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Biochimica et Biophysica Acta 1463 (2000) 254–266

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells

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Received 18 May 1999; received in revised form 30 September 1999; accepted 19 October 1999

Abstract

It was previously demonstrated that fluid liposomal-encapsulated tobramycin, named Fluidosomes, was successful in eradicating mucoid *Pseudomonas aeruginosa* in an animal model of chronic pulmonary infection, whereas free antibiotic did not reduce colony-forming unit (CFU) counts (C. Beaulac et al., Antimicrob. Agents Chemother. 40 (1996) 665–669; C. Beaulac et al., J. Antimicrob. Chemother. 41 (1998) 35–41). These liposomes were also shown to be bactericidal in in vitro tests against strong resistant *P. aeruginosa* strains. To define the mechanism by which Fluidosomes work, negative staining, immunoelectron microscopy, fluorescence activated cell sorting (FACS) and lipid-mixing studies were performed. All the lines of evidence suggest that Fluidosomes fuse with bacterial cells leading to a marked increase of tobramycin in the cytoplasm of a resistant bacteria (minimal inhibitory concentration (MIC) >64 μg/ml). The time needed to reach the maximal fusion rate was about 5 h for the resistant strain comparatively to much shorter time for the sensitive strain. The specific characteristics of Fluidosomes could help overcome bacterial resistance related to permeability barrier and even enzymatic hydrolysis considering the importance of synergy in the whole process of antibiotic resistance.

Keywords: Liposome; Fusion; Bacteria; *Pseudomonas aeruginosa*; Flow cytometry; Immunocytochemistry

1. Introduction

Chronic pulmonary infections with *Pseudomonas aeruginosa* and other related strains like *Burkholderia cepacia* are considered as the most important factor determining the prognosis of cystic fibrosis (CF) patients [1–3]. These bacteria are known for their very low non-specific permeability and/or the presence of membrane-associated energy-driven efflux systems that make them resistant to the majority of antibiotics [4]. Other mechanisms of resistance, like enzymatic inactivation of the drug and alteration of its molecular target, can work synergistically with previous mechanisms [5–8]. Another cause of resistance to antibiotics that is largely underestimated is a transitory and unstable phenomenon of decreased membrane permeability that appears and is maintained only in the presence of the antibiotic [9]. Conse-

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0005-2736/00/$ – see front matter
PII: S0005-2736(99)00217-5

quently, efficient therapy against *P. aeruginosa* in CF remains difficult and controversial leading to progressive pulmonary damage and, eventually to death [2,3].

To increase the bactericidal efficacy of antibiotics, different liposomal formulations were developed in our laboratory with the aim of promoting effective interactions between bacteria and encapsulated drugs, increasing the lifetime of the encapsulated antibiotics and reducing systemic drug absorption [10]. Tobramycin encapsulated in a negatively charged liposomal formulation presenting a low gel–liquid crystalline transition temperature (T_C) ≤ 37°C succeeded, for the first time, in eradicating mucoid *P. aeruginosa* in an animal model of chronic pulmonary infection [11]. This fluid liposomal encapsulated tobramycin, named Fluidosomes, was later shown to be effective against all the other bacterial strains tested: *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Escherichia coli* and *Staphylococcus aureus* [12]. To investigate further how Fluidosomes interact with bacteria, we proceeded in performing negative staining, immunoelectron microscopy, fluorescence-activated cell sorting (FACS) and lipid-mixing studies.

2. Materials and methods

2.1. Bacterial strains

A clinical and a reference strain were used in this study. *P. aeruginosa* 429 is a clinical strain obtained from a patient with cystic fibrosis (Sainte-Justine Hospital, Montréal, Qué., Canada) which has an minimal inhibitory concentration (MIC) for tobramycin of 64 μg/ml. A *P. aeruginosa* 429 susceptibility profile with the 24 aminoglycosides for which MICs were performed shows resistance values between 64 and 512 μg/ml. This strain was also probed negative by DNA hybridization to all 14 aminoglycoside resistance genes tested (Antibiotic Screening and Evaluation, Schering-Plough, New Jersey, USA) suggesting permeability resistance that was confirmed by disk tests. Strain 429 shows small colony morphology and a rate of growth corresponding to only about 29% of that of the reference strain. The reference strain, *P. aeruginosa* ATCC 25619, was obtained from the American Type Culture Collection (ATCC), Rockville, MD). This strain is not resistant to tobramycin (MIC <1 μg/ml). Both strains were stored in brain heart infusion broth supplemented with 10% glycerol. For the experiments, a 17-h culture of these organisms in proteose peptone no. 2 broth (Difco Laboratories, Detroit, MI) was prepared.

2.2. Fluidosomes

Fluidosomes are liposomes composed of dipalmitoyl phosphatidylcholine (DPPC) and dimiristoyl phosphatidylglycerol (DMPG) (Avanti polar lipids, Alasbaster, AL) in a ratio of 18:1 (w/w) which have an overall low gel–liquid crystalline transition temperature ($T_C < 37$°C). They are prepared by Theralipids (Ville Mont-Royal, Qué., Canada) using the dehydration–rehydration method as previously described [11,12]. Briefly, appropriate amounts of both phospholipids are dissolved in chloroform in a round-bottom flask. A lipid film is then obtained by rotoevaporation (Büchi Rotavapor) at 65°C under vacuum (Büchi vacuum controller). The lipids are then re-dissolved in phosphate-buffered saline (PBS) 1:20, freeze-dried at 4°C (Virtis Genesis 12 EL Console freeze dryer, Canberra Parkard, Toronto, Canada) and kept at −70°C until use. When Fluidosomes containing tobramycin are needed, 3.75 ml of tobramycin sulfate (Nebcin, Eli Lilly, Toronto, Ont., Canada) at a concentration of 40 mg/ml was added to the freeze-dried liposomal preparation. The solution was incubated 60 min at 65°C with vigorous vortexing every 10 min and extruded successively through polycarbonate membranes of 1.0, 0.6 and 0.4 μm. Fluidosomes without tobramycin were prepared similarly, but PBS was used instead of the antibiotic. Unencapsulated tobramycin was then removed by centrifugations ($2 \times 5000 \times g$, 30 min 4°C) and the final liposomal pellet was resuspended in PBS. The quantification of tobramycin in Fluidosomes was performed by HPLC analysis as previously described [11,12].

2.3. Antibiotic penetration assessment by immunoelectron microscopy

For these experiments, the *P. aeruginosa* strain 429

was used. Briefly, a 17-h culture of this clinical strain was collected and a solution with an optical density (OD) of 0.04 (660 nm) was made in fresh proteose peptone no. 2. Amounts of 29 ml of this solution were then added to 50-ml tubes. Fluidosomes or free tobramycin at a final concentration of 30 μg/ml (half the MIC of the bacteria) or PBS as a control were then added and incubated at 37°C with agitation (250 rpm). Two-ml samples were taken after 0, 1, 2, 3 and 6 h of incubation and processed for electron microscopy. Briefly, the samples were centrifuged and the pellet resuspended in a solution of electron microscopy grade glutaraldehyde and paraformaldehyde (Sigma, St. Louis, MI) at percentages of 0.1 and 4%, respectively, prepared in 0.1 M of cacodylate buffer, pH 7.0. The cells were incubated for a period of 2 h at room temperature in the fixation solution. After fixation, the cells were washed in cacodylate buffer 0.1 M containing 3% sucrose (w/v) and processed for embedding in Spurr resin. Ultra-thin cuts of the samples were made and prepared for immunogold labeling using a standard procedure [13]. To detect the presence of tobramycin inside the bacteria, antibodies against tobramycin (Cerderlane, Ont.) were used and revealed with colloidal gold (10 nm) coupled to protein A/G prepared as previously described [14]. Samples were analyzed using a Hitachi transmission electron microscope (Hitachi 7100).

2.4. Analysis of fusion between bacteria and Fluidosomes by negative staining

Bacteria (*P. aeruginosa* 429) were incubated with Fluidosomes following the same procedure as described above. A sample was taken 1 h after incubation and processed for visualization by negative staining. A drop of the bacterial suspension was layered on formvar-coated copper grid and incubated 1 min at room temperature to allow the bacteria to adhere to the grid. Excess liquid was absorbed and a drop of phosphotungtenic acid (PTA) at 1% (Electron Microscopy Sciences, Fort Washington, PA) was added. The grid was incubated 1 min, excess liquid was absorbed and the sample was visualized by electron microscopy.

2.5. Bacteria and liposome fusion as measured by lipid-mixing assay

Fluidosomal fusion with bacteria was monitored by a lipid-mixing assay based on the extent of resonance energy transfer (RET) between the lipid headgroup-labeled probes, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine Rhodamine B sulfonyl) (Rh-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), as described by Struck et al. [15]. All fluorescence measurements were carried out with a Perkin-Elmer LS-50B fluorescence spectrophotometer. In a typical experiment, the NBD/Rh-labeled Fluidosomes were prepared as the method described previously containing both NBD-PE and Rh-PE at 0.2 mol% each. The NBD/Rh-labeled vesicles (36 μM) were mixed with bacteria ($OD_{660} = 0.6$) and incubated at 37°C under agitation (250 rpm). RET efficiency was monitored by measuring wavelength scans of the reaction mixtures fluorescence. At different time points, 100-μl aliquots were mixed with an equal volume of HEPES buffer (pH 7.4, 20 mM HEPES and 150 mM NaCl) in a 96-well plate, and the fluorescence intensity was determined in a wavelength range from 500 to 610 nm under steady-state excitation at 475 nm (maximal excitation peak for NBD). The fusion of NBD/Rh-labeled Fluidosomes with bacteria resulted in probe dilution (lipid-mixing) and an increased distance between the NBD-PE and Rh-PE, thereby decreasing RET efficiency and decreasing Rh-fluorescence intensity.

The Rh-fluorescence decrease due to fusion was measured and used for calculation of degree of fusion. In brief, the NBD/Rh-labeled Fluidosomes were prepared according to the method described previously containing both NBD-PE and Rh-PE at 0.2 mol% each. The NBD/Rh-labeled vesicles (58 μM) were quickly mixed with bacteria ($OD_{660} = 0.6$) in a cuvette under controlled temperature and continuous stirring. The final incubation volume in all measurements was 2 ml. Continuous monitoring of rhodamine fluorescence (590 nm) were done at an interval of 1 min under steady-state excitation at 475 nm (NBD maximal excitation). The

final fluorescence intensity (F_{max}) which represents maximal fluorescent lipid probe dilution in each sample was determined following the solubilization of vesicles with SDS detergent (0.2% volume). The percentage of fusion (or lipid dilution) was calculated using the following equation:

$$\% \text{ Fusion} = \frac{F_t - F_o}{F_{max} - F_o} \times 100$$

where F_t is the fluorescence intensity at each time point; F_o is the initial fluorescence intensity. Each experiment for the given conditions was repeated three times.

2.6. Labeling of Fluidosomes with PHK2-GL

To evaluate fusion of Fluidosomes with bacteria by flow cytometry, Fluidosomes were labeled with PKH2-GL using the PKH2-GL labeling kit (Sigma, St. Louis, MI). PHK2-GL is a probe often used to study interactions between biological membranes [16,17]. This probe is a fluorescent cell linker dye containing aliphatic carbon tails which insert themselves in membranes. It possesses similar spectral characteristics as FITC. Briefly, freeze-dried Fluidosomes were rehydrated with phosphate buffered saline (PBS), pH 7.2 as previously described [11,12]. The final liposomal pellet was then used for the labeling procedure. Labeling was effectuated following the manufacturer's instructions, but with small modifications. A 1-ml aliquot was taken and centrifuged 30 min at $6000 \times g$ under 4°C. The pellet was then resuspended in 1 ml of diluent A (Sigma, St. Louis, MI). Diluent A is a solution contained in the kit used for the labeling procedure. Eight μl of PKH2-GL was then added for a final concentration of 4×10^{-6} M. The volume was then completed to 2 ml with diluent A. The PKH-liposomal solution was incubated for 5 min at room temperature with circular agitation. Following the incubation, 2 ml of bovine serum albumin at 1% (w/v) in PBS was added and the solution was further incubated for 1 min with agitation to stop the labeling reaction. Fluidosomes were then washed twice with PBS by centrifuging at $5000 \times g$ for 30 min under 4°C to remove any free PKH. Labeling efficiency was on average 95% as determined by flow cytometric analysis.

2.7. Flow cytometric analysis of fusion using PKH2-GL

Integration of Fluidosomes to bacterial cells was demonstrated in fluorescence-activated cell sorting (FACS) analysis, using the fluorescent marker PKH2-GL. *P. aeruginosa* 429 or ATCC 25619 were incubated for 17 h in proteose peptone broth no 2. A solution of 0.3 of OD (660 nm) was then made. The solution was centrifuged and the cell pellet resuspended in RPMI supplemented with 2.5% fetal calf serum for washing. The final cell pellet was resuspended in RPMI supplemented with 2.5% fetal calf serum to obtain the same initial concentration. RPMI was used because it was determined by earlier experimentation to be the best medium for reducing background fluorescence of *P. aeruginosa* due to siderophore production. Aliquots of 18 ml were then taken and transferred to conical tubes of 50 ml (Sarstedt, St. Laurent, Qué., Canada). Bacteria were incubated either with 200 μl of Fluidosomes labeled with PHK2-GL, 200 μl of PBS (negative control) or 80 μl of free PKH-2GL (positive control) to obtain a final concentration of 4×10^{-6} M. PBS was then added to complete the volume to 20 ml and the tubes were incubated at 37°C with agitation (250 rpm). Tobramycin was not encapsulated in Fluidosomes in order to avoid bacterial cell killing. Based on preliminary studies, 2-ml samples were taken after 10 min, 0.5, 1, 2, 3, 4, 5 and 6 h of incubation. Fluidosomes and free PKH2-GL were separated from the bacteria by centrifugation through a sucrose cushion of 21% (w/v) in PBS. The recovered bacterial pellet was washed twice in PBS and the final cell pellet was fixed with 300 μl of 2% paraformaldehyde diluted in PBS for FACS analysis.

3. Results

3.1. Determination of tobramycin penetration with immunocytochemistry

To try to understand how Fluidosomes succeed in showing strong bactericidal activity against resistant bacteria with a quantity of antibiotic $\leq 50\%$ of the MIC [12], kinetic studies with *P. aeruginosa* 429 were performed as previously described [12] to investigate

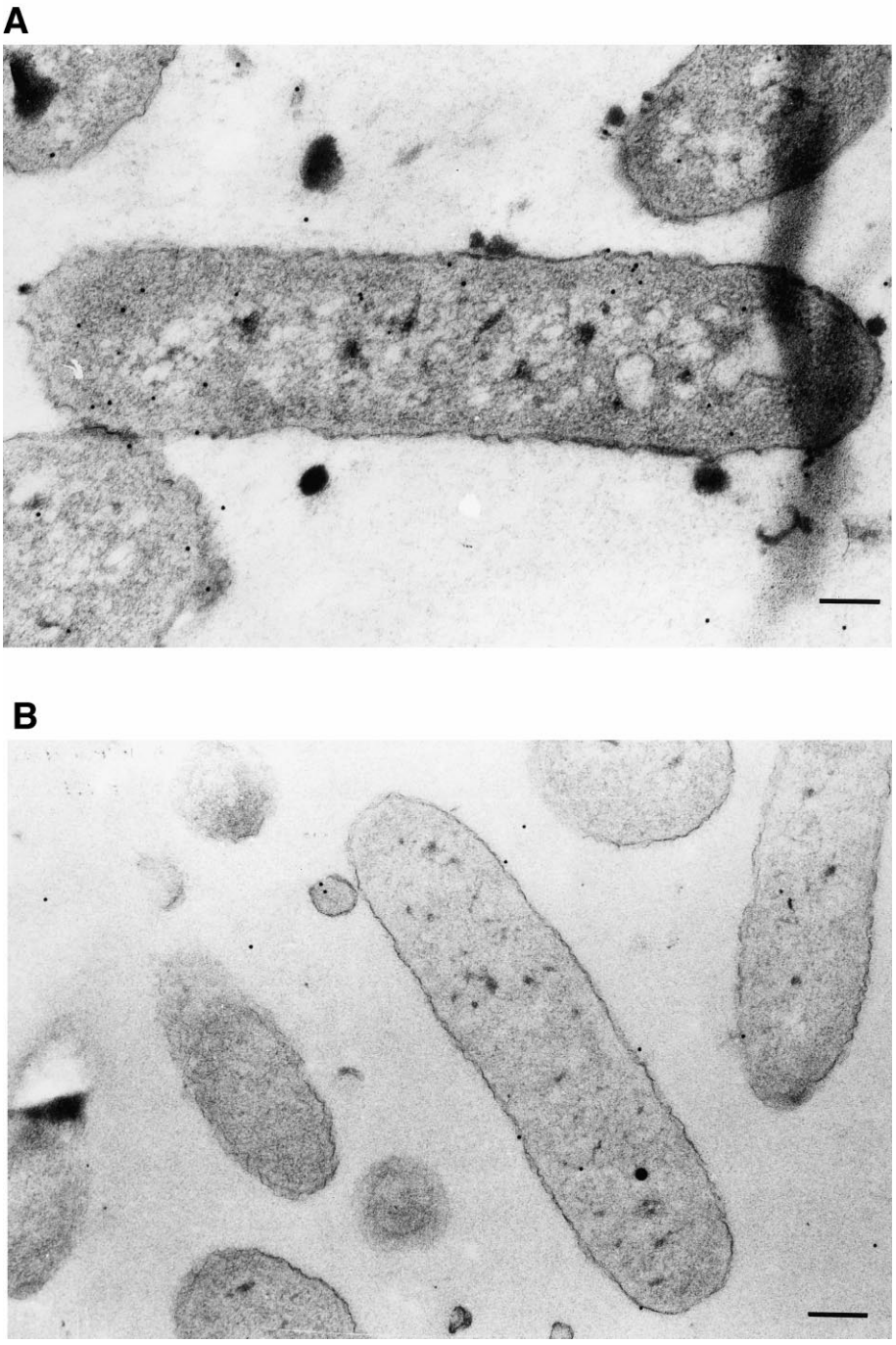

Fig. 1. Detection of tobramycin inside bacterial cells by immunogold labeling. (A) *Pseudomonas aeruginosa* 429 incubated 6 h with Fluidosomes. (B) *Pseudomonas aeruginosa* 429 incubated 6 h with free tobramycin. Magnification: A, ×41 126; B, ×36 720.

the mode of interaction of Fluidosomes. This strain was selected because of its strong permeability resistance to tobramycin corresponding to 64 μg/ml. Following electron microscopic observations performed on negative stained samples 1 h after contact of Fluidosomes with *P. aeruginosa* 429, it could be observed that Fluidosomes interact very closely with the outer membrane of the bacteria (Fig. 2, dark spheres). Thickenings of the outer membrane were observed suggesting fusion of the liposomes with

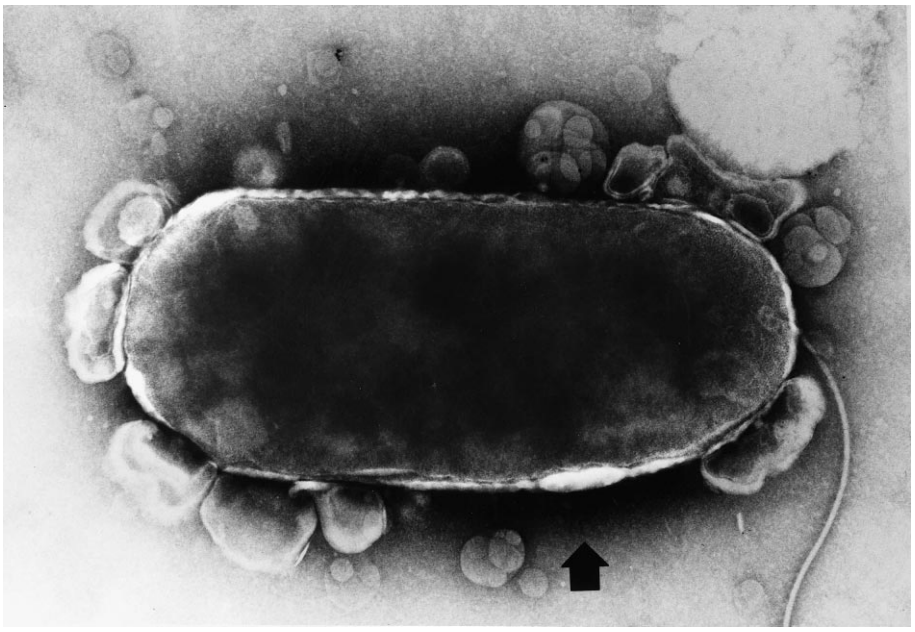

Fig. 2. Interaction of Fluidosomes with *Pseudomonas aeruginosa* 429 cells as observed by negative staining with PTA. Magnification: ×50 561.

the bacterial membranes which could explain the increased penetration of antibiotic with Fluidosomes inside bacterial cells (see arrow on Fig. 2).

To assess the penetration of tobramycin inside the bacterial cells, strain 429 was incubated with free tobramycin, Fluidosomes or PBS. Samples were taken after 0, 1, 2, 3 and 6 h post-treatment. Immunogold techniques were used to reveal the presence of tobramycin. No significant differences were observed in the penetration of antibiotic between encapsulated and free tobramycin after 0–3 h of incubation (data not shown). However, after 6 h of incubation, a noticeable difference could be seen. In longitudinal bacterial sections, up to 23 gold particles could be detected in bacteria exposed to Fluidosomes (Fig. 1A), whereas less than a mean of two gold particles were found in the cytoplasm of cells treated with free antibiotic (Fig. 1B). Statistical analysis of colloidal gold labeling after 6 h of incubation in longitudinal bacterial sections showed an average of 9.54 ± 4.34 gold particles/μm^2 in bacteria exposed to Fluidosomes, whereas only 1.20 ± 1.22 gold particles/μm^2 were enumerated in longitudinal sections of bacteria incubated with free tobramycin ($P < 0.001$). The PBS control gave an average labeling of 0.51 ± 0.82 gold particles/μm^2 in longitudinal sections of bacterial cells. Because transversal sections represent only about 33% of the longitudinal section surface of *P. aeruginosa* cells, only longitudinal sections were selected for enumeration of gold particles to assure the reproducibility of the counts between the different groups of cells. Background labeling, which consisted of gold particles not associated with bacteria or liposomes, was, respectively, of 1.96 ± 0.19, 0.25 ± 0.12 and 0.02 ± 0.02 gold particles/μm^2 in thin sections for Fluidosomes, free tobramycin and PBS control.

3.2. Analysis of fusion by flow cytometry

To verify whether the interaction of Fluidosomes with the bacteria implicates an integration of liposomal phospholipids in bacterial membranes by fusion, FACS studies using PKH-2GL, a fluorescent lipophilic marker, were performed. Labeled Fluidosomes without antibiotic, were incubated with *P. aeruginosa* 429 (MIC 64 μg/ml) and the reference strain ATCC 25619, displaying no resistance to tobramycin. Antibiotic was not encapsulated in Fluidosomes to avoid bacterial cell killing. After 10 min, 0.5, 1, 2, 3, 4, and 6 h of incubation, bacteria were separated by centrifugation through a sucrose cushion and washed. Fu-

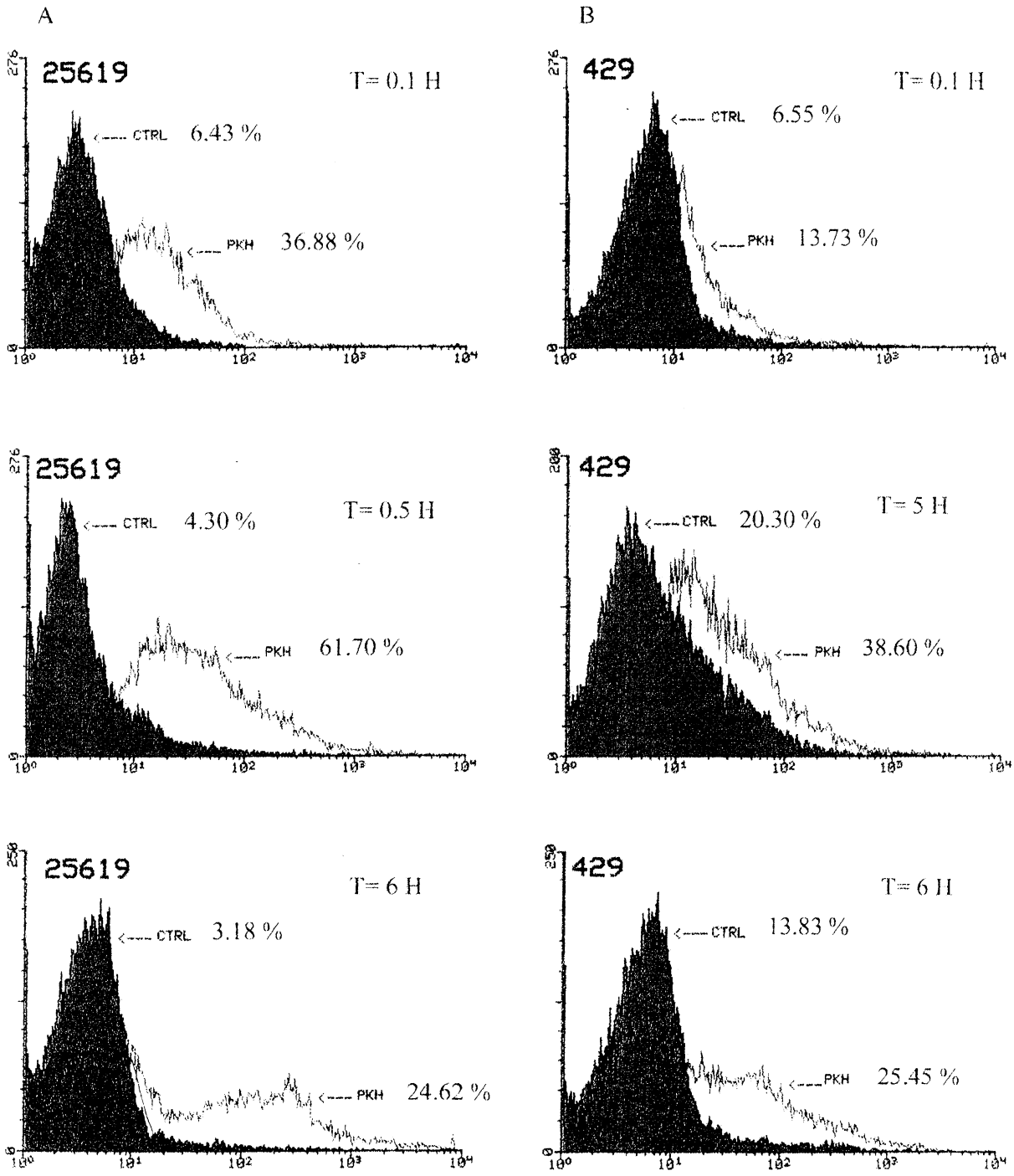

Fig. 3. Flow cytometry histograms of *Pseudomonas aeruginosa* strains ATCC 25619 (A) and 429 (B) incubated with Fluidosomes. T, time (h); CTRL, negative control; PKH, Fluidosomes labeled with PKH. The percentages of fluorescent cells for the negative control and bacteria exposed to PKH2-GL are indicated on the figure. The *x*-axis represents fluorescence intensity and the *y*-axis cell number.

sion was assessed by the integration of phospholipid-PKH2-GL in bacterial cells as demonstrated by flow cytometry studies (Fig. 3). Interaction with the non-resistant strain was observed within 10 min of contact with Fluidosomes reaching a maximum signal of 61.70% of average fluorescent cells after 30 min and decreasing afterwards (Fig. 3A). The pattern observed with the resistant strain, *P. aeruginosa* 429,

was strongly delayed. Indeed, after 10 min of contact, no significant difference was observed between the control (bacteria not exposed to labeled Fluidosomes) and bacteria incubated with liposomal PKH2-GL. As time progressed, the percentage of fluorescent bacteria increased gradually to reach a maximum of 38.60% after 5 h of incubation with liposomal PKH2-GL (Fig. 3B), strengthening the idea of fusion between the liposomes and the bacterial cells as suggested by electron microscopy studies. Positive controls, which consisted of bacteria marked with free PKH2-GL, were not put on the figures for simplicity, but were in the range of 70–95% of fluorescence for the duration of the experiments. This control assured us that PKH2-GL was compatible with bacterial membrane. All studies were repeated at least three times with the same results for a given strain.

3.3. Monitor and quantify fusion by lipid-mixing assay

It has been reported that the lipid-mixing assay, in general, gives a more reliable measurement of membrane fusion since the fluorescence intensity changes are directly related to the distance between NBD and Rh, but not to vesicular aggregation and lipid-exchange [15,18,19]. This assay has been widely used for study of membrane fusion [20–22]. Therefore, the fusion of Fluidosomes with bacteria was further confirmed by the lipid-mixing assay with Rh-PE and NBD-PE as fluorescent probes. Fig. 4 represents the typical fluorescence emission scan profiles of Fluidosome–bacteria fusions monitored by spectrofluorometer with a constant excitation at 475 nm (excitation peak of NBD). As time increased, fluorescence intensity of Rh (590 nm) decreased and NBD signal (520 nm) was increased. It indicates that a significant fusion occurred when Fluidosomes were mixed with bacteria and incubated at 37°C. In control experiments with either Fluidosomes or bacteria, or a mixture of NBD/Rh-labeled Fluidosomes and non-labeled Fluidosomes, no fluorescence signal changes were detectable under the same experimental conditions as for fluidosomal fusion with bacteria (data not shown).

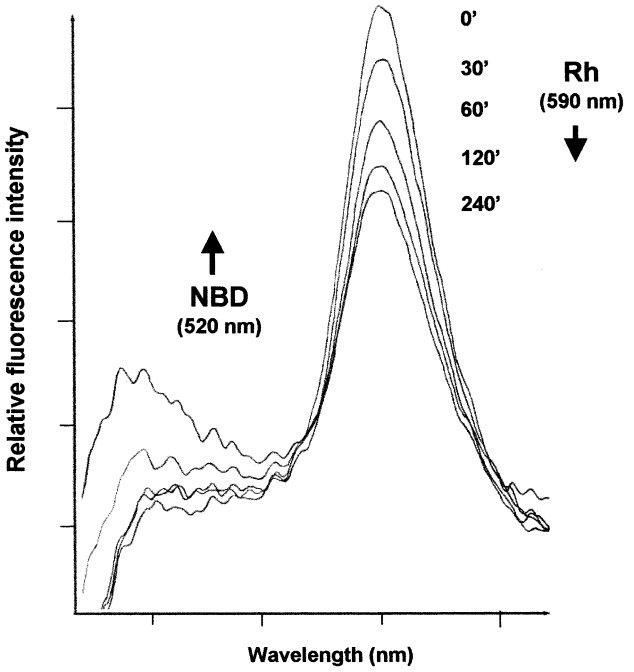

Fig. 4. Fluorescence emission scans of a mixture of Fluidosomes and intact bacteria (*P. aeruginosa* 25619) at 37°C in a wavelength range of 510–610 nm. Fusion of Fluidosomes with bacteria resulted in NBD fluorescence increase and Rh fluorescence decrease (resonance energy transfer efficiency decrease during fusion). Bacteria grown to an OD_{660} of 0.6 in LB medium were then mixed with 36.5 μM of NBD/Rh-labeled Fluidosomes. Fluorescence emission intensity was monitored at different time points using an LS-50b spectrofluorometer (excitation at 475 nm, slit width 7 nm). The emission scans were corrected from bacterial background at the corresponding time points.

The fusion of Fluidosome–bacteria was temperature dependent. There were no significant Rh-fluores-

Table 1
Permeability, MIC and degree of fusion of wild-type and resistant *P. aeruginosa* strains with Fluidosomes

Strain	Permeability	MIC to free tobramycin (μg/ml)	(%) Fusion of Fluidosomes	
			FACS	Lipid mixing
25619	High	0.1	61.7	55 ± 8
429	Low	64	38.6	29 ± 5

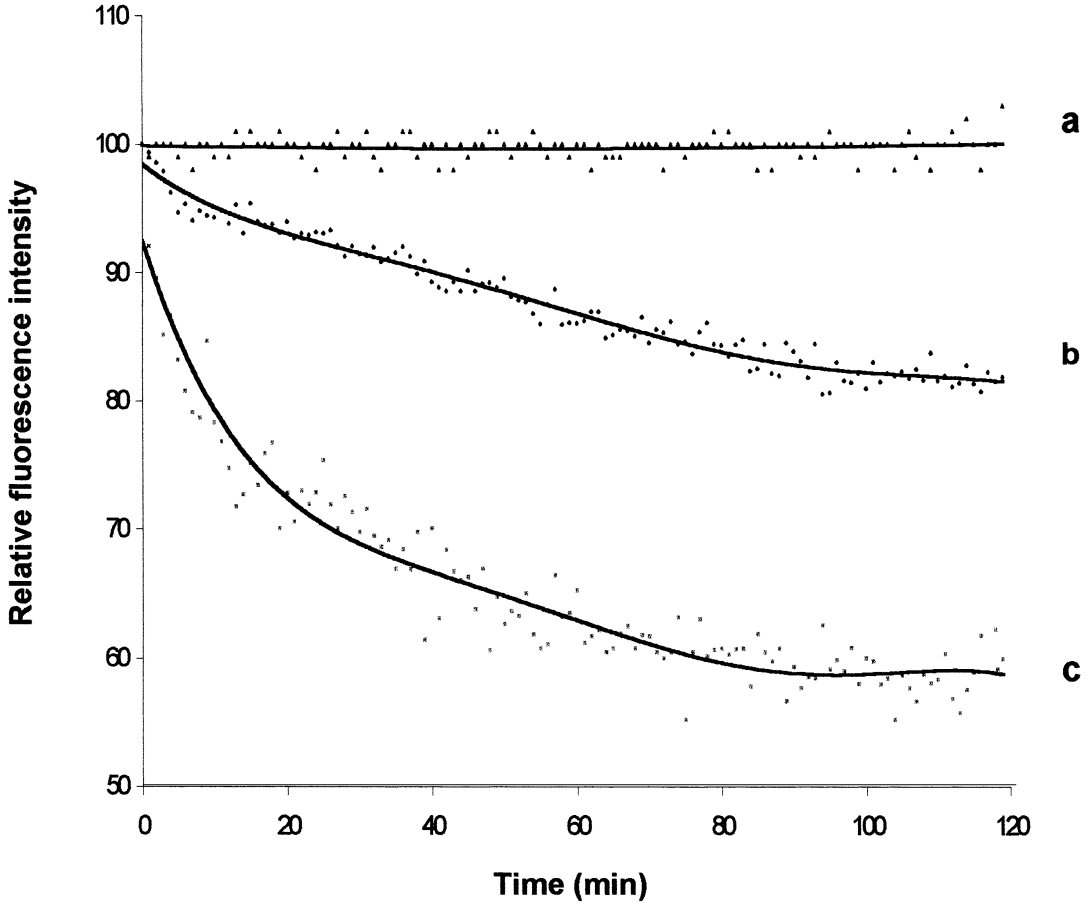

Fig. 5. Fusion of Fluidosomes with intact bacteria monitored by rhodamine fluorescence decrease at 590 nm resulting from resonance energy transfer efficiency decrease (see text). Bacteria grown to an OD_{660} of 0.6 in LB medium were then mixed with 82.5 μM of NBD/Rh-labeled Fluidosomes. Rhodamine emission at 590 nm (slit width 5 nm) was continuously monitored using an LS-50b spectrofluorometer (excitation at 475 nm, slit width 5 nm). Dots were experimental measurements and the solid lines were calculated by regression. A, at 4°C for *P. aeruginosa* 25619 (wild-type); B, at 37°C for *P. aeruginosa* 429 (resistant strain); C, at 37°C for *P. aeruginosa* 25619.

cence decrease at 4°C for both wild-type and drug-resistant bacteria (Fig. 5, plot A; a similar observation obtained for *P. aeruginosa* strain 429, but data not shown). The rate and degree of fusion were also dependent on the nature of bacterial strain. The liposomal fusion with non-resistant strain, such as *P. aeruginosa* 25619, was relatively fast, occurring earlier in accelerating phase at 1 h and reaching slower phase at 2 h (Fig. 5, plot C). While for the drug-resistant strain of *P. aeruginosa* 429, the fusion was delayed substantially and was much lower (Fig. 5, plot B). Table 1 summarizes the degree of fluidosomal fusion with both non-resistant and resistant *P. aeruginosa* strains with two different assays (FACS and lipid mixing).

4. Discussion

Using *P. aeruginosa* 429, it was previously demonstrated that the use of a quantity of tobramycin corresponding to only 50% of the MIC of this bacteria, succeeded in reducing the number of CFU by a factor of 84 times compared to the same quantity of free tobramycin 6 h after the addition of antibiotic [12]. These results strongly suggest that the mode of action of Fluidosomes is related to an uncommon way of penetration in bacterial cells, since a normal diffusion process cannot explain why a quantity of encapsulated antibiotic corresponding to only 50% of the MIC can kill bacteria, whereas the same quantity of free antibiotic is ineffective. Unfortunately, it was

impossible to reproduce such killing curves with strain ATCC 25619 used as control in FACS studies, since this latter strain is sensitive to tobramycin. This strain cannot stay alive in the presence of a minimal concentration of antibiotic, which is essential for measuring a modification of growth following contact with free and encapsulated antibiotic. On the other hand, the sensitivity of the control strain to tobramycin was essential to perform comparative FACS studies with the resistant strain.

Using the following methodologies, negative staining coloration for EM, immunoelectron microscopy, FACS and lipid-mixing assay, we reproduced, in the present work, the kinetic study with strain 429 with the aim to elucidate its mode of interaction with Fluidosomes. EM observations of negative staining coloration of bacterial cell grown in the presence of Fluidosomes (dark spheres in Fig. 1A) show that these latter interact very closely with bacterial membranes (dark spheres in Fig. 1A). Immunogold coloration of ultra-thin sections done with strain 429 showed the presence of greater quantities of tobramycin in bacterial cells incubated with Fluidosomes compared with the free antibiotic ($P < 0.001$) demonstrating increased penetration of the antibiotic when encapsulated (Fig. 1A,B).

Even if the difference of labeling of bacteria exposed to Fluidosomes and free tobramycin was very significant ($P < 0.001$), one could expect a more important labeling if we take into account the strong bactericidal efficacy of Fluidosomes compared to free tobramycin in previous studies [12]. In fact, we found only a few gold particles into each Fluidosomes (data not shown), this means that only a low percentage of molecules of tobramycin are revealed by antibodies/colloid gold particles coupled to protein A/G. Despite this fact, a very significant difference in labeling could be observed between bacteria exposed to Fluidosomes and free tobramycin.

To investigate further the interaction of Fluidosomes with bacterial cells and to determine if the increased penetration of tobramycin was associated with incorporation of liposomal phospholipids in bacterial cells, PKH2-GL was used in cytometry studies. PKH2-GL has the characteristic of inserting itself into membranes due to its aliphatic properties by use of Zyn-linker technology [16,17]. Because of its inherent insolubility in aqueous environments, the probe is trapped once incorporated into the membrane, making it a reliable tracer for fusion and/or integration analysis. Since the probe cannot by itself disincorporate or diffuse from the liposomes to the bacteria using the aqueous environment as an intermediate, this implies that the results observed can only be explained by a direct incorporation of liposomes or liposomal phospholipids in the outer membrane. The cytometry results show that the speed of incorporation of the markers in the two bacteria was very different. The integration of Fluidosomes in the sensitive bacteria (ATCC 25619) was already important 10 min after the addition of liposomes, reaching a maximum value of 61.70% of bacteria containing liposomal-PHK2-GL after 30 min (Fig. 3A). The maximal incorporation was achieved after only 5 h of incubation with the resistant strain (*P. aeruginosa* 429), at which time 38.60% of bacteria contained liposomal-PKH2-GL (Fig. 3B). It is significant that for the resistant strain, optimal interaction took place after 5 h of incubation (Fig. 3B) which is in accordance with the beginning of the bactericidal effect observed after 6 h in previous kinetic studies following interaction of Fluidosomes with *P. aeruginosa* 429 [4]. The FACS study also showed that after 6 h of incubation, two different populations of cells could be distinguished in relation to the incorporation of liposomal-PHK2-GL (Fig. 3A,B). This phenomenon was probably due to the fact that the concentration of liposomal-PKH2-GL stayed the same throughout the whole experiment, whereas as time progressed, liposomal-PKH2-GL was incorporated or fused with bacteria. A fluorescent population of bacteria appeared, meanwhile bacterial growth proceeded and an increasing number of non-fluorescent bacteria repopulated the non-fluorescent pool. This eventually created two populations of cells, which were seen after 6 h of incubation in the two strains tested. Eventually, if additional liposomal-PKH2-GL would be available in the system, one could hypothesize that all bacteria present would be fluorescent. The different levels of liposomal-PKH2-GL incorporations in function of time between the two strains imply that membrane impermeability affects the initiation time, the rapidity and the rate of fusion. But even if the interaction was slowed with the resistant strain, the processes of fusion between the liposomes and the bacterial cells was still important and suffi-

cient to explain the bactericidal activity previously observed with Fluidosomes and *P. aeruginosa* 429 [12]. Finally, the necessity to perform the FACS study in the presence of liposomes free of tobramycin to maintain the integrity of the bacterial cells allowed the observation to be made that tobramycin is not necessary for fusion to occur.

The lipid-mixing assay confirms that the increased fluorescence incorporated into bacterial population observed with liposomal-PKH2-GL is mainly a contribution of fusion, but not aggregation. As mentioned above, the lipid-mixing assay is based on fluorescence resonance energy transfer between the headgroup of NBD and Rh [15]. Dilution of fluorescent lipids from liposomes into the bacterial membranes could either occur through a process of membrane fusion or through transfer of individual molecules. It have been shown that NBD-PE and Rh-PE are non-exchangeable [15,18,19], suggesting that the interaction observed involves membrane fusion. The non-exchangeable nature of Rh-PE and NBD-PE were also confirmed in this study. When a mixture of Rh/NBD-labeled Fluidosomes and non-labeled Fluidosomes was incubated at 37°C for 4 h, there was no significant fluorescence change. In principle, aggregation of liposomes to bacteria can also be ruled out because it does not involve lipid dilution [15,18,19]. Hence fusion between bacteria and Fluidosomes resulting in dilution of fluidosomal lipids into bacterial membrane would be increasing the distance between NBD and Rh and thus decreasing the energy transfer efficiency. It is therefore expected that fusion would lead to an increase in NBD emission and a decrease for rhodamine. A clear fusion pattern is shown in Fig. 4, where rhodamine fluorescence intensity decreased and NBD intensity increased as the incubation time increased. Similar fluorescence emission scan (an increase in the NBD emission and a decrease in rhodamine emission) resulted from a fusion of liposomes with sperm cells has recently been observed by Garrett and co-workers [22]. The observed fluorescence changes were not due to possible biodegradation since there was no significant rhodamine fluorescence decrease when rhodamine emission was monitored under rhodamine excitation at 530 nm instead of NBD excitation at 475 nm by-passing RET (data not show).

Additionally, degrees of fluidosomal fusion with both antibiotic-sensitive (*P. aeruginosa* 25619) and resistant (*P. aeruginosa* 429) strains were compared by kinetically monitoring the decrease of the rhodamine intensity at 590 nm (excitation at 475 nm) with NBD/Rh-labeled Fluidosomes. A substantial rhodamine signal decrease was observed at 37°C for *P. aeruginosa* 25619. On the other hand, rhodamine fluorescence slowly decreased for *P. aeruginosa* 429. The degree of fusion is therefore correlated with the predicted permeability and MIC values of both strains as shown in Table 1. In a control experiment under 4°C, there was no significant decrease in Rh intensity for both strains in presence of Fluidosomes.

The ability of Fluidosomes to increase penetration of antibiotics in bacterial cells by a fusion mechanism would be dependent on the T_C and negative charge of liposomal phospholipids [10,11]. At body temperature, the liposomal phospholipids are in equilibrium between the gel and fluid phases. Membrane merging could be possible due to the formation of domains in the liposomal membrane at temperatures equal or above 37°C. Due to membrane merging, the antibiotic could easily penetrate inside the bacteria allowing the increased bactericidal efficacy observed with the drug, thus circumventing the normal pathway of penetration [11,12]. Earlier studies have shown that empty liposomes do not promote uptake of free tobramycin [12]. This is the first time that fusion between liposomes and bacterial cells based on low T_C of liposomes has been demonstrated to enhance the efficacy of the encapsulated therapeutic agent against resistant strains [11,12]. Usually, drugs entrapped in rigid liposomes may be more effective than free drugs in vivo, but they have generally low bactericidal activity against extracellular bacteria in vitro [23–25]. The superior therapeutic effect of liposome-encapsulated drugs may result from liposomes being delivered to the infection site where they are degraded, releasing their contents. Experimental models of infections in which antimicrobial agents encapsulated in rigid liposomes have been successful in both in vivo and in vitro studies all involved infections of cells of the mononuclear phagocyte system (MPS) by facultative or obligate intracellular pathogens [26,27]. In these studies, uptake of rigid liposomes by phagocytes was essential to obtain a superior therapeutic effect. However, this is not the case with Fluidosomes and efficacy is displayed both in

vitro and in vivo [11,12] against extracellular bacteria and without the involvement of macrophages.

The treatment of bacterial infections is a growing problem. Bacteria are becoming increasingly resistant to a wide variety of antibiotics making their eradication more difficult. The use of fluid liposomes for the treatment of persistent or difficult to treat infections could be an answer to this growing problem of resistance. Fluidosomes possess a unique bactericidal activity against resistant strains and can be used with different drugs (unpublished data). The present work strongly suggests a fusion process between liposomal and bacterial membranes to explain the bactericidal efficacy of Fluidosomes that has been demonstrated in previous studies [11,12]. This demonstration is particularly striking since *P. aeruginosa* possesses one of the most efficient impermeability barrier against antibiotics. The data confirm that Fluidosomes are very promising for patients suffering from infections hard to treat that resist conventional antibiotic therapy. Pre-clinical and clinical studies are in progress to test the efficacy of Fluidosomes in the treatment of cystic fibrosis. Many other applications of Fluidosomes are currently in progress.

Acknowledgements

We thank Robert Alain for his help in electron microscopy analysis and Drs. E.A. Cohen and G. Szatmari for their constructive criticism. This work was supported by a grant from the Canadian Cystic Fibrosis Foundation.

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