Improved oligonucleotide uptake and stability by a new drug carrier, the SupraMolecular BioVector (SMBV)

Myriam Berton a, Sophie Sixou a, Roger Kravtzoff b, Claire Dartigues b, Laurent Imbertie b, Cuider Allal a, Gilles Favre a, *

a EA / UPRES 2048, Laboratoire d’Oncologie Cellulaire et Moléculaire, Faculté des Sciences Pharmaceutiques and Centre Claudius Regaud, 20–24 rue du Pont St Pierre, 31052 Toulouse Cedex, France
b Biovector Therapeutics, Chemin du Chêne Vert, BP 169, 31676 Labège Cedex, France

Received 21 March 1996; revised 8 July 1996; accepted 9 July 1996

Abstract

Antisense oligodeoxynucleotides are potential therapeutic agents, but their development is still limited by both a poor cellular uptake and a high degradation rate in biological media. The strategy that we propose to face these problems is to use small synthetic carriers, around 30 nm diameter, the SupraMolecular BioVectors (SMBV).

We used positively charged SMBV and settled the ionic incorporation of negatively charged oligonucleotides into these carriers. A minimal leakage of 10% of total incorporated oligonucleotides was then measured during two months.

Both protection and uptake of oligonucleotides were then analyzed. On the one hand, we showed that the incorporation of oligonucleotides into the selected SMBV allows to significantly increase, 8 times, their half-life, in cell growth medium. On the other hand, the internalization of the SMBV, into cells, by an endosomal pathway has been characterized. The essential point is that the SMBV uptake elicits the simultaneous oligonucleotide uptake. The oligonucleotide amount that goes through cells within 5 h can be up to 30 times higher than for free oligonucleotides and the fraction of oligonucleotides that is present in the cytosol is increased up to 10 fold after incorporation into the SMBV. This study demonstrates the ability of SMBV to improve oligonucleotide cellular behaviour.

Keywords: Antisense oligodeoxynucleotide; Cellular uptake; Drug carrier; Stability; SupraMolecular BioVector

1. Introduction

Antisense oligodeoxynucleotides (ODN) are designed to inhibit gene expression in a sequence-specific manner [1]. Because of this property, they represent useful tools to study gene function and they may be efficient therapeutic agents [2]. ODN have been demonstrated to act as specific inhibitors of gene expression in a variety of in vitro models [3] and of cell proliferation in vivo [4,5]. The potential action sites of ODN are numerous on pre-mRNA or...
They may act either by a physical blocking or by the cleavage of the RNA strand of RNA/DNA duplexes by cellular ribonuclease H (RNase H). The usefulness of oligonucleotides is limited by their polyanionic nature that leads to non specific interactions with many extracellular cationic molecules [6,7] and their poor diffusion through the plasma membrane [8–10]. Other difficulties that limit their efficiency are then their intracellular trafficking [9] and the non specific binding to intracellular cationic molecules [2,10]. Moreover, nucleases, particularly 3’-exonucleases, induce fast ODN degradation.

Many attempts have been performed to circumvent these drawbacks, mainly chemical modifications of ODN [11] and the use of carrier systems [12]. Concerning the former approach, the most promising structural analogs of native phosphodiester ODN are phosphorothioate ODN. They are resistant to cleavage by nucleases and do not inhibit RNase H cleavage [13]. These advantages quickly led to cellular and pharmacokinetic studies [3,14,15] and to clinical tests of these compounds as antitumoral and antiviral agents [16]. However, the evidence of effects that are not sequence-specific has moderated the enthusiasm for their therapeutic use [2,17,18]. The ODN carrier strategy appears then as a better approach to improve both the transport and the stability of unmodified ODN, while conserving their target specificity. ODN have been efficiently encapsulated into small liposomes as immunoliposomes [19,20] or pH-sensitive liposomes [21]. It has been shown that encapsulation allows the protection of ODN against nucleases and increases their delivery into specific cells. Besides these advantages, the degradation of ODN and their low efficiency of encapsulation into many liposomes [22] are major drawbacks for the targeted delivery approach. Besides, it has been proposed an interaction of cholesterol conjugated ODN with natural LDL that revealed prolonged plasma half-life (from 1 to 10 min) [23] and an in vitro improvement of antisense efficacy [24]. Nevertheless, the obtention of LDL (from human plasma) and the weak stability of the encapsulated ODN remain major handicaps to their therapeutic use. Synthetic cationic lipids such as DOTMA or DOTAP, first used for DNA transfection, are attractive ODN carriers too [25,26]. Their efficiency has been proved and especially, ODN/DOTAP complexes allow an enhancement of ODN uptake as well as a decrease of extra- and intracellular ODN degradation [26]. Nevertheless, the cellular toxicity of these complexes limit their use for in vitro experiments [12,27] or local administration [28]. More recently, the ODN adsorption on polyalkylcyanoacrylate nanoparticles led to an increased protection against nuclease degradation [29]. Inhibition of neoplastic growth in nude mice was obtained with a concentration of ODN adsorbed on nanoparticles, 100 times lower than for free ODN [30]. But systemic use of these carriers has not yet been shown. In brief, considering the main current carriers, none of them fill yet all the requirements for therapeutic use of ODN [12].

The SupraMolecular BioVectors SMBV appear then as new promising ODN carriers. They are multi-layered particles, around 30 nm diameter, composed of an internal core of cross-linked polysaccharides, externally grafted with fatty acids and surrounded by a lipid layer. Their synthesis can be modulated and controlled to allow an efficient drug cellular uptake and intracellular trafficking [31–34]. SMBV have been shown to efficiently entrap drugs, such as gentamicin and doxorubicin, with entrapment yields of, respectively, 95 and 70% [33]. Moreover, association of interleukin-2 with SMBV allows the stabilization and an enhancement of the biological activity of the cytokine [35]. Besides the incorporation of these positively charged drugs, the SMBV structure has been adapted to the requirements both of the incorporation of anionic ODN into a cationic core and of their intracellular release.

The aim of this study is to evaluate the ability of the SMBV to be ODN carriers. We optimized the incorporation efficiency of ODN into selected SMBV and were allowed to show a dramatic enhancement of both ODN protection and cellular uptake, after incorporation of ODN into SMBV.

2. Materials and methods

2.1. Materials

RPMI 1640 growth medium and FBS were obtained from Gibco (France) and palmitoyl chloride from Fluka (France), cholesterol, chloroquine, de-
oxyglucose, monensin, nonidet P-40, N-propylgallate, sodium azide and MTT from Sigma (France), EYPC from Lipoid (Germany), \( \gamma[^{32}P] \)-ATP from ICN (France) and Hecamel from Vegatech (France).

2.2. Synthesis and labelling of ODN

We used 18 mer phosphodiester oligodeoxynucleotides (ODN) with a sequence complementary to the AUG initiation site of the chloramphenicol acetyl transferase (CAT) mRNA \( 5'\)-AGT GAT TTT TTT CTC CAT-3' \), according to Bunnell [36]. Unlabelled ODN and fluorescent ODN, bearing a fluorescein moiety at the 5’-end, were synthesized and HPLC purified by Genset (France).

Radioactive ODN were 5’-end labelled with \( \gamma[^{32}P] \)-ATP using a T4 polynucleotide kinase Boehringer Mannheim, France at 37°C for 1 h, purified by exclusion column, ethanol precipitated and resuspended in water.

2.3. SMBV composition and ODN incorporation

SMBV were provided by Biovectors Therapeutics. They were synthesized as already described [32–34]. Briefly, they were polysaccharides cross-linked with 2–3 epoxy-chloropropane epichlorhydrin and cationically functionalized with glucidyl trimethylammonium chloride. Polysaccharide core PSC were acylated as previously described. The acylated cores (5 mg) were dispersed in 1 ml of lipid solution (5 mg of EYPC/cholesterol, 80/20 (w/w) in 50 mM Hecameg). The resulting mixture was then quickly diluted with 4 ml of water under ultrasonic probe (Vibracell, Bioblock, France). Resulting SMBV were finally extensively dialysed against water, at 4°C, during 48 h. SMBV were sterilized on 0.22 μm filters (Nalgene, France). Charge determination was performed by titration.

For fluorescent SMBV synthesis, acylated cores were labelled with DTAF (Dichloro Triazo Amino Fluorescein, Molecular Probes, UK) using a DTAF/acylated core ratio of 2.5% (w/w) [37]. SMBV concentrations are expressed as μg/ml of PSC. All SMBV stock solutions were adjusted to 1 mg/ml of PSC.

For ODN incorporation, ODN solution in water was slowly added to PSC or SMBV suspension (1 mg/ml) and kept at 45°C, under magnetic stirring, during 5 h. ODN were added, according to the tested incorporation yield (2.5 to 10%, w/w). At the end of the experiment, free and incorporated ODN were separated by ultrafiltration on microsep (300 kDa, Filtron, France). Free ODN concentrations were measured in the ultra filtrate by UV spectrophotometry at 262 nm.

2.4. ODN / SMBV complex characterization

2.4.1. Stability in PBS

In order to evaluate the stability of ODN incorporation, 0.9 ml of ODN/SMBV suspension were mixed with 0.1 ml of PBS, 10 times concentrated. The suspension was then incubated at 37°C and ultrafiltered on microsep, in order to separate free and incorporated ODN. Free ODN concentrations were then measured as described above.

2.4.2. Size determination

The size of SMBV was measured with a photon correlation laser light-scattering method using a Coulter N4ND submicron analyser (Coultronics, France).

2.5. Determination of the ODN protection against degradation, by SMBV components

2.5.1. Degradation by phosphodiesterase

Radioactive ODN were either incorporated into acylated cores or incorporated into SMBV (100 μg/ml ODN final concentration) or associated to lipids (5 mg/ml of EYPC/cholesterol, 80/20, w/w, diluted, sonicated and dialysed as for SMBV preparation). Samples were then incubated, at 37°C, in presence of \( 4 \cdot 10^{-3} \) U of phosphodiesterase (3’-exonuclease, Boehringer-Mannheim, France), for 1 h. The reaction was stopped by a 10 min incubation of samples at 65°C. They were then analyzed using 20% non-denaturing PAGE. Degradation patterns were observed by autoradiography.

2.5.2. Degradation in various media

Radioactive ODN, free or incorporated into SMBV (10% incorporation yield), were incubated at 37°C, either with RPMI 1640 medium containing 10% FBS or with RPMI 1640 medium containing 10% heat-inactivated FBS or with human plasma (collected and
pooled from donors of ‘Centre de Transfusion Sanguine de Toulouse’, tested HIV-negative by ELISA kit, Abbott, France). Final concentrations were respectively 62.5 μg/ml and 6.25 μg/ml for SMBV and ODN. After various incubation times, aliquots of each sample were collected, incubated for 1 h at 65°C, to stop any nuclease activity of the serum, and stored at 4°C. Samples were then analyzed as described above and autoradiographs were quantified by scanning, using a Kodak DCS 200 numeric camera and an Optimas 4.8 image analysis software. Mean grey values and area of non degraded ODN were determined for each sample. Results were expressed as percentages of the sample collected at initial time (100%).

2.6. Cell culture

The human adenocarcinoma breast cell line (MCF-7) was grown routinely in RPMI 1640 growth medium, supplemented with 10% FBS and containing 4.5 g/l glucose. When specified, FBS was heat-inactivated at 65°C during 1 h. All cells were incubated at 37°C in a humidified 5% CO₂ incubator.

2.7. Uptake studies

2.7.1. Fluorescent SMBV uptake analyzed by flow cytometry

2·10⁵ MCF-7 cells were seeded in 35-mm Petri culture dishes (Nunc, France). 24 h later, cells were washed once with PBS. Then 1 ml of growth medium containing 10% heat-inactivated FBS, with or without 0.1 mM chloroquine and with or without 10 mM sodium azide and 10 mM deoxyglucose, was added per dish. After 1-h incubation at 4°C or at 37°C, fluorescent SMBV, loaded or not with ODN, were added at various concentrations. Cells were again incubated at 4°C or at 37°C during various incubation times, then washed twice with PBS and collected by trypsinization. Cells were centrifuged (120 × g, 5 min, 15°C) washed twice with PBS and resuspended in PBS. An aliquot of each sample was treated during 40 min at room temperature in 20 μM final concentration of monensin. Cells were then analyzed by flow cytometry, on a Facsscan flow cytometer (Becton Dickinson, France) with a 488 nm laser excitation and a 530 nm emission filter. Data was acquired on 10⁴ viable cells and mean fluorescence values were collected.

2.7.2. Fluorescent ODN uptake analyzed by flow cytometry

Cells were treated and analyzed according to the protocol described above, excepted that they were incubated in presence of various concentrations of either free fluorescent ODN or unlabelled SMBV loaded with fluorescent ODN. Monensin was added in all samples at 20 μM final concentration.

2.7.3. Confocal microscopy analysis

2·10⁵ MCF-7 cells were grown in monolayers by plating them on 18 × 18 mm² sterile glass coverslips, set in 35-mm Petri dishes (Nunc, France). After 48 h, cells were washed once with PBS and 1 ml of growth medium containing 10% heat-inactivated FBS was added in each dish, 1 h before SMBV treatment. The labelled SMBV were then added at various concentrations. After a 3-h incubation, cells were fixed with 4% formaldehyde in PBS and permeabilized in 1% Nonidet P-40 in PBS. Glass coverslips were then mounted on glass slides and observed by confocal analysis (Microsystems LSM, Zeiss, France), with an inverted microscop (Axiovert 100, Zeiss, France). For fluorescence analysis, a 488 nm laser excitation was used, with a 510-525 nm short bandpass emission filter. Interferential contrast pictures were obtained by Nomarsky analysis. In both cases, the magnification was 63 (immersion lens) plus 2.6 (electronic zoom).

2.7.4. Radioactive ODN uptake

3·10⁵ MCF-7 cells were seeded in 60-mm Petri culture dishes. 24 h later, cells were washed once with PBS and 1 ml of growth medium containing 10% heat-inactivated FBS was added in each dish, 1 h before SMBV treatment. Then, at various times, labelled ODN, free or incorporated into SMBV were added (10⁶ cpm total radioactivity with 6.25 μg/ml of ODN and 62.5 μg/ml of SMBV). The culture medium was then removed and cells were washed twice with PBS. The cells were collected by trypsinization, centrifuged (120 × g, 5 min, 15°C), washed twice with PBS and then resuspended in PBS. Cells were either submitted to a fractionation, as described below, or lysed by a 1-h incubation in a
0.5% SDS solution and radioactive ODN were counted by liquid scintillation.

2.7.5. Cell fractionation

MCF-7 cells, treated with radioactive ODN, trypsinized and washed as described above, were resuspended in 0.5 ml of 10 mM Tris/HCl, pH = 7.4. All procedures were carried out at 4°C. Cells were disrupted with 40 strokes in a Dounce homogenizer and centrifuged (10000 × g, 10 min). Supernatants were centrifuged at 100000 × g for 60 min (Kontron instruments, Centrikon T-1075 ultracentrifuge, 70 Ti rotor, France). The supernatant and pellet obtained refer to predominant cytosolic and microsomal fractions respectively (as tested by acid phosphatase activity). Radioactive ODN were either counted by liquid scintillation or phenol/chloroform extracted, ethanol precipitated and analyzed on a 20% non denaturing PAGE. The degradation pattern was visualized by autoradiography.

2.7.6. Cytotoxicity assay

The quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) colorimetric assay [38] was used to determine the toxicity of the SMBV towards MCF-7 cells. 2500 cells were seeded per well, in 96-well plates and grown during 24 h at 37°C. Cells were then incubated, during 3 to 12 h, with increasing concentrations of SMBV, up to 62.5 μg/ml of SMBV in 200 μl final volume of growth medium containing 10% FBS. The SMBV containing medium was then removed and replaced with 50 μl of a 1 mg/ml MTT solution in RPMI medium, without phenol red. The cells were incubated during 3 h at 37°C, in a humidified 5% CO₂ incubator. The MTT containing medium was then removed, 150 μl of DMSO were added and cells were incubated during 30 min in the same conditions. The blue MTT formazan product was quantified by measuring the optical density of the cell extract at 540 nm. For each concentration, average viability value was calculated from the data of 6 wells and expressed as percentages, compared to untreated cells (100%). The IC20 values were then determined, as the SMBV concentrations that allow 80% of cell survival.

3. Results

3.1. Characterization of ODN / SMBV complexes

Our initial goal was to select SMBV compositions that allow ODN efficient and stable incorporation. The rational for ODN incorporation is based on ionic interactions between cationically functionalized polysaccharide cores (PSC) and anionic ODN. Three different PSC with similar sizes, 32.8 ± 5 nm, and with increasing charges 0.6 to 1.6 meq/g have been tested. The leakage after 18 h was either increased to more than 70% (PSC I and II) or maintained around 46% (PSC III). We selected thus, in a first approach, the PSC III that exhibit a stable incorporation.

Similar experiments were performed with complete SMBV, synthesized with PSC III. SMBV allow a 100% incorporation yield, significantly different from incorporation into PSC III alone (99.9% confi-
Fig. 1. Size analysis of ODN/SMBV complexes according to time. ODN incorporated into SMBV were incubated at 4°C in water. At different times (0 to 60 days), particle size distribution was determined on submicron nanosizer. Average particle diameters are summarized in the insert, according to time.

dence limits by two-sample independant-groups t test). A minimal leakage in PBS (10%) is observed after a 30 min incubation and was maintained at 15% after 18 h. We checked that this minimal leakage was roughly constant over 2 months. All previous experiments were performed with 2.5% ODN/SMBV incorporation ratios (w/w). With a 10% ratio, 100% incorporation yield and similar stability results were obtained. In addition, we tested the effect of the dilution factor upon the ODN leakage in PBS. The high stability of the incorporation can be due to the minimal dilution factor (1/1 for previous standard conditions), even if the ODN phosphate/PBS phosphate ratio is low (1 to 4 \(\times\) 10\(^{-2}\), depending on the initial ODN/SMBV incorporation ratio). However, the stable measured leakage was confirmed for dilutions, up to 10-fold, of the ODN/SMBV complexes in PBS (data not shown). This result and the effect of the nature of the charge of PSC on ODN incorporation indicate that the incorporation of ODN is driven by strong ionic interactions.

Concerning the ability of the loaded SMBV to be stored in water, the Fig. 1 summarizes the results obtained in term of particle size analysis. ODN/SMBV complexes (10% incorporation ratio), stored at 4°C, have an initial mean size of 27 ± 9 nm. The solution, clear and without precipitates, exhibits no signal above 50 nm, testifying the absence of

![Graph showing size analysis](image)

Fig. 2. Role of SMBV components for ODN protection against phosphodiesterase. ODN, free or incorporated, were incubated for 1 h with phosphodiesterase and analyzed by 20% PAGE and autoradiography. ODN were free or incorporated into SMBV or incorporated into acylated cores or associated to EYPC/cholesterol (80/20, w/w).
aggregate formation. Size measurements performed over 2 months show that the mean size of the complexes is roughly constant (Fig. 1, insert). Again, aggregate formation was not observed during this period of time. ODN leakage was not increased, with a constant free ODN concentration lower than 3%, during the 2 months.

3.2. Determination of ODN protection by SMBV

We then determine if SMBV elicit the ODN protection against nucleases. The Fig. 2 shows that free ODN are degraded within 1 h in presence of phosphodiesterase while the incorporation of ODN into SMBV allows their complete protection. To determine if this protection is the result of an inhibition of the nuclease by one of the SMBV components, we checked on the one hand, the degradation pattern of ODN associated to acylated cores and on the other hand the degradation pattern in presence of the lipid suspension (EYPC/cholesterol) (Fig. 2). The results show that the acylated cores do not protect ODN against nuclease degradation, while the presence of complete SMBV is necessary for ODN protection.

We then determined if, in the same way, SMBV can protect ODN against degradation in various cell growth media. The Fig. 3A shows that free ODN are quickly degraded in growth medium containing FBS (minute scale), while SMBV (Fig. 3B) efficiently protect ODN, whose degradation begins only after 1 h. The quantitative analysis of this experiment (Fig. 3C) confirms that free ODN start to be degraded at 10 min and are completely degraded at 20 min, while ODN incorporated into SMBV are only slightly degraded at 2 h. The complete degradation occurs after 6 to 8 h of incubation. The half-life of ODN shifts then from 25 min to 225 min (3 h and 45 min), after SMBV incorporation. In growth medium containing 10% heat-inactivated FBS, neither free ODN nor incorporated ODN were degraded during 6-h incubation. When incubated in human plasma, free ODN were only slightly degraded after 1 h and 90% degraded after 3 h, while ODN incorporated into SMBV show the same degradation profile as in the growth medium containing 10% FBS. In this latter condition, the half-life of ODN shifts from 120 min to 225 min. These results prove that SMBV allow a significant protection of ODN in human plasma and in cell growth medium, increasing markedly their life time (half-lives increased 2 to 8 times). In both tested conditions, the degradation of SMBV protected ODN is completed only after an 8-h incubation.
3.3. SMBV cellular uptake

Flow cytometry analysis (Fig. 4) shows that fluorescent SMBV do enter into MCF-7 cells, in a dose-dependent manner. The fluorescence level is markedly higher than the signal of free fluorescein, used as control. This process is saturable above 10 μg/ml SMBV concentration (Fig. 4, insert). A constant increase of the mean cell fluorescence is observed during 24 h in these standard conditions (37°C). It is noteworthy that ODN incorporation, with a 10% ratio, do not impair SMBV cellular uptake.

To analyze the mechanisms involved in SMBV uptake and trafficking, similar experiments were carried out in low energy conditions and in presence of lysosomotropic agent. At 4°C, the SMBV uptake is weak, with fluorescence mean values lower than at 37°C: 2 times lower after a 7-h incubation (Fig. 5) and 6 times lower after a 24-h incubation (data not shown). At 4°C, a plateau is reached after 1 h. The presence of sodium azide and deoxyglucose, that inhibits ATP synthesis at 37°C, induces a SMBV uptake similar to the 4°C one and with a fluorescence level 1.5 time lower than at 37°C in standard conditions, after a 7-h incubation. We then used chloroquine, that prevents the fusion of endosomes and lysosomes, to study the intracellular fate of SMBV. The addition of chloroquine induces an increased fluorescence signal. Fluorescein fluorescence being pH-dependent, SMBV that go into the acid lysosomal compartment (pH = 4.7–4.8 [39]) lead to a low fluorescence signal. In presence of chloroquine, SMBV are blocked within the less acidic endosomal compartment (pH = 5.4 to 6, [40]) and the fluorescence signal is higher. We confirmed this hypothesis by incubating aliquots of cells with monensin [41], an ionophore that suppresses the pH gradient inside the cells. Such a treatment leads to the same increased fluorescence signal (data not shown), confirming that SMBV are located in an intracellular acidic compartment. These results indicate that the SMBV uptake is not saturable over 24 h, is energy-dependent and that SMBV are internalized by cells up to lysosomes.

To complete these quantitative data, we performed fluorescence confocal microscopy studies. At 37°C
(Fig. 6A), many small, highly fluorescent punctuations are observed inside the cytoplasm, characteristic of endocytosis, with nucleus exclusion. At 4°C (Fig. 6B), these punctuations are not present and a slight fluorescence diffuses in the cells, besides membrane fluorescence. At last, in presence of chloroquine (Fig. 6C), fluorescent cytoplasmic punctuations are present, with a pattern similar to that observed at 37°C, again with nucleus exclusion. These results confirm, in turn, that the SMBV are internalized by an endosomal pathway.

### 3.4. ODN uptake

We performed similar flow cytometry experiments with non labelled SMBV and fluorescent ODN, to determine if the association of ODN to SMBV is strong enough to allow the simultaneous ODN/SMBV uptake. As a control, free ODN were incubated with cells and only minimal uptake was observed, the fluorescence signal being similar to the background cell fluorescence level (Fig. 7). The up-
take of fluorescent ODN mediated by the SMBV is markedly increased, compared to free ODN.

When samples were treated with monensine, the fluorescence level is increased (data not shown), as well as for SMBV uptake, testifying of the presence of ODN, free or associated to SMBV, in acidic compartment. All samples have, in turn, been treated with monensine to normalize the fluorescence signals [41], Fig. 7. As shown in the insert of Fig. 7, the SMBV incorporated ODN uptake is a dose-dependent process, less saturable than for SMBV uptake. According to the SMBV concentration (1 to 62.5 μg/ml), ODN cumulative uptake is increased 2 to 30 times compared to free ODN at 5 h. A plateau is reached after a 5-h incubation, in contrast to the SMBV uptake that slowly increases during the 24-h uptake. It is noteworthy that when ODN are degraded inside the cells, free fluorescein is poorly permeant and stays mainly inside the cells [40]. The level of fluorescence quantified in this experiment is then representative of the cumulative amount of fluorescent ODN (degraded or not) that go through the cells. The ODN uptake saturation might be explained by the fact that, over 5 h, ODN incorporated into SMBV are progressively degraded in presence of growth medium and cells and do not enter cells anymore.

We used radioactive ODN to confirm the increased uptake of ODN incorporated into SMBV, knowing that radiolabelled nucleotides can exit cells, after ODN degradation [42]. Radioactivity was counted from 3 · 10^5 MCF-7 cells, seeded in each Petri culture dish. We observed that after a 1-h incubation, ODN incorporated into SMBV are 10 times more concentrated in cells than free ODN (25 523 cpm/2559 cpm). This increased uptake is 4.9 times higher after a 5-h incubation (20625 cpm/4211 cpm) and non significant after 24 h (14 630 cpm/14 290 cpm). This agrees with a SMBV driven uptake of ODN, followed by a release of ODN from cells, after intracellular progressive degradation.

The subcellular location of ODN is crucial for the biological effects. We then looked for the presence of radioactive ODN in cell cytosol, after 1- and 5-h incubations (ODN uptake being maximum at 5 h). We compared the amounts of radioactive ODN in this fraction, with and without SMBV incorporation. Experiments were triplicated and mean ± S.E. are expressed. We observed that ODN incorporated into SMBV are 11.5 times more concentrated in cell cytosols than free ODN, after a 1-h incubation (7192 ± 1689 cpm/626 ± 114 cpm) and 7.3 times, after a 5-h incubation (7907 ± 2863 cpm/1041 ± 237 cpm).

We looked for the presence of intact radioactive ODN, in these cytosolic fractions after the longer incubation (Fig. 8). Equal total cpm amounts have been loaded on the gel, for both samples: cytosols of cells treated either with free ODN or with SMBV incorporated ODN. The proportion of intact ODN is much higher for SMBV incorporated ODN than for free ODN (respectively, lanes 3 and 2 of Fig. 8).

3.5. IC20 determinations

The cytotoxicity of SMBV on MCF-7 cells was studied in order to determine the SMBV concentrations usable for cellular study. For this, IC20, SMBV concentrations that allow 80% of cell survival, were determined for 3-, 6- and 12-h incubations. IC20 are respectively 7, 2.3 and 0.8 μg/ml of SMBV.

4. Discussion

The aim of this work is to demonstrate the ability of SMBV to protect ODN against nuclease degradation and to allow their cellular uptake. We first show that SMBV exhibiting a charge of 1.6 meq/g are able to stably incorporate 10% of ODN (w/w). Such a strong association is relevant for an efficient transport of ODN to cells and then for their uptake inside cells, before ODN are released from the carrier.
Our results show that complete SMBV, composed of the internal acylated core and the lipid layer, allow ODN protection. The ODN half-life is increased 8 fold in cell growth medium, guaranteeing the presence of intact ODN up to the 8 h following cell treatment. The ODN degradation must finally be due to either the ODN release from SMBV or to the SMBV degradation. It is important to note that this half-life of incorporated ODN is close to 4 h whereas efficient ODN carriers such as DOTAP only allow to reach a 40-min half-life [26]. According to the manufacturer’s recommendations and publications [43,44], actual cationic liposomes (DOTMA, GIBCO or DOTAP, Boehringer) are often used in serum-free or at least reduced-serum media. In the biologically relevant case of human plasma, ODN protected by the SMBV exhibit a half-life as long as the one observed in cell growth medium.

Besides, any ODN carrier must exhibit both an efficient cellular uptake and the simultaneous ODN uptake. On the first hand, we demonstrate that SMBV are internalized into MCF-7 cells. First, we prove the existence of an active cellular process, independent of a simple adsorption: the fluorescence signal observed is decreased both at 4°C and at 37°C in presence of sodium azide, compared to standard conditions at 37°C. Moreover, characteristic endocytosis punctuations, energy-dependent, are observed. Besides, the fluorescence signal is altered in presence of lysosomotropic agent (chloroquine) or of ionophore (monensin), indicating that SMBV would go through endosomes and lysosomes inside cells. Incorporation of 10% ODN into SMBV (W/W), that leads to neutralization of 20% of the SMBV positive charges by the anionic ODN, does not modify the cellular uptake of SMBV.

On the other hand, we demonstrate that SMBV dramatically increase the ODN uptake. When free ODN are incubated with cells, intact ODN are not observed inside cells, many hours after the administration. This is only possible if ODN are driven into cells by carriers, such as DOTAP or nanoparticles [26,30]. In our study, the cumulative uptake of ODN incorporated into SMBV is plateauing after a relatively short incubation time, 5 h. At this time, a significant proportion of ODN is still present in cells. These ODN are, at least partially, localized in the cell acidic compartments as monensin shifts the fluorescence levels after fluorescent ODN uptake. Up to now, it is not known how free ODN have access to cell cytoplasm or nucleus [45,46]. Nevertheless, when free ODN (highly concentrated phosphodiester or phosphorothioate) are endocytosed by cells, most of the molecules must be trapped inside lysosomes. At least a small part escapes and reaches the cytosol to allow the antisense effects already described [3,5]. Similarly, solid carriers such as nanoparticles are supposed to promote, via endocytosis, the cellular uptake of the associated ODN [47]. Consequently, any ODN carrier that efficiently drives ODN through cell membranes and then allows at least part of them to escape from lysosomal compartments may be performant [9]. For SMBV, the ODN/SMBV association must allow the release of ODN inside cells, with minimal degradation by lysosomal enzymes. Our results show that ODN present in cell cytosol are up to 11 fold concentrated after SMBV incorporation, compared to free ODN. Moreover, the proportion of intact ODN in the cytosol is significantly higher for SMBV incorporated ODN than for free ODN. It must be noted that intact ODN are analyzed but we cannot distinguish ODN still incorporated into SMBV from dissociated ODN. The intracellular dissociation of the SMBV/ODN complexes can be rapid or progressive. It can occur either during the endocytic pathway (before the complex is in the lysosomes) or only after escaping lysosomes, once the complex reaches the cytosol. According to this intracellular dissociation, we can expect various release kinetics of ODN that will enhance differently the antisense effects.

Taken together, these results show that ODN incorporated into SMBV penetrate cells and cytosol during the 5 h following the treatment, while being still partially intact. The interval between two consecutive administrations could be as short as 5 to 6 h and, in any case, should be adjusted experimentally and according to the target mRNA and target protein half-lives. At the lowest SMBV concentration we tested (1 μg/ml of SMBV, i.e. around 20 nM of ODN for the 10% incorporation rate), the SMBV incorporated ODN uptake is already increased 3 times compared to free ODN uptake and their life-time is prolonged 8 times, in culture growth medium containing 10% bovine serum. Cytotoxicity results show that SMBV administrated at this concentration are not toxic on MCF-7 cells: for a 3- or 6-h incubation,
more than 80% of cells are viable. Because cationic liposomes (DOTMA, GIBCO or DOTAP, Boehringer) are usually incubated during 3 to 12 h with cells [43,44], we tested the longer 12-h incubation for SMBV and showed that the 1 μg/ml is always in the range of the non-toxic concentrations.

As a conclusion, SMBV appear as efficient ODN carriers that greatly increase both ODN protection and ODN intracellular uptake. Moreover, the ODN are stably incorporated into SMBV, at least over 2 months in water, predicting optimal storage conditions of the ODN/SMBV complexes. We are currently testing the efficiency of these carriers for cellular antisense effect.

Acknowledgements

Thanks are due to Dr. Toulas for having initiated this work, to Prof. Gas and Dr. Teissié for preliminary fluorescence studies and to Mrs. Berg for cell culture facilities. Confocal microscopy analysis has been performed in the laboratory of Dr. Duprat (CNRS, Toulouse), with the valuable help of Dr Cochard. This work was supported by grants from the French `Ministere de la Recherche et de l’Enseignement Superieur’, the ‘Comites departementaux de la Ligue contre le cancer’ and the ‘Federation Nationale des Centres de Lutte contre le Cancer’ and the ‘Ligue Nationale Contre le Cancer’, for M.B. and the UPRES laboratory.

References

[33] Demiguel, I., Ioulalalen, K., Bonnefous, M., Peyrot, M., Nguyen, F., Cervilla, M., Soulet, N., Dirson, R., Rieumajou,