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Synthesis and characterization of supramolecular biovector (SMBV) specifically designed for the entrapment of ionic molecules

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Abstract

Supramolecular biovectors (SMBV) are nanoparticular drug carriers composed of an internal crosslinked solid core externally grafted with fatty acids and surrounded with a phospholipid layer. We show in this paper that the internal core can be derivatized with anionic ligands such as phosphate in order to allow the efficient entrapment of cationic molecules through a process akin to ion exchange. Synthesis of SMBV involved first a cross linking and derivatization step of polysaccharides followed by a homogenization, a drying and a regioselective acylation step. Acylated polysaccharide cores are thus obtained which can be loaded with drugs and wrapped with a phospholipid layer. The SMBVs obtained are characterized through their size, 20 nm, and their ability to filter through 0.22 μm pore size membrane. Gel permeation chromatography experiments performed with various phospholipid/acylated cores ratios indicate that SMBVs form entities distinct from liposomes and that the optimum phospholipid/acylated cores ratio for this specific type of SMBVs is close to 100%. The supramolecular structure of SMBVs and in particular the spatial proximity between acylated cores and phospholipids is demonstrated through resonance energy transfer experiments. The drug loading capability of SMBVs is illustrated by the preparation of gentamicin and doxorubicin loaded SMBV. The therapeutic potential of SMBVs is then discussed notably in the light of a possible biomimetism with low density lipoproteins (LDL).

Keywords: Nanoparticle; Polysaccharide; Drug carrier; Phospholipid; Gentamicin; Doxorubicin

1. Introduction

The therapeutic interest of nanoparticular and nanovesicular drug carriers for the targeting of pharmacologically active drugs has been largely documented in the general and patent literature [1]. Although an impressive amount of work has been devoted to the study of these systems and notably of liposomes [2,3], practical applications are still extremely limited. This situation stems from the fact that existing carriers still present a number of serious shortcomings such as shelf and plasmatic stability, lack of reproducibility, low drug entrapment capability, inadequate pharmaceutical definition and, in some cases, lack of biocompatibility. In addition, none of the existing synthetic carriers have been shown to have a limited ability to cross the blood vessel linings and therefore, their targeting capabilities following intravenous injections are mainly restricted to the blood compartment.

To solve this last problem, the use of natural carriers such as LDLs has been suggested [4]. Indeed, LDLs present some very interesting properties such as systemic diffusion from the blood stream and the fact that they can be internalized in specific cells through a high affinity receptor mediated endocytosis [5]. We [6–8] and others [9–11] have proposed LDLs as a useful discriminatory vehicle for the delivery of cytotoxic drugs to tumour cells on the basis of a higher uptake of LDLs by these tissues.

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Because of their lipidic structure, LDLs have however a drug entrapment capability which is restricted to lipophilic molecules. In addition, attempts to achieve drug loading ratios compatible with therapeutic applications have generally led to alterations of the physical integrity of the LDLs and loss of their original diffusion and targeting properties [12]. It would be, therefore, particularly interesting to dispose of biomimetic synthetic particles with the ability to entrap efficiently hydrophilic molecules while maintaining the outstanding metabolic properties of LDLs. We have reported [13] previously the design and the synthesis of a new family of synthetic nanoparticles biomimetic of the lipidic part of LDL, the supra molecular biovectors (SMBVs).

We wish now to address the problem of ionic drugs entrapment in SMBVs and we are describing in this paper, the synthesis and characterization of SMBVs specially designed for this purpose. The SMBVs are nanoparticles composed of an internal core of cross linked polysaccharide externally grafted with fatty acid and surrounded by a layer of phospholipids. SMBVs are then constituted by a hydrophilic internal core surrounded by a lipophilic external layer and have thus the theoretical ability to entrap both hydrophilic and lipophilic molecules. The internal volume of small diameter particles is however very small and conventional entrapment techniques are thus bound to be poorly efficient in terms of entrapment yield and entrapment ratio. We have thus selected instead to apprehend the problem of drug entrapment in terms of partition coefficient of the drug between SMBVs and the surrounding aqueous medium. The selection of SMBVs with suitable partition coefficient may be approached using chromatographic considerations. Chromatographic matrices are usually constituted of microporous particles derived with specific ligands. Numerous ligands have been employed for the synthesis of these matrices which therefore exhibit a large array of possibilities of physico-chemical interactions. The chromatographic behaviour of a molecule and notably its retention on a column is directly linked to its partition coefficient, K, between the chromatographic matrix and the surrounding medium, i.e., the mobile phase [14]. This is notably expressed by the measure of the capacity factor k' which is equal to $K \times (V_{sp}/V_{mp})$ where V_{sp} is the volume of the stationary phase and V_{mp} the volume of the mobile phase, k' is also equal to $(V_r - V_0)/V_0$, where V_r is the elution volume of the solute and V_0 is the void volume of the column. Materials with suitable partition coefficient, in a given medium, may, then, be easily deduced from chromatographic data.

Analysis of drug chromatographic data [15] indicates that, indeed, water soluble ionic drugs are, as expected, only weakly retained on hydrophobic reverse phase columns and would have, accordingly, unfavourable partition coefficients in LDLs. Those drugs, however, when sufficiently ionic, are found to be strongly retained on opposite charge ion exchange columns. For those drugs, the use of materials analogous to ion exchange matrices might then solve the problem of the entrapment efficiency. This principle has, in fact, already been used for the entrapment of ionic cytotoxic agents and notably doxorubicin within ion exchange microspheres [16,17] and acrylic acid nanoparticles [18].

We have then undertaken to synthesize a specific type of SMBVs characterized by the presence of ionic ligands grafted upon the polysaccharide network. We describe here the synthesis of anionic SMBVs, their characterization and their potential for cationic drugs entrapment.

2. Materials and methods

2.1. Chemicals

Soluble starch (Prolabo, France); $POCl_3$ (Prolabo, France), palmitoyl chloride (Fluka); egg yolk phosphatidylcholine (EYPC) (Lipoid, Germany); cholesterol (Sigma, France); octyl glucopyranoside (OGP) (Sigma); rhodamine isothiocyanate (Molecular Probe); rhodamine dipalmitoylphosphatidylethanolamine (Molecular Probe); NBD dipalmitoylphosphatidylethanolamine (Molecular Probe); dicyclohexylcarbodiimide (DCCI) (Fluka); [^{14}C]palmitic acid 53.7 mCi/mmol (CEA Saclay, France); 4-dimethylaminopyridine (DMAP) (Fluka, France); scintillation liquid cocktail (Ready safe Beckman, France); gentamicin (Sigma); doxorubicin (Carlo Erba). All others chemicals and solvents were commercially available and at least reagent grade.

2.2. Equipments

Radioactivity measurements were performed on a Packard Tri-Carb 460 CD (Packard Instrument SA, France). The HPLC system was composed of a model 302 Gilson pump, a Rheodyne model 71-25 injector and a model 802 C Gilson manometric module. Detection was performed either with a refractive index detector Gilson, a fluorescent detector Shimadzu (RF 551) or an evaporating light scattering detector (DDL 21 Eurosep Instrument). Energy transfer experiments were performed with a F 2000 Hitachi spectrofluorimeter (Tokyo, Japan). A Coulter N4 MD nanoparticles analyser (Coultronics, Margency, France) was used to determine the diameter of liposomes and SMBVs. Bath sonications were performed with a Transonic 460 (Elma) and probe sonications with a Vibracell Instrument (Bioblock). High pressure homogenization was carried out with a Minilab (Rannies, Denmark).

2.3. Preparation of SMBVs

SMBVs are synthesized as described by Samain et al. [19].

Step 1: preparation of 20 nm anionic polysaccharide particles

100 g of soluble starch are introduced in a 3 l reaction vessel fitted with mechanical stirring. 500 ml of NaOH 2 M are then introduced in the flask and the mixture is stirred until complete homogenization is obtained. The temperature of the flask is then adjusted to 4° C and $POCl_3$ (50 g, 0.32 M) is added dropwise together with a solution of NaOH 10 M (150 ml). When the addition is completed, the reaction is stirred for an additional 15 min then brought to pH 7 through the addition of chlorohydric acid. The resulting gel is diluted in 2 l of distilled water and homogenized at 800 bars with a high pressure homogenizer, until particles with a diameter around 20 nm are obtained. The polysaccharide particles are then precipitated by the addition of 2 l of EtOH, filtered, washed with 2 × 500 ml of EtOH, suspended in 4 l distilled water or 7 l of a 20 g/l NH_4HCO_3 solution and freeze-dried to afford 70 g of dry polysaccharide cores.

Step 2: preparation of acylated cores

10 g of the dry polysaccharide cores obtained previously are introduced in a 100 ml round bottom flask fitted with magnetic stirring and a reflux condenser and containing 1.7 g of palmitoyl chloride dissolved in 30 ml of CH_2Cl_2. The reaction is stirred energetically and refluxed overnight. The solvent is then evaporated under vacuum with a rotary evaporator. The residue is suspended in 50 ml of diethyl ether, filtered, washed with 2 × 50 ml of ethanol and dried under vacuum to afford 10.3 g of acylated particles. 10 g of the acylated cores are then resuspended in 300 ml of distilled water or in 300 ml of a 20 g/l ammonium bicarbonate solution, freeze-dried, resuspended in 50 ml of CH_2Cl_2 and reacylated as described previously. The material obtained is then submitted to a third and a fourth acylation cycle.

The rhodamine labelled acylated cores are synthesized from acylated cores as follows. Rhodamine isothiocyanate (1 mg) dissolved in 10 μl of DMF is added to acylated cores (100 mg), dispersed in 2 ml of Na_2CO_3, 50 mM, pH 10 buffer. The reaction mixture is maintained for 15 hours at room temperature in the dark. The rhodamine labelled acylated cores are then precipitated with excess EtOH, filtered, washed with EtOH until complete elimination of excess free rhodamine and dried under vacuum.

For the preparation of the ^{14}C-radiolabelled core, DCCI (0.93 mg) dissolved in (500 μl) of CH_2Cl_2 is added to [^{14}C]palmitic acid (500 μCi) in a well-stoppered 5 ml flask fitted with magnetic stirring. The reaction is then stirred vigorously for 24 h at 4° C to afford [^{14}C]palmitic acid anhydride which is used without further purification. DMAP (0.55 mg) and acylated cores (100 mg) are added to the anhydride solution and the reaction mixture is stirred vigorously at room temperature for 24 h. The ^{14}C-acylated cores are then filtered, washed with 3 × 5 ml of diethyl ether and 3 × 5 ml of ethanol, resuspended in water and freeze-dried.

Step 3: establishment of the phospholipid layer

The acylated cores (5 mg) are mixed with phospholipids (5 mg) and thoroughly dispersed in 2 ml of 50 mM OGP. The resulting mixture is then diluted rapidly with 4 ml of water and submitted to extensive dialysis at 4° C. For the control experiments, liposomes are prepared through the same process.

Fluorescent labelled SMBVs are prepared by the detergent dialysis technique using the previously obtained rhodamine labelled acylated cores or a phospholipid composition containing 0.1% (w/w) of NBD phosphatidylethanolamine or rhodamine phosphatidylethanolamine.

^{14}C-radiolabelled SMBVs are prepared from ^{14}C-radiolabelled acylated cores, using the same detergent dialysis technique as the one described for the preparation of cold SMBVs.

After extensive dialysis, the fluorescent and ^{14}C-labelled SMBVs are submitted to a last purification step through Sephadex PD10 chromatography.

2.4. Analytical characterization of SMBVs

Determination of the ratio of phosphate grafting

The ratio of phosphate grafting is determined by titration of the polysaccharide particles with 0.1 M NaOH, using phenolphthaleine as a pH indicator.

Quantification of fatty acids

The percentage of fatty acids covalently linked to the polysaccharide cores is determined by HPLC after saponification. 100 mg of acylated cores are first dispersed by sonication in 10 ml dichloromethane. 10 ml of methanol and 2 ml of NaOH 2 M are then added. The suspension obtained is then stirred during three days. Hydrolyzed fatty acids are extracted by dichloromethane after acidification by HCl 2 M. The organic phase is evaporated and the residue is solubilized in 10 ml of methanol. Quantification is achieved by HPLC with evaporating light scattering detection. The HPLC system used is a reverse phase system (Nucleosil C18 5 μm, 15 cm) with a mobile phase 0.1% TFA in methanol. The flow rate is 1 ml/min.

Size determination

The size of SMBVs is measured with a photon correlation laser light-scattering method. Mean value and standard deviation for the overall particle size distribution are determined. It has not proved possible to measure the size of fluorescent-labelled SMBV and doxorubicin loaded SMBV due to the interference of the fluorescence with the measurement.

Gel permeation chromatography

The rhodamine fluorescent labelled SMBVs and the rhodamine fluorescent labelled liposomes are analyzed by

column chromatography on a TSK PW 6000 column 30 cm (Tokyo Soda, Tokyo, Japan) as previously described [20,21]. All chromatographic runs are made at room temperature and with a spectrofluorometric detector. The mobile phase consisted of Tris-HCl 10 mM pH 7.4, NaCl 120 mM, sodium azide 0.2% with a 0.3 ml/min flow rate. Samples are detected in the eluate by fluorescence emission at 580 nm after excitation at 520 nm.

Resonance fluorescence energy transfer study

These studies have been carried out on SMBVs prepared from rhodamine labelled acylated cores and from NBD labelled phospholipids. The amount of energy transfer between the two fluorophores is measured by comparison with mixtures of rhodamine labelled acylated cores and liposomes prepared independently from NBD labelled phospholipids. The fluorescence emission of NBD at 530 nm following excitation at 470 nm, was measured for the various preparations of SMBVs, acylated cores and liposomes. The efficiency of energy transfer (E) was calculated using the following equation: $E = (1 - F/F_0) \times 100$ where F is the fluorescence of the NBD in the assay in presence of rhodamine and F_0 is the fluorescence of NBD in a similar assay in absence of rhodamine. The energy transfer was also followed through the appearance of the rhodamine fluorescence emission at 580 nm following NBD excitation at 470 nm.

2.5. Drugs entrapment within SMBVs

Gentamicin

Gentamicin (10.5 mg) is added to 35 mg of anionic acylated cores, dispersed in 1 ml distilled water. The preparation is stirred overnight, and then dispersed in 14 ml of 50 mM OGP containing 28 mg of EYPC and 7 mg of cholesterol. The resulting mixture is then brought to pH 8.5, through the addition of diluted NaOH, and diluted rapidly with 28 ml of pH 8.5 aqueous solution. Non entrapped gentamicin and the detergent are then removed by sequential ultrafiltration. The amount of non entrapped gentamicin is determined by difference through the measure of the amount of free gentamicin determined by antimicrobial assay. Briefly, the assay is performed through the measure of the area of inhibition of the growth of BS (ATCC6633) grown on an agar medium (bio-gelytone 6 g, yeast extract 3 g, beef extract 1.5 g, agar 15 g for a liter of distilled water. The pH of the medium is then adjusted to 7).

Doxorubicin

Doxorubicin (200 mg) dissolved into 10 ml of distilled water is slowly added to a suspension of anionic acylated cores (500 mg) in 10 ml of ethanol. The mixture obtained is then stirred for two hours at room temperature and protected from light. Ethanol (10 ml) is then added to the suspension, the acylated cores are recovered by centrifugation (10000 × g-15 min) and dried. The dry residue of doxorubicin loaded acetylated cores is taken up with 175 ml of 50 mM OGP containing 375 mg of 80:20 (w/w) EYPC/cholesterol and dispersed in an ultrasonic bath. The suspension obtained is then injected under an ultrasonic probe into 700 ml of distilled water. The SMBVs loaded with doxorubicin thereby formed are dialyzed extensively at 4° C to remove the detergent and then sterilized by filtration on 0.2 μm filters. The doxorubicin is assayed by HPLC as previously described [22] except that the detection is performed with an UV detector at 240 nm. The incorporated Doxorubicin is determined after release by incubation of the SMBVs in a 2 M NaH_2PO_4/ethanol (70:30) solution and ultrafiltration through a 50 kDa membrane.

3. Results

3.1. Synthesis of SMBVs

The crosslinking of soluble starch, characterized by a mean molecular weight of 4000, with $POCl_3$ followed by a homogenization step, gives rise with 70% yield to a monodispersed population of about 20 nm particles (Fig. 1). The ionic functionalization (1.53 mmol/g) of these polysaccharide cores was determined by titration (data not shown) [23]. These results indicate the presence of a phosphate group for every 3.5 glucose residues.

The acylation of these polysaccharide cores was carried out in CH_2Cl_2 after freeze drying performed conventionally or in the presence of NH_4HCO_3. The polysaccharide cores were submitted to several cycles of acylation separated by hydrating and drying steps. The results are presented in Fig. 2 and indicate that only a slight percentage of fatty acids can be grafted in the absence of NH_4HCO_3, even after several acylation steps. When the drying, however, is conducted in presence of NH_4HCO_3, the resulting percentage of fatty acids is higher and increases with the

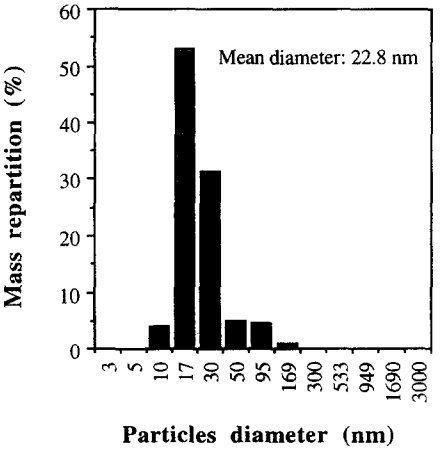

Fig. 1. Size histogram of anionic polysaccharide cores.

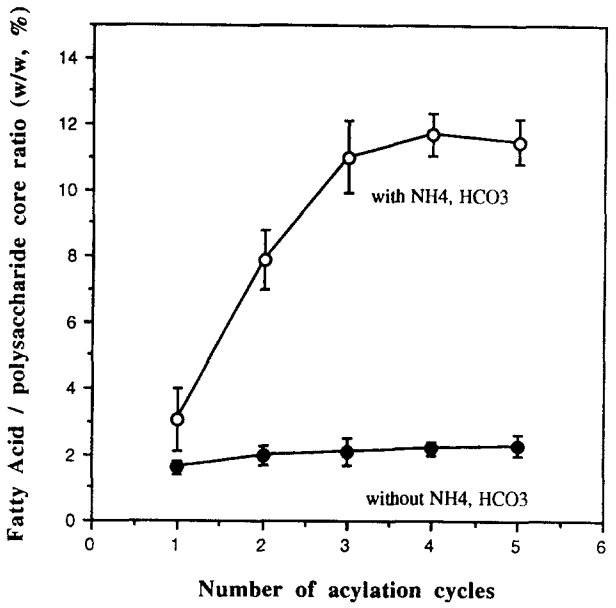

Fig. 2. Dependence of the fatty acid/polysaccharide cores ratio upon the number of acylation cycles and upon the presence of NH_4HCO_3 during drying. After the acylation step, the particle are washed and the amount of fatty acids grafted upon the polysaccharide cores was determined by HPLC following extensive saponification of the acylated particles as described in Materials and methods. Presented data are averages of triplicate measurement.

number of acylations until reaching a plateau after the third.

The phospholipid layer was established through the detergent dialysis technique using the dialysable detergent, octyl glucopyranoside [24]. The resulting preparations obtained with various phospholipids/acylated cores ratios, were then analyzed by photon correlation light scattering and by gel permeation chromatography.

3.2. Determination of the optimum phospholipid / acylated core ratio

The diameter of SMBVs prepared with various polysaccharide/acylated core ratios and control liposomes prepared with the same technique was measured by photon correlation light scattering. The results presented in Table 1 indicate that, in the presence of phospholipids, 20 nm species are obtained. Liposomes prepared in the same conditions with the same phospholipidic composition, exhibit however also a 20 nm diameter and therefore may then be also present in the preparation. It is thus not possible to deduce from this experiment the amount of phospholipids actually associated with the acylated cores.

The chromatographic analysis of SMBV was performed on a TSK PW 6000 column able to include particles with a diameter range between 10 and 500 nm. Fluorescent labelled SMBVs and fluorescent labelled liposomes were prepared by using phospholipid composition containing 0.1% of rhodamine phosphatidylethanolamine. The results

Table 1
Study of SMBVs as a function of the phospholipid/acylated core ratio

Phospholipid/acylated core ratio (w/w)	Size diameter (nm)
0	> 1000
0.50	18 ± 4
0.75	19 ± 5
1.00	18 ± 3
1.50	19 ± 5
2.00	19 ± 5
2.50	20 ± 5
Control liposomes	20 ± 5

SMBVs were prepared with various phospholipid/acylated core ratios and the diameter of the resulting preparations was measured by photon correlation light scattering. The phospholipid composition used was EYPC/cholesterol (80:20, w/w). The results indicate that the presence of phospholipids is necessary for the obtention of 20 nm entities ($n = 3$).

are presented in Fig. 3. The trace A indicates that liposomes are eluted as a major peak with a 33 min retention time. The results presented on trace E with SMBVs prepared with a phospholipid/acylated cores ratio of only 0.5 indicate that the 33 min peak of liposomes is not observed

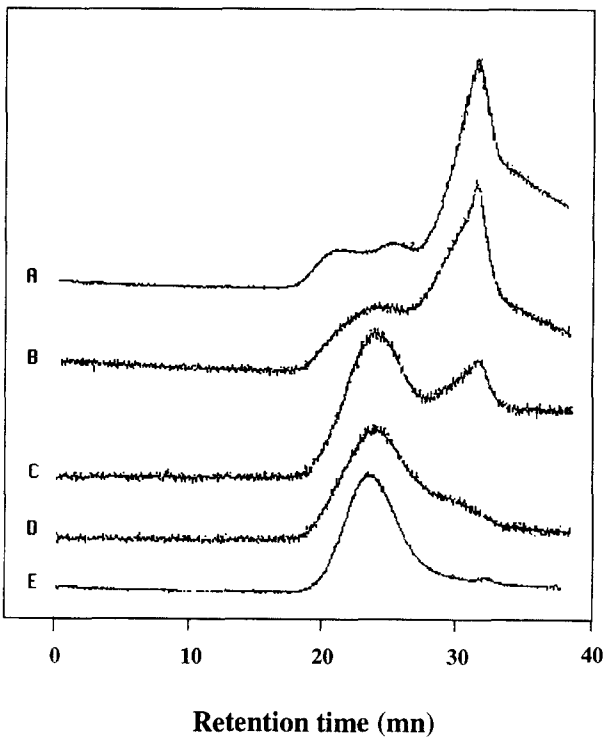

Fig. 3. Gel permeation analysis of fluorescence labelled SMBVs. SMBVs prepared with various rhodamine labelled phospholipids/acylated cores ratios and control liposomes are analyzed by high performance gel permeation chromatography. Column TSK G 6000 PW; mobile phase: Tris-HCl 10 mM, NaCl 120 mM, azide 0.2 g/l, pH 7.4, fluorescence detection (exc. 540 nm, em. 580 nm). In order to allow a better comparison, the various chromatographic profiles are stacked upon each other. Trace A, control liposome. Trace B, acylated core ratio: 3. Trace C, acylated core ratio: 2. Trace D, acylated core ratio: 1. Trace E, acylated core ration: 0.5. The results indicate that liposomes appear above a phospholipid/acylated cores ratio of 1.

Table 2
Filtration yields on 0.45 and 0.22 μm membrane of ^{14}C-labelled SMBVs

Before filtration		After filtration	
		0.45 μm	0.22 μm
SMBV ^{14}C (dpm)	3055 ± 155	3010 ± 175	2687 ± 128
SMBV recovery (%)	–	100	89.5

^{14}C-labelled SMBVs were filtered through 0.45 and 0.22 μm membranes. The radioactivity of the preparations was measured before and after filtration ($n = 3$).

and that, instead, a single peak is observed with a 25 min retention time. These results suggest that liposomes are not present in this preparation and that only SMBVs are observed. These results are confirmed by the study of traces D, C and B which analyzed SMBVs prepared with increasing phospholipid/acylated cores ratios. As expected, increased liposome peak areas corresponding to the excess of phospholipids are observed in these preparations. Trace D with a phospholipid/acylated cores ratio of 1 shows the presence of a small amount of liposomes. Traces C and B with a phospholipid/acylated cores ratio of 2 and 3 show the presence of a much larger amount of liposomes. These results suggest that the optimal ratio between phospholipids and acylated cores for these specific SMBSs is close to 1.

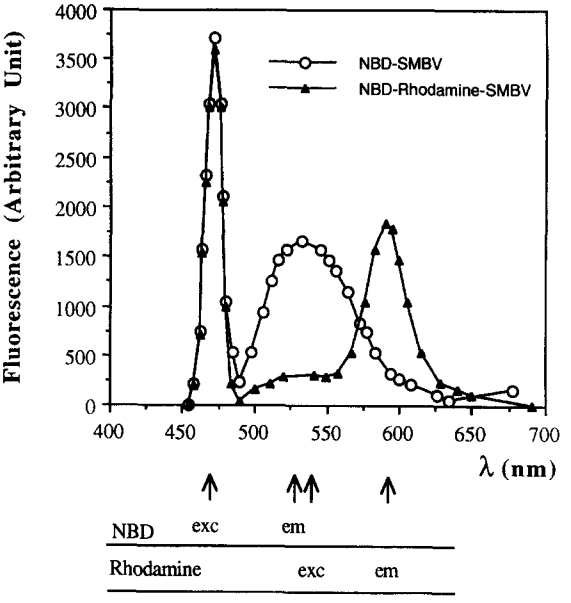

Fig. 4. Resonance energy transfer experiments. NBD-SMBV are prepared from acylated cores and NBD phospholipids. NBD-rhodamine-SMBV are prepared from rhodamine acylated cores and NBD phospholipids. Excitation of NBD-SMBV at 470 nm leads to the normal NBD emission at 530 nm while excitation of NBD-rhodamine-SMBV leads to a quenching of NBD emission and to a rhodamine emission at 580 nm. Presented data are typical of triplicate experiments.

3.3. SMBVs characterization

The SMBVs obtained previously were characterized by their ability to filter through 0.45 and 0.22 μm membrane, their stability and by fluorescence energy transfer studies.

The filtration yield of SMBVs on a 0.45 and 0.22 μm membrane has been investigated through the use of radiolabelled acylated cores. The labelling was carried out by performing an additional acylation step with [^{14}C]palmitic acid. The filtration yield was determined by measuring the radioactivity of the preparation before and after filtration on the membranes. The results are presented in Table 2 and indicate an almost quantitative filtration yield. These results suggest that filtration through 0.22 μm membranes may be used for the obtention of sterile SMBVs preparations.

SMBVs and liposomes samples were prepared and sterilized by 0.22 μm filtration and kept at 4 and 20° C. The size of the dispersions was measured at regular intervals and the results are presented in Table 3. At both temperatures, SMBVs are remarkably stable showing no sign of size increase or aggregation over time. On the other hand, liposomes appear to aggregate heavily after a few months.

Fluorescence energy transfer studies have been carried out on SMBVs prepared from rhodamine labelled acylated cores and from NBD labelled phospholipids. The amount of energy transfer between the two fluorophores is quantitatively measured by following the decrease in the NBD

Table 3
Comparative SMBVs and liposomes stability as a function of time

		Size diameter (nm); time (months)							
		1	2	3	5	6	7	8	12
4° C	SMBVs	18 ± 4	18 ± 4	19 ± 3	18 ± 4	19 ± 5	19 ± 3	20 ± 4	23 ± 4
	liposomes	21 ± 5	31 ± 5	32 ± 6	Agg [a]				
25° C	SMBVs	19 ± 4	19 ± 3	18 ± 3	n.d. [b]	n.d. [b]	22 ± 4	20 ± 4	n.d. [b]
	liposomes	22 ± 3	22 ± 4	Agg [a]					

SMBVs and liposomes were prepared with the same phospholipid composition EYPC/cholesterol (80:20, w/w), the same lipidic concentration (0.5 mg/ml), the same process (dialysis of detergent). The preparations were measured at regular intervals by photon correlation light scattering. The phospholipid/acylated core ratio used for the preparation of SMBVs was 1.5 (w/w). SMBVs appear to exhibit a superior stability compared to liposomes ($n = 3$).

[a] Aggregates.

[b] Not determined.

emission at 530 nm following NBD excitation at 470 nm. The results are compared with those obtained with mixtures of rhodamine-labelled acylated cores with liposomes prepared from NBD labelled phospholipids. The results obtained indicate that up to 12% of energy transfer is observed with SMBVs while only 1.2% is observed with the mixture of acylated cores and liposomes. These results are in good agreement with the appearance of the rhodamine emission at 580 nm following NBD excitation at 470 nm (Fig. 4)

3.4. Drugs entrapment

Drug entrapment is performed through a single incubation step between acylated cores and the drug in aqueous or ethanol/water solutions. The incubation step is followed by a codispersion with phospholipids and OGP, then by a rapid dilution under the critical micellar concentration (CMC) of the detergent and finally by an extensive dialysis. The results obtained with gentamicin and doxorubicin are presented in Table 4. These results indicate that an almost quantitative entrapment yield can be obtained with gentamicin with a 14% entrapment ratio. Doxorubicin, on the other hand, is entrapped with a lower efficiency with a 70% yield and with a 10% entrapment ratio.

4. Discussion

The design of a drug carrier intended for therapeutic uses and notably for parenteral administration, has to follow a strict set of guidelines concerning toxicity, possibility of industrial manufacturing, structure characterization, stability and drug entrapment efficiency. The results obtained with SMBVs will then be discussed with these considerations in mind.

Although several starting materials, such as acrylic acid [18] or cellulose [23] are available to prepare ionic matrices, we decided, early on, that the use of those materials would inevitably arises concerns about their toxicity and biodegradability and we have accordingly selected a known biocompatible material, starch, for the synthesis of 20 nm ionic cores. Beside its biocompatibility, starch, like all polysaccharides, exhibits a high number of reactive hydroxyl groups allowing easy chemical derivatization [25] such as cross linking or acylation and appears then to be a particularly appropriate material for the synthesis of SMBVs. Starch is composed of homopolymers of glucose with various degree of branching [26] and thus cross linking is necessary in order to achieve a stable three-dimensional structure. Numerous methods have been described to achieve cross linking of polysaccharides, the best known being through the use of epichlorhydrin [27] employed notably in the preparation of Sephadex particles. Epichlorhydrin gives rise to a glycerol-ether link between two glucose units and concern may be raised about the biodegradability of the ether linkage. In addition the resulting product is neutral and has to undergo another reaction for the grafting of ionic ligands. We have thus selected, instead, $POCl_3$ which can act both as a cross linking and a functionalizing agent (Fig. 5).

This reaction gives rise to both phosphodiester and monophosphate bonds. These bonds exhibit good chemical stability while remaining susceptible to enzymatic hydrolysis [28]. The resulting material has therefore good chemical and physical stability while remaining biodegradable.The reaction performed in alkaline conditions gives rise to a gel of polymerized starch which structure is directly dependent upon the nature of the starting polysaccharide and upon the cross linking conditions. The mechanical strength, the mesh size and the ionic character can thus be adjusted in view of a specific application. The conditions selected give rise to a polymerized gel which affords directly monodispersed 20 nm population, after a high pressure homogenization step, avoiding then the need for any further sizing treatment. The 20 nm particles obtained can be easily washed free of salts and secondary material by precipitation with methanol. Both the cross linking reaction of starch and the high pressure homogenization step present strong similarities with analogous reactions performed in the modified starch [29] and dairy industries [30]. This similarity augurs favorably of the ruggedness of the process and of the availability of industrial processing equipment.

Acylation reactions of polysaccharides are normally carried out in polar solvents such as pyridine, DMF or DMSO [31] which have the ability to solubilize both the polysaccharide and the fatty acid reagent. In these conditions, the acylation is homogeneous within the polysaccharide matrix and is not restricted to the outer part of the

Table 4
Efficiency of the gentamicin and doxorubicin entrapment within SMBV

	Drug [a] (mg)	Ac. core [a] (mg)	Phospholipids [a] (mg)	Non entrappeddrug (mg)	Entrapment yield (%)	Drug/SMBV (w/w)	SMBVs diameter (nm)	
							before loading	after loading
Gentamicin	10.5	35	35	< 0.5	95	0.14	21 ± 5	21 ± 5
Doxorubicin	150	500	500	45 ± 3	70	0.11	21 ± 5	n.d. [b]

[a] Amount initially involved.
[b] Not determined.

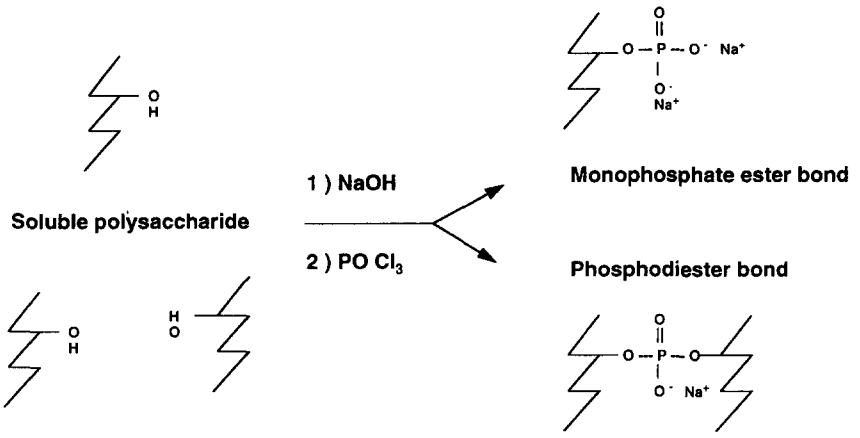

Fig. 5. Reaction of polysaccharides with $POCl_3$. The reaction gives rise to both monophosphate and phosphodiester bonds.

particle. If, on the other hand, the reaction is carried out in a non polar solvent, with the ability to solubilize the fatty acid reagent, but not the polysaccharide, then the reaction is obviously limited to the surface of the polysaccharide particle. This is notably confirmed by the results of the acylation performed in CH_2Cl_2 with polysaccharide particles dried with conventional techniques and which indicate a very low amount of grafted fatty acids even after several acylation steps (Fig. 2). This very low amount can be explained, on one hand, by the inability of the non-polar solvent to solubilize the polysaccharide particles and on the other hand by the fact that the particles, when dried conventionally, are heavily aggregated and the solvent is thus prevented to have access to the entire surface area of the particles (Fig. 6). Non-aggregative drying obtained through the addition of NH_4HCO_3 allows a better access of the solvent to the surface of the particles and gives rise to a corresponding higher percentage of fatty acid grafted, notably after several acylation steps.

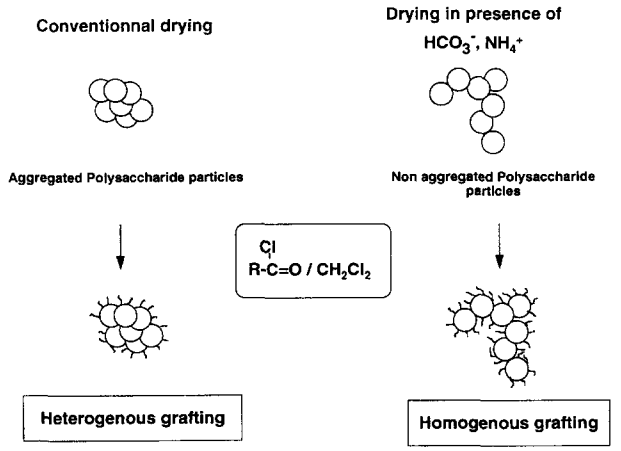

Fig. 6. Schematic representation of external acylation of polysaccharide cores performed after conventional drying or after drying in the presence of NH_4HCO_3.

The lack of stability has proved to be a major difficulty in the use of liposomes [32]. We therefore undertook a study to evaluate the stability of SMBVs, notably by comparison with liposomes prepared in the same conditions with the same technique and with the same phospholipid composition. The increased stability of SMBVs compared to liposomes is probably due to the structuring effect of the lipidic layer of the polymeric core [33] and augurs favourably for their pharmaceutical applications.

SMBVs are supramolecular assemblies involving an internal acylated core and an external phospholipidic layer. The key issue is then to establish that these two components are indeed associated together and not merely present as separate entities. A first information can be drawn from the size measurement of SMBVs as a function of the phospholipids/acylated cores ratio. These results indicate that in the absence of phospholipids, acylated cores alone aggregate heavily. The presence of amphiphilic phospholipids is thus essential to ensure the dispersion of SMBVs. These results are confirmed by the resonance energy transfer experiments. These experiments rely upon the interactions which occur between two fluorophores if the emission band of one, the energy donor, overlaps with the excitation band of the second, the energy acceptor. When the two probes exist in close spatial proximity, the energy from a photon absorbed by the energy donor can be transferred to the energy acceptor which will then fluoresce as though it had been excited directly. The efficiency of the fluorescence energy transfer between two matching fluorophores is dependent upon their spatial separation [34] and the measure of this efficiency provides thus a means to evaluate the distance between the fluorophores. This approach has notably been used successfully to monitor membrane fusion [35]. In our case, the results obtained with SMBVs bearing simultaneously a rhodamine fluorophore grafted upon the acylated cores and a NBD fluorophore incorporated in the phospholipid layer indicate

that rhodamine fluorescence at 580 nm is indeed observed after NBD excitation at 470 nm. They also indicate that the amount of energy transfer measured through the decrease in the NBD emission is much larger than the amount of energy transfer observed with a simple mixture of rhodamine acylated cores and NBD liposomes. These results confirm that the acylated cores and the phospholipids are actually in close spatial proximity, in agreement with the proposed SMBV structure. These results, however, do not guarantee that some phospholipids may not be present as liposomes. Indeed the surface of the SMBVs is obviously limited and, in the case of the use of an excess of phospholipids, liposomes should necessary also be present in the preparations. The results obtained with the gel permeation analysis of SMBVs as a function of the phospholipids/acylated core ratio, clearly show that liposomes are significantly absent from the preparations when the phospholipid/acylated core ratio is lower than 100% and that they appear above this value.

SMBVs have been designed through their internal ionic core to achieve good drug entrapment. The SMBVs described in this paper are derived with anionic phosphate groups and are thus particularly well suited for the entrapment of cationic drugs. Similarly, positively derivatized SMBVs will be appropriate for the entrapment of anionic drugs. Because of the similarity between this entrapment mechanism and ion exchange [16], the aptitude of a drug to be satisfactorily entrapped within SMBVs and the stability of the entrapment could be initially approached through the analysis of its ion exchange chromatographic behaviour. Actual entrapment capability and stability within SMBVs are bound however to differ somewhat according to the nature of the drug because of the presence of the lipidic membrane which brings the possibility of hydrophobic interactions. Numerous drugs of various size and nature can be entrapped efficiently within SMBVs.

The work performed with gentamicin, indicate that the drug, because of its polycationic structure, has a very strong affinity for the anionic cores of SMBVs, and gives rise to an extremely efficient entrapment. It has indeed proved difficult to quantatively release gentamicin from SMBVs and the determination of the entrapment had to be done by measuring the amount of non entrapped drug. It is also interesting to note that the loading of the drug does not modify the diameter of the biovector and hence should not affect its biodistribution. The strength of the interactions between gentamicin and SMBVs will probably ensure satisfactory entrapment stability of the antibiotic in plasm and its slow release only after cell internalization and lysosomal degradation of the carrier. Because of the high toxicity of aminoglycosides and because of their poor efficiency for achieving high intracellular concentration, this property could be used advantageously in the treatment of aminoglycoside sensitive intracellular disease [36]. The work performed with doxorubicin indicated that this monocationic drug can also be readily entrapped within SMBVs, although with a lower affinity compared to gentamicin. Interestingly, doxorubicin can be released from SMBVs using high ionic strength buffer and HPLC analysis did not indicate any degradation of the molecule.

The use of vesicular and particular drug carriers has been hampered for a long time by their rapid recognition and removal by cells of the mononuclear phagocytic system (MPS). Although sterically stabilized liposomes with long circulation time have been described [37], the presence of phospholipids with special head groups such as PEG PE [38–42] may interfere with the recognition process of ligands anchored at the surface of the particles. It is known however that size is a key parameter in phagocytosis and that larger particles are taken up at a much higher rate than smaller particles [39]. SMBVs with their small diameter comparable to LDL, might provide a way to circumvent this problem and to allow the escape from the MPS while ensuring efficient targeting.

Another central aspect regarding the feasibility of particles as drug carriers is whether they are able to cross the anatomical barriers such as the capillary walls, which separate the extravascular compartment from the blood stream. Blood capillaries are classified into three different groups, continuous, fenestrated and sinusoidal. Only the sinusoidal ones offer sufficiently large gaps for drug carriers such as liposomes to penetrate the endothelium. Indeed, the ability of some liposome formulations to accumulate in transplantable human tumors has been attributed to increased microvascular permeability [40]. Thanks to their small size analogous to LDLs, SMBV might be capable of diffusing through fenestrated capillaries as well, increasing the probability of targeting cells in the extravascular compartment.

The similarity between SMBV and the lipidic part of LDL should also allow the preparation of ApoB-SMBV conjugates. Indeed, it has been reported that ApoB could be extracted from LDL through a cold hexane and detergent technique while retaining its native conformation and properties after conjugation with liposomes [41]. It is anticipated that since LDL/antineoplastic drug complexes have proven to be an effective drug carrier in vivo and in vitro [35–37], SMBV Apo B should also prove to be an useful carrier for ionic drug targeting.

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