto, and M. A. R. B. Castanho. Structural characterisation of organized systems of polysaccharides and phospholipids by light scattering spectroscopy and electron microscopy. *Carbohydr. Res.* **300**:31–40 (1997).
14. K. Gao and L. Huang. Solid core liposomes with encapsulated gold particles. *Biochim. Biophys. Acta* **897**:377–383 (1987).
15. T. Jin, P. Pennefather and P. I. Lee. Lipobeads: a hydrogel anchored lipid vesicle system. *FEBS Letters* **397**:70–74 (1996).
16. I. De Miguel, K. Ioualalem, M. Bonnefous, M. Peyrot, F. Nguyen, M. Cervilla, N. Soulet, R. Dirson, V. Rieumajou, L. Imbertie, C. Soler, S. Cazes, G. Favre, and D. Samain. Synthesis and characterization of Supramolecular Biovectors (SMBV™) specifically designed for the entrapment of ionic molecules. *Biochim. Biophys. Acta* **1237**:49–58 (1995).
17. D. Samain, E. Cohen, F. Nguyen, M. Peyrot, and J. L. Bec. Particulate vector useful for the transport of molecules with biological activity and process for its preparation *Eur. Pat. EP 344040 (PCT WO89/11271), Chem. Abstr. N° 112 (26) 240518 z.*
18. G. Barratt. Characterisation of colloidal drug carrier systems with zeta potential measurements. *Pharm. Tech. Eur.* **11**:25–32 (1999).
19. K. Makino, T. Yamada, M. Kimura, T. Oka, H. Ohshima, and T. Kondo. Temperature and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. *Biophys. Chem.* **41**:175–183 (1991).
20. H. Ringsdorf, B. Schlarb, and J. Venzmer. Molecular architecture and function of polymeric oriented systems: models for the study of organisation, surface recognition, and dynamics of biomembranes. *Angew. Chem. Int. Ed. Engl.* **27**:113–158 (1988).
21. A. A. Yaroslarov, E. A. Kiseliova, O. Y. Udalykh, and V. A. Kabanov. Integrity of mixed liposomes contacting a polycation depends on the negatively charged lipid content. *Langmuir* **14**:5160–5163 (1998).
22. M. N. Jones. The surface properties of phospholipid liposome systems and their characterisation. *Adv. Colloid. Interface. Sci.* **54**:93–128 (1995).
23. D. K. Struck, D. Hoekstra, and R. E. Pagano. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20**:4093–4099 (1981).
24. P. Vanderwerf and E. F. Ullman. Monitoring of phospholipid vesicle fusion by fluorescence energy transfer between membrane-bound dye labels. *Biochim. Biophys. Acta* **596**:302–314 (1980).
25. B. K. K. Fung and L. Stryer. Surface density determination in membranes by fluorescence energy transfer. *Biochemistry* **17**:5241–5248 (1978).