Université de Montréal

# Liposomes et polyethylenimine : deux nouveaux outils contre la résistance bactérienne aux antibiotiques

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# Sommaire

*Pseudomonas aeruginosa*, une bactérie pathogène opportuniste par excellence, colonise le tractus respiratoire de la majorité des patients atteints de fibrose kystique. Ce type d'infection s'avère particulièrement résistant aux antibiotiques en raison de l'imperméabilité marquée de la membrane externe de cette bactérie. Ceci fait de *Pseudomonas aeruginosa*, la bactérie responsable du plus haut taux de mortalité chez les patients atteints de fibrose kystique. Dans le but d'accroître le potentiel des antibiotiques, notre laboratoire a développé durant les dernières années une formulation liposomale fluide capable d'accroître le passage des antibiotiques à travers la paroi bactérienne.

Les travaux présentés dans ce mémoire sont divisés en deux volets, tous deux dans l'optique de vaincre la résistance par imperméabilité membranaire. Tout d'abord nous avons adapté une méthode de fluorescence afin de mettre en évidence le phénomène de fusion entre les liposomes fluides et les bactéries Gram négatives. Les résultats obtenus confirment les études de cytométrie en flux et de microscopie électronique effectuée par un autre étudiant de l'équipe. Une fois ce phénomène de fusion démontré, il nous restait à mettre en évidence les différents paramètres qui sont impliqués dans la fusion. A l'aide du transfert d'énergie de fluorescence par résonance, nous avons évalué l'importance de la température, de différents cations divalents, de la fluidité, du pH et finalement des composants de la membrane bactérienne.

Les résultats ont démontré que la fusion entre les liposomes fluides et les bactéries Gram négatives est dépendante de tous les facteurs cités précédemment. Une perturbation de la fluidité membranaire par l'ajout de seulement 10% de cholestérol diminue énormément le niveau de fusion entre les liposomes et les bactéries. Les cations divalents dans la séquence $Fe^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$ ainsi que les pH basiques et acides augmentent le degré de fusion alors que des températures inférieures au Tc des liposomes (dipalmitoyl phosphatidylcholine DPPC : dimiristoyl phosphatidylglycerol DMPG 9:1 mol/mol) diminue leur capacité de fusion.

Le deuxième volet des travaux présentés concerne l'utilisation d'agents polycationiques, en l'occurrence le polyethylenimine (PEI), pour vaincre l'imperméabilité membranaire. Nous avons mis en évidence pour la première fois que le PEI avait un caractère synergique avec de nombreux antibiotiques, autant hydrophiles qu'hydrophobes. Des études de CMI (concentration minimale inhibitrice), de courbe de mortalité et de CFI (concentration fractionnelle inhibitrice) ont été effectuées pour évaluer l'effet synergique du PEI.

Nous avons tout d'abord mis en évidence que le PEI augmente de 5 à 50 fois l'effet bactéricide des β-lactams testés. Ceci représente la première démonstration de l'effet synergique d'un agent polycationique avec des antibiotiques hydrophiles et non pas hydrophobes.

Finalement, nous avons évalué le spectre d'action du PEI sur une vaste gamme d'antibiotiques couramment utilisés. Cette étude a permis de démontrer que le PEI est un antagoniste d'antibiotiques chargés positivement, mais qu'il peut être synergique avec des substances autant hydrophiles qu'hydrophobes.

Le traitement des infections bactériennes est un problème sans cesse grandissant. Nous avons étudié deux nouveaux outils qui pourraient constituer une solution à ce problème. Les liposomes fluides fusionnent avec les bactéries livrant leur contenu. Cette fusion peut être optimisée en variant les différents paramètres mis en évidence dans ce travail. D'un autre côté, le PEI permet d'augmenter l'effet bactéricide de nombreux antibiotiques en créant des imperfections dans la membrane.

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# LISTE DES ABBRÉVIATIONS

| | |
|---|---|
| Atb : | Antibiotique |
| ATCC : | "American type culture collection" |
| CFU : | "colony forming unit" |
| CL : | Cardiolipines |
| DMPG : | dimiristoyl phosphatidylglycérol |
| DNA : | acide déoxyribonucléique |
| DPPC : | Dipalmitoyl phosphatidylcholine |
| EDTA : | Ethylenediaminetetraacetic acid |
| FACS : | "fluorescence activated cell sorting" |
| FIC : | "fractional inhibitory concentration" |
| LPS : | Lipopolysaccharide |
| LUV : | "Large unilamellar vesicles" |
| M : | Molaire |
| ME : | Membrane externe |
| MH : | Mueller hinton |
| MIC : | "minimal inhibitory concentration" |
| MLV : | "multilamellar vesicles" |
| mM : | Millimolaire |
| mol% : | Pourcentage molaire |
| nm : | Nanomètre |
| N-NBD-PE : | (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl |
| N-Rh-PE : | (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl) |
| O.D. : | "optical density" |
| OM : | "outer membrane" |
| PBS : | Tampon phosphate saline |
| PC : | Phosphatidylcholine |

| | |
|---|---|
| PE : | Phosphatidylethanolamine |
| PEG : | Poly(ethyleneglycol) |
| PEI : | Polyethylenimine |
| PG : | Phosphatidylglycérol |
| PMBN : | Polymixin B nonapeptide |
| PS : | Phophatidylsérine |
| RET : | "resonance energy transfer" |
| SD : | "standard deviation" |
| SM : | Sphingomyéline |
| SUV : | "small unlamellar vesicles" |
| Tc : | Température de transition de phase" |
| TEFR : | Transfert d'énergie de fluorescence par résonance |
| TSB : | "tryptic soy broth" |
| $V_E$ : | Forces de répulsions électrostatiques |
| $V_T$ : | Somme des forces |
| $V_W$ : | Forces d'Attractions de Van der Waals |

# CHAPITRE 1- Revue de la littérature

## 1.1 La résistance bactérienne aux antibiotiques

### 1.1.1 Historique

La découverte des antibiotiques a constitué une véritable révolution dans le domaine des maladies infectieuses. L'antibiothérapie a sauvé un très grand nombre de vies et l'on a cru que les maladies infectieuses seraient toutes un jour jugulées. C'était occulter la capacité d'adaptation des micro-organismes et leur potentiel de devenir résistants aux drogues. Le phénomène de résistance bactérienne aux antibiotiques mit fin à cette « fatale illusion ». En 1943, Florey et collaborateurs ont publié la première étude clinique démontrant l'efficacité de la pénicilline chez des blessés de guerre durant la campagne d'Afrique du Nord (Florey and Cairns, 1943). On avait découvert le « magic bullet » capable de tuer le parasite sans affecter l'hôte, déjà recherché dès le 19$^{e}$ siècle par le chimiste allemand Paul Ehrlich. Peu de temps après que les hôpitaux aient commencé à utiliser la pénicilline, des souches de *Staphylococcus aureus* résistantes ont été isolées. En 1950, ces souches étaient la principale cause d'infections nosocomiales dans les hôpitaux où la pénicilline était utilisée.

### 1.1.2 Principaux mécanismes de résistance

Les bactéries utilisent principalement trois stratégies de résistance : (1) la modification de la perméabilité membranaire, (2) la modification de la cible, et (3) la modification de l'antibiotique. Elles modifient leur perméabilité soit en devenant imperméables aux antibiotiques, soit en excrétant ces derniers activement (Nikaido et al., 1985). Comme alternative, elles peuvent également modifier la structure de la cible moléculaire de l'antibiotique, souvent une enzyme essentielle au métabolisme de la bactérie, et échapper ainsi à l'effet toxique de l'antibiotique. Finalement, elles peuvent produire des enzymes capables d'altérer l'antibiotique et l'inactiver. Les bactéries ont également développé des systèmes de transfert et d'accumulation de gènes de résistance extrêmement efficaces ce qui permet une dissémination rapide de la résistance.

La résistance aux antibiotiques peut se diviser en trois catégories : la résistance naturelle ou intrinsèque, la résistance transitoire et finalement la résistance acquise. La résistance intrinsèque est présente dans toutes les souches de l'espèce considérée et préexiste à l'usage des antibiotiques. Elle constitue une caractéristique propre à l'espèce et délimite le spectre d'activité des antibiotiques. Elle comprend les mécanismes de résistance cités précédemment. La résistance transitoire fait suite à une exposition de la souche à des conditions stimulantes comme l'exposition aux antibiotiques. Elle n'entraîne aucun changement observable dans le génotype bactérien. Une telle résistance est réversible et la

souche qui l'exprime retrouvera son caractère sensible lorsque les conditions stimulantes ne seront plus présentes. En revanche la résistance acquise n'est présente que chez quelques souches d'une espèce normalement sensible et apparaît à la suite de l'utilisation des antibiotiques. Cette forme de résistance est portée le plus souvent par des éléments mobiles (plasmides ou transposons) (Bidwell et al., 1981).

Dans ce présent travail, l'emphase sera mise sur les mécanismes de résistance attribuable à l'imperméabilité membranaire des bactéries Gram négatives, plus particulièrement celle de *Pseudomonas aeruginosa*.

## 1.2 Rôle de la membrane externe des bactéries Gram négatives dans la résistance aux antibiotiques.

### 1.2.1 Introduction

Les bactéries Gram négatives sont en général beaucoup plus résistantes que les Gram positives à un large spectre d'antibiotiques. Une étude a récemment démontré que plus de 90% des antibiotiques naturels testés étaient inefficaces contre *Escherichia coli*, alors qu'ils étaient efficaces contre des bactéries Gram positives (Vaara, 1993). L'importance de la membrane externe (ME) chez les bactéries Gram négatives est de plus en plus évidente. Elle permet à ces dernières d'être résistantes à des facteurs de défense de l'hôte tel les lysozymes, la β-lysine

et diverses protéines leucocytaires qui sont très toxiques envers les bactéries Gram positives (Donaldson et al., 1974; Patterson-Delafield et al., 1980; Rest et al., 1977). Chez les bactéries Gram négatives entériques, qui vivent dans le tractus intestinal des animaux, la ME confère une protection extrêmement efficace contre les sels biliaires et la dégradation par les enzymes digestives (Nikaido and Nakae, 1979). La ME agit également comme une barrière imperméable et ralentit l'entrée d'une multitude d'antibiotiques dans les cellules bactériennes (macrolides, novobiocin, rifamycins, lincomycin, clindamycin, acide fusidique, etc...) (Nikaido, 1984; Nikaido, 1985). Même si la diffusion de l'antibiotique n'est que ralentie, la bactérie peut alors inactiver la faible quantité ayant pénétré au lieu d'essayer d'inactiver toutes les molécules présentes dans le milieu. Ceci permet d'atteindre des niveaux de résistance incroyablement élevés chez les bactéries Gram négatives (Richmond and Curtis, 1974). Ces phénomènes sont manifestement reliés à la prévalence des infections nosocomiales causées par des bactéries Gram négatives.

### 1.2.2 La membrane externe en tant que barrière

#### 1.2.2.1 Structure

Les bactéries Gram négatives possèdent une ME recouvrant la couche de peptidoglycane et la membrane cytoplasmique. Habituellement, les membranes lipidiques sont très perméables aux substances hydrophobiques (Stein WD, 1967).

Cependant chez les bactéries entériques telle que *Escherichia coli* cette ME est une barrière extrêmement efficace contre l'entrée d'agents hydrophobiques tel l'actinomycin D, la novobiocine, la rifamycine, les macrolides et divers détergents (Nikaido, 1976). La ME possède une composition chimique distincte de celle des membranes biologiques habituelles. Sa structure est une double couche dont le feuillet interne à une composition ressemblant à celle de la membrane cytoplasmique. Son feuillet externe, par contre, possède un constituant unique à la place des phospholipides (Figure 1). Ce composé, le lipopolysaccharide bactérien ou LPS, est une molécule complexe qu'on ne trouve pas ailleurs dans la nature (Kamio and Nikaido, 1976; Nikaido and Vaara, 1985). Le résultat est que les feuillets de cette membrane sont asymétriques, et que les propriétés de cette double couche inhabituelle diffèrent considérablement de celles d'une membrane biologique normale.

### 1.2.2.2 Les lipopolysaccharides

Les lipopolysaccharides sont constitués de trois parties, un lipide appelé lipide A, une courte série de sucres constituant la partie centrale et une longue chaîne glucidique appelée antigène O (Figure 2) (Galanos et al., 1977; Lüderitz et al., 1982; Nikaido, 1973). Le Lipide A ancre le LPS dans le feuillet externe de la membrane. C'est un glycolipide inhabituel, dans la mesure où les acides gras qui sont attachés au disaccharide sont plus courts que de coutume (une longueur de 14 carbones au lieu des 16 à 18 communément rencontrés). Tous ces acides gras sont

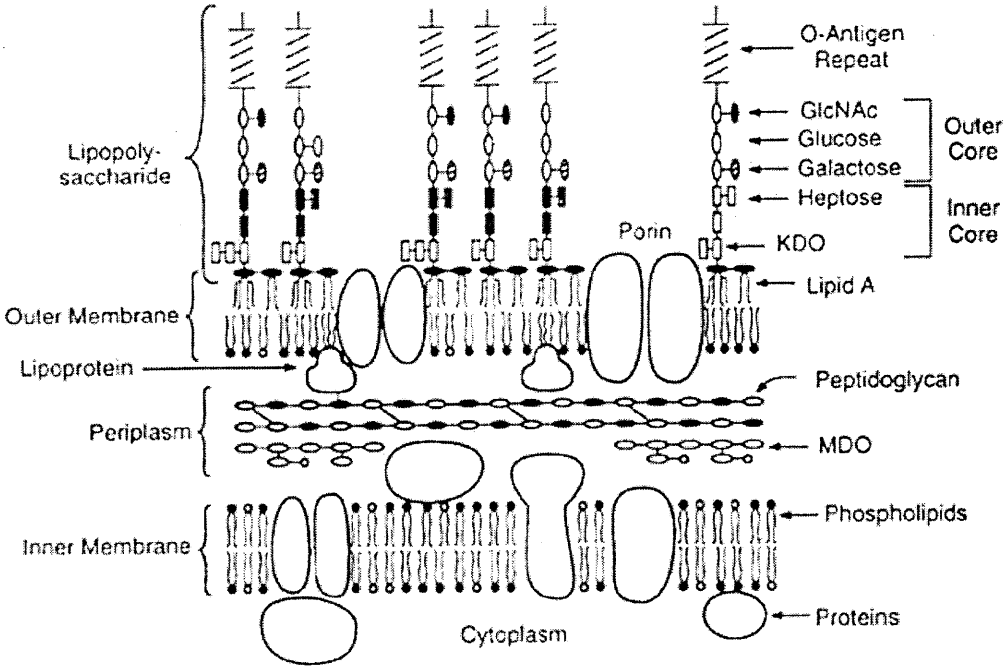


**Figure 1**. Illustration de l'organisation de la membrane externe des bactéries gram négatives (Voet et Voet, 1990)

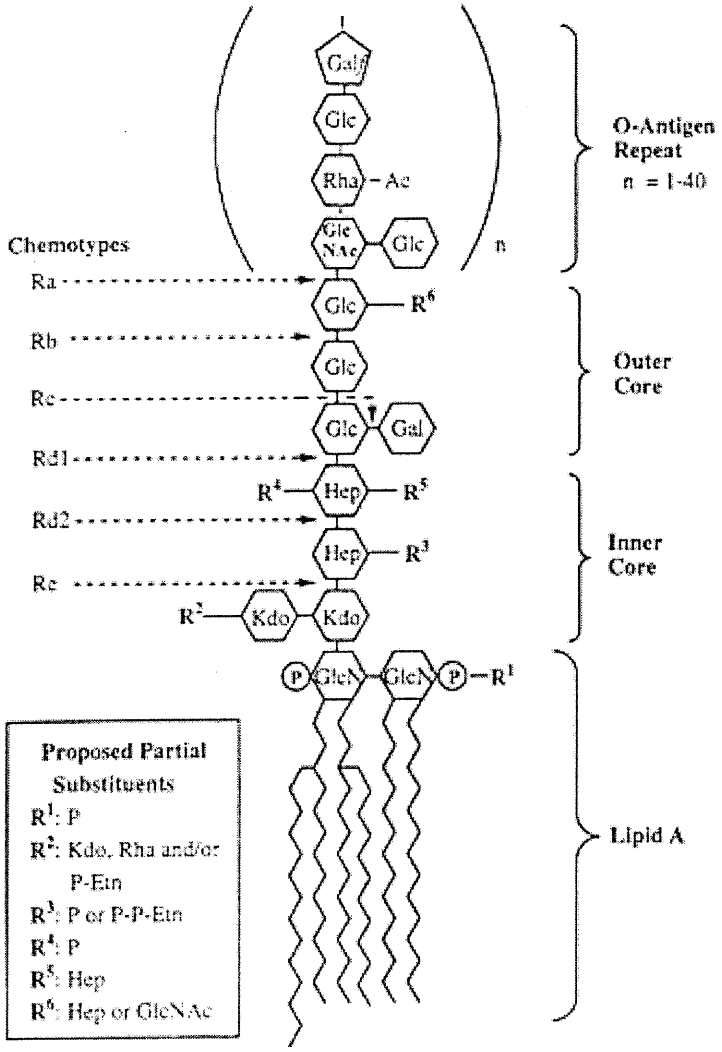


**Figure 2.** Structure moléculaire d'un lipopolysaccharide (LPS) bactérien (Voet and voet, 1990)

saturés et sont substitués par des groupements hydroxyles. Contrairement aux phospholipides qui ne possèdent que deux chaînes d'acide gras, les LPS possèdent six ou sept chaînes d'acide gras reliées au disaccharide. De plus des groupes phosphate sont attachés au disaccharide. Cette conformation inhabituelle des acides gras leur confère une structure beaucoup moins fluide, quasi cristalline, comparativement aux phospholipides, et peut donc servir de barrière (Labischinski et al., 1985; Nikaido et al., 1977).

La partie centrale ou « core » possède une structure relativement constante parmi les bactéries Gram négatives. Elle comprend deux sucres caractéristiques, l'acide céto-désoxyoctonoïque (KDO) et un heptose. Ces deux régions, le lipide A et la partie centrale, contiennent énormément de charges négatives. En présence de cations divalents, ces structures forment alors un réseau entrelacé extrêmement stable (Nikaido and Vaara, 1985). Ces groupements sont cependant, en même temps, le talon d'Achille des bactéries Gram négatives en présence d'agents polycationiques comme nous le verrons plus loin (Vaara, 1992).

La dernière partie des LPS, l'antigène O, est constituée d'une longue chaîne glucidique, dont la longueur peut atteindre 40 sucres. Ces chaînes glucidiques recouvrent toute la surface bactérienne. Bien qu'il s'agisse d'une structure assez lâche, elle est très efficace pour repousser les composés hydrophobes. Les mutants qui ne forment pas d'antigène O deviennent sensibles à des composés comme les sels biliaires et certains antibiotiques auxquels la souche

sauvage est résistante (Nikaido, 1976).

La solution particulière que les bactéries Gram négatives ont apporté au problème de la protection de la membrane cytoplasmique a des conséquences biologiques inattendues. Le LPS de la ME est hautement réactif lorsqu'il est introduit dans l'animal. La partie lipide A possède un grand nombre d'activités biologiques, dépendamment de sa concentration. A petites doses, il provoque de la fièvre et active une série d'évènements immunologiques et biochimiques qui conduisent à la mise en alerte des mécanismes de défense de l'hôte. A fortes doses, ce composé, également connu sous le nom d'endotoxine, peut provoquer un état de choc et même la mort (Voet et al., 1990).

Si le feuillet externe de la ME était composé uniquement de LPS, très peu de molécules (nutriments essentiels) seraient capables de la franchir pour rejoindre la membrane cytoplasmique. C'est pourquoi, plusieurs protéines, en particulier des porines, sont parsemées dans la ME modifiant ainsi sa perméabilité (Benz, 1985; Nikaido and Vaara, 1985).

### 1.2.2.3 Les porines

Toutes les bactéries Gram négatives produisent probablement des porines (Nikaido and Vaara, 1985; Vachon et al., 1985; Bavoil et al., 1984). Généralement, les porines possèdent un poids moléculaire entre 30000 et 40000 et permettent la diffusion passive de composés hydrophiles comme les sucres, les

acides aminés, et certains ions. Elles sont souvent sous forme de trimères, bien que récemment une forme dimérique ait été rapportée (Zalman and Nikaido, 1985). Les canaux des protéines sont étroits, d'une taille permettant juste le passage de molécules de masse inférieure à 600-700 daltons. Les bactéries possèdent un nombre extrêmement élevé de porines. Une unique cellule d'*Escherichia coli* peut contenir, par exemple, $10^5$ porines. Il n'existe aucune évidence jusqu'à présent que les porines permettent uniquement la diffusion de molécules spécifiques (Nikaido and Vaara, 1985).

### 1.2.3 Rôle dans la résistance aux antibiotiques par diminution de la perméabilité

#### 1.2.3.1 Altération des protéines de la membrane externe

Komatsu et collaborateurs ont démontré qu'une diminution de la perméabilité de la ME entraînait une augmentation du niveau de résistance aux β-lactams. En altérant la production des porines OmpF et/ou OmpC au niveau du locus *ompB,* ils ont observé une augmentation du niveau de résistance au cefoxitin, un membre de la famille des β-lactams (Komatsu et al., 1981).

#### 1.2.3.2 Modification des lipopolysaccharides

Une altération au niveau de la structure des LPS peut également entraîner

un changement dans le niveau de résistance aux antibiotiques. Ceci a beaucoup été étudié dans le cas de mutants de *Salmonella typhimurium* ayant des LPS tronqués (Ames et al., 1974; Janzer et al., 1981; Koplow and Goldfine, 1974; Nikaido, 1976; Nikaido and Nakae, 1979).

Les changements dans le LPS sont également associés à une augmentation de la résistance intrinsèque chez *Pseudomonas aeruginosa*. En 1976, Nakae démontre que la pénétration des β-lactamines à travers la membrane externe, est inhibée chez les mutants de *Pseudomonas aeruginosa* ayant des altérations au niveau du LPS. Une diminution des sucres aminés des chaînes antigéniques des LPS réduit le passage des β-lactamines. Ces variations sont souvent accompagnées d'une perte de spécificité pour le sérotype et une susceptibilité aux phages (Livermore, 1988). La signification clinique de ces modifications en tant que mécanismes de résistance acquise n'a pas été encore étudiée.

### 1.2.3.3 Acquisition d'un phénotype muqueux

La relation entre la production d'exopolysaccharides et la résistance aux antibiotiques a été énormément étudiée chez *Pseudomonas aeruginosa*. Les infections pulmonaires chroniques à *P. aeruginosa* chez les patients souffrant de fibrose kystique sont associées principalement au phénotype muqueux. Cette mucosité confère à cette bactérie un avantage sélectif par rapport aux autres microorganismes et même au *P.aeruginosa* phénotype lisse. Une fois acquise elle

permet à *P.aeruginosa* de persister au niveau des poumons et signe généralement un mauvais pronostic. Cette mucosité, appelée aussi biofilm, mucoexopolysaccharide, « slime » ou encore glycocalyx est composée principalement d'alginate, un polymère acétyle d'acide β D-mannuronique et α L-guluronique (Costerton et al., 1981; Pedersen et al., 1989). Cet exopolysaccharide, associé à une grande quantité d'eau et de cations, aboutit à la formation d'une vraie coque autour des bactéries. Dans cette coque les bactéries n'auront pas accès à tous les nutriments et vont voir leur croissance et leur métabolisme ralentis et par conséquent vont former des microcolonies (Lam et al., 1980; Costerton et al., 1987). Cette couche d'alginate va empêcher le système immunitaire de l'hôte de combattre la bactérie bien que la réponse immunitaire générale soit normale (Bryan et al., 1983). L'alginate inhibe l'opsonophagocytose en empêchant l'adhérence des anticorps sur la bactérie (Pier, 1989) et la qualité de l'opsonisation est détériorée une fois l'infection installée (Tosi et al., 1995). L'alginate aurait également un rôle dans l'adhérence de *P. aeruginosa* aux cellules épithéliales buccales (Doig et al., 1987) et au niveau du tractus respiratoire favorisant ainsi la colonisation de ce dernier (Baker and Svanborg-Eden, 1989). L'alginate constitue une véritable barrière à la pénétration de plusieurs antibiotiques, il entrave non seulement la diffusion de la tobramycine (Nichols et al., 1988) mais aussi celle des β-lactamines (Bolister et al., 1991). Étant donné la tendance de ces *P. aeruginosa* à former des microcolonies dans lesquelles la croissance des bactéries est ralentie, l'action de plusieurs antibiotiques sur ce mode de croissance s'avère inefficace (Stewart, 1994).

### 1.2.3.4 Combinaison de l'imperméabilité membranaire avec d'autres mécanismes de résistance

Les mécanismes de résistance ne sont pas mutuellement exclusifs et ils travaillent souvent en synergie pour accroître la résistance aux antibiotiques. Dans le cas de la résistance aux β-lactamines, par exemple, la diminution de la perméabilité agit en conjonction avec les β-lactamases bactériennes qui sont localisées dans l'espace périplasmique (Bidwell and Reeves, 1981; Richmond, 1978). Le ralentissement de l'entrée des antibiotiques permet d'aboutir à un état où le taux d'hydrolyse des β-lactames est plus rapide que l'entrée empêchant ainsi l'antibiotique d'atteindre sa cible (Ohmori et al., 1977).

L'imperméabilité membranaire peut également être associée à un reflux actif des molécules d'antibiotiques accumulées à l'intérieur de la cellule. Ces pompes à reflux ont été démontrées récemment comme très importantes dans la résistance de *P. aeruginosa* aux antibiotiques (Poole et al., 1993). La famille des pompes RND (« résistance-nodulation-division »), souvent retrouvées chez *P. aeruginosa* agit sur une multitude de composés incluant la tétracycline, le chloramphénicol, les quinolones, la novobiocine, les macrolides, le triméthoprime et apparemment les β-lactames et inhibiteurs de β-lactamases (Poole et al., 1993; Gotoh et al., 1995; Li et al., 1995; Li et al., 1998; Kohler et al., 1996). Ces pompes sont composées de trois sous-unités : un transporteur qui se retrouve dans la

membrane cytoplasmique de la bactérie et qui pompe les composés vers l'extérieur, une porine située dans la membrane externe et qui sert de sortie à l'agent expulsé par la pompe, et finalement une protéine accessoire qui sert de pont entre la protéine interne (pompe) et externe (porine) (Figure 3).

## 1.3 Les liposomes

### 1.3.1 Perspective historique

Les liposomes, décrits en 1965 pour la première fois par Bangham, sont des structures sphériques lipidiques, unies ou multilamellaires séparées par des espaces aqueux. Utilisés d'abord par les biologistes comme modèles pour l'étude de la perméabilité des membranes cellulaires, les liposomes sont maintenant très largement étudiés comme vecteurs possibles de substances médicamenteuses. Les liposomes permettent d'encapsuler, dans des volumes extrêmement faibles, une grande variété de composés hydrophiles ou lipophiles sans qu'il soit nécessaire de créer une liaison covalente entre le médicament et son transporteur (Bangham et al., 1965; Rahman et al., 1973; Gregoriadis, 1976; Gregoriadis, 1977; papahadjopoulos, 1978). Depuis, les chercheurs incorporent dans les liposomes des protéines tels des anticorps (Alving and Richards, 1983), des agents anti-inflammatoires (de Silva et al., 1979), des antibiotiques (Nacucchio et al., 1985; Swenson et al., 1988; Beaulac et al., 1996; Beaulac et al., 1998) (Nacucchio et al., 1988) et des antifongiques (Lopez-Berestein, 1987; Lopez-Berestein et al., 1985).

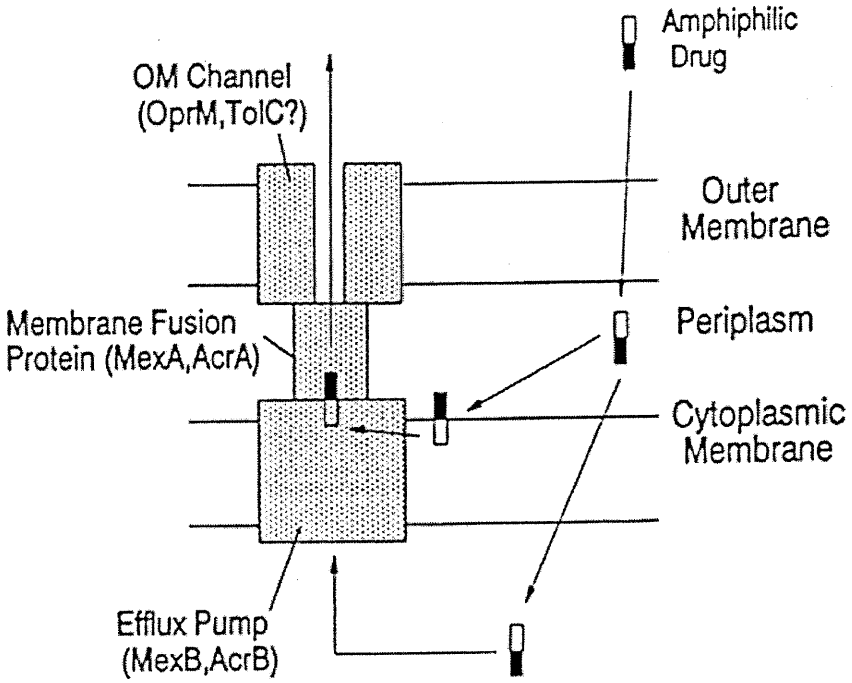


**Figure 3.** Modèle de pompe à reflux chez les bactéries Gram négatives (Nikaido, 1996)

### 1.3.2 Polymorphisme des phospholipides

Les phospholipides sont des molécules amphiphiles constituées d'une zone polaire ou chargée, à forte affinité pour l'eau, et d'une zone apolaire hydrophobe constituée de chaînes aliphatiques. De cette particularité structurale, découle une propriété fondamentale : leur tendance à s'auto-associer pour former des édifices supramoléculaires. Cette structure spontanée en présence d'eau conduit, en général, à des bicouches dont les propriétés varient très largement avec de nombreux paramètres tels que le taux d'hydratation, la température, le pH et la présence d'ions. Ces structures ne sont stabilisées que par des interactions faibles entre les différents constituants, les molécules au sein de l'édifice gardant donc une liberté de mouvement très importante.

La figure 4 représente la structure générale des phospholipides naturels, deux chaînes d'acide gras estérifiant les fonctions alcool du glycérol en positions 1 et 2. Les divers groupes polaires fixés sur le phosphate estérifiant la position 3 du glycérol conduisent aux diverses classes des glycérophospholipides. D'autres phospholipides s'écartent de cette structure, c'est le cas des cardiolipines (CL) qui possèdent quatre chaînes, mais aussi des sphingomyélines (SM). Ces phospholipides ne possèdent plus un squelette glycérol : c'est une chaîne sphingosine qui est amidée par un acide gras sur le groupe NH.

Les phospholipides présentent, en général, un double polymorphisme caractéristique : ce sont des composés lyotropes (phases de structures différentes selon les quantités d'eau) mais également thermotropes (changement de phase par

variation thermique).

### 1.3.3 Classification

L'hydratation des phospholipides a pour conséquence l'apparition de nombreuses phases de structures variées. Trois types de liposomes ou de vésicules peuvent être obtenus. Les vésicules ou liposomes multilamellaires (MLV, multilamellar vesicles) ont été le premier type de liposomes décrit par Bangham; ils sont composés de plusieurs compartiments aqueux concentriques. Leur taille est généralement comprise entre 100 nm et 1 μm et comporte cinq lamelles ou plus. Les petites vésicules unilamellaires (SUV, small unilamellar vesicles) comportent une seule paroi et une seule cavité aqueuse et leur diamètre est de l'ordre de 25 nm. Les grosses vésicules unilamellaires (LUV, Large unilamellar vesicles) ont une dimension similaire aux MLV mais sont entourées d'une seule bicouche lipidique (Puisieux, 1983; Marchal-Heussler et al., 1990).

### 1.3.4 Propriétés physico-chimiques

La composition chimique des liposomes, et donc le choix des phospholipides utilisés pour les fabriquer, dicte les propriétés physico-chimiques de ceux-ci. Les principaux facteurs en jeu sont, la température de transition de phase, la fluidité, la charge, et le pH.

Chaque phospholipide se caractérise par une température de transition de

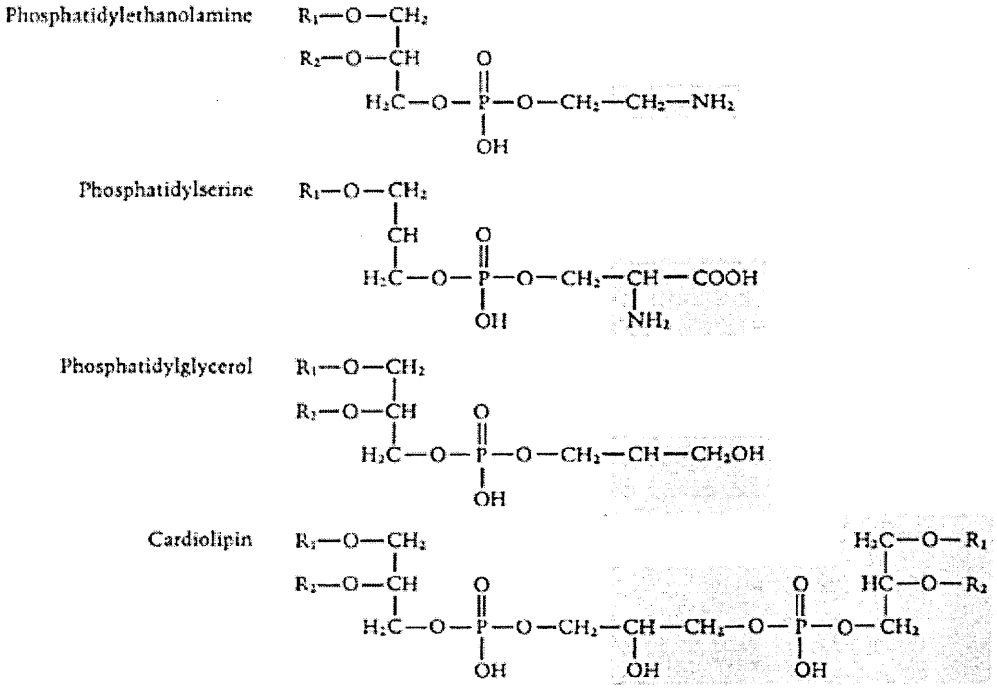


**Figure 4.** Structure des phospholipides naturels les plus répandus. R1 et R2 représentant des chaînes d'acide gras (Neidhardt et al., 1990)

phase (Tc), qui se définit comme la température minimale requise pour permettre à l'eau de pénétrer entre les couches de lipides (New, 1995). A cette température, la chaîne d'acide gras « fond », le mouvement moléculaire s'accélère et l'épaisseur de la membrane ainsi que l'espace entre les molécules augmente. A une température < Tc, la membrane est dans un état de « gel » et possède peu de mouvements moléculaires. A une température > Tc, il y a une dissociation du réseau ionique par l'entrée d'eau, et la membrane entre dans un état fluide caractérisé par un mouvement rapide des molécules. La Tc dépend de la nature de la chaîne d'acide gras et de la région polaire (New, 1995). La Tc est en général abaissée par la diminution de la longueur de la chaîne acyle, par l'existence de doubles liaisons entre deux carbones, par la ramification de la chaîne et par la présence de noyaux aromatiques (New, 1995; Szoka, Jr. and Papahadjopoulos, 1980). L'ajout de cholestérol aux liposomes permet d'augmenter ou de diminuer la fluidité membranaire. Le cholestérol réduit la fluidité membranaire au-dessus de la température de transition de phase, ce qui est directement corrélé à une diminution de la perméabilité. Par contre l'ajout de cholestérol augmente la fluidité membranaire en dessous de la température de transition de phase ce qui peut aboutir à une instabilité (New, 1995).

La charge des liposomes est également un facteur qui dépend de la composition en phospholipides. Le phosphatidylglycérol (PG) et la phosphatidylsérine (PS) sont des exemples de phospholipides chargés négativements. La phosphatidylcholine (PC) et la phosphatidyléthanolamine (PE) sont par contre des exemples de lipides neutres. La charge est importante dans

l'élaboration d'une formulation liposomale, car elle influence directement l'interaction des liposomes avec des membranes biologiques ou artificielles et avec l'agent thérapeutique encapsulé (New, 1995).

Le pH a une influence importante sur les propriétés physico-chimiques des liposomes. L'incorporation de phospholipides ayant une tête polaire ionisable (ex . PE) confère une instabilité à la membrane à certains pH. Cette instabilité peut conduire à une fuite du contenu, fusion ou rupture du liposome. Cette sensibilité au pH a ses avantages et ses inconvénients. Des liposomes sensibles au pH sont souvent utilisés comme véhicules pour cibler le cytoplasme tandis que des liposomes résistants au pH sont utilisés pour cibler les lysosomes (Chu et al., 1990).

### 1.3.5 Les liposomes fluides

Une formulation liposomale fluide, appelée Fluidosomes$^{TM}$, a démontré sa capacité de faciliter le passage des antibiotiques à travers la paroi bactérienne. Cette formulation liposomale, développée dans notre laboratoire, est caractérisée par sa fluidité. Il a été démontré lors de tests *in vitro* et *in vivo* qu'une activité bactéricide fortement amplifiée contre *Pseudomonas aeruginosa* et d'autres bactéries pouvait être obtenue lorsqu'un antibiotique était encapsulé dans les fluidosomes comparativement à sa forme libre (Beaulac et al., 1997; Beaulac et al., 1998; New, 1995).

Les Fluidosomes$^{TM}$ permettent également une présence prolongée de l'antibiotique au niveau pulmonaire et une réduction de l'accumulation de l'antibiotique dans les reins (Beaulac et al., 1996; Beaulac et al., 1997; New, 1995).

Les liposomes fluides sont composés de deux phospholipides : du dipalmitoylphosphatidylcholine (DPPC) et du dimiristoyl phosphatidylglycérol (DMPG) dans un ratio molaire de 10 :1-18 :1. Ils possèdent une température de transition de phase de 37-40°C, ce qui leurs confèrent un caractère fluide aux températures corporelles.

## 1.4 La fusion membranaire

Les liposomes sont souvent utilisés comme véhicule pour introduire des molécules dans les cellules. Cependant, la majorité de ces études concernent des cellules eucaryotes (Gregoriadis, 1976; Gregoriadis, 1977). Seulement quelques études ont été publiées sur l'interaction des liposomes avec des cellules procaryotes. La fusion de liposomes avec la ME de *Salmonella typhimurium* (Jones and Osborn, 1977a; Jones and Osborn, 1977b), le transfert de plasmides encapsulés dans *Escherichia coli* (Fraley et al., 1979), et *Mycoplasma capricolum* (Nicolau and Rottem, 1982) sont quelques uns des rares exemples.

### 1.4.1 Principe de la fusion membranaire

Le processus de fusion entre deux vésicules peut être décortiqué en trois étapes. Tout d'abord, il doit y avoir une agregation ou adherence des vesicules qui est sujette à trois différentes forces d'interactions (Nir and Andersen, 1977; Rand, 1981; McIntosh and Simon, 1986). Ces forces sont : l'attraction ou répulsion électrostatique, l'interaction de Van der Waals et finalement les forces d'hydratation. Les deux premières forces sont dites de longue portée, elles agissent à des distances beaucoup plus grande que 1 nm. Dans le cas de deux particules de même charge, la force électrostatique sera répulsive et diminuera exponentiellement avec la distance, tandis que la force de Van der Waals (ou électrodynamique) est attractive et augmente lorsque les vésicules se rapprochent. Lorsque les deux vésicules sont amenées à se rapprocher, il y a création d'une imposante force d'hydratation répulsive. Ceci est représenté par l'énergie nécessaire à la dissociation des molécules d'eau liées fermement aux têtes polaires des phospholipides (Figure 5) (Rand, 1981; LeNeveu et al., 1976). Les forces d'hydratation deviennent significatives à des distances de séparation de l'ordre de 2-3.5 nm selon la nature des lipides en jeu. La figure 6 représente l'impact de ces trois différentes forces sur le rapprochement de deux vésicules.

La force d'hydratation est donc la principale barrière que deux vésicules doivent surmonter pour pouvoir fusionner. Ceci explique pourquoi des agents déshydratants comme le poly(ethylèneglycol) (PEG) possèdent la capacité d'induire une fusion membranaire (MacDonald, 1985). Les cations divalents tels le calcium ou le magnésium possèdent également une activité déshydratante et donc fusogénique (Wilschut et al., 1981).

L'agrégation des vésicules est un phénomène nécessaire, mais insuffisant par soi même, pour une fusion membranaire. Une perturbation locale de l'organisation des phospholipides est également nécessaire (Hui et al., 1981). Cette perturbation fait suite à la déshydratation des têtes polaires comme le montre la figure 7. Ce réarrangement des phospholipides aboutit finalement à un mélange du contenu aqueux des deux vésicules après une réorganisation des phospholipides du feuillet interne.

### 1.4.2 Méthodes de mesure de la fusion

Plusieurs méthodes ont été développées pour mesurer le mélange de phospholipides (i.e. la fusion) entre deux membranes. Une des plus connues est basée sur le transfert d'énergie de fluorescence par résonance (TEFR) entre une paire de donneurs fluorescents et accepteurs (Figure 8). Le couple de molécules le plus utilisé pour le TEFR est constitué de deux dérivés de phosphatidylethanolamine (PE), marqués au niveau de leur têtes polaires : N-NBD-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) et N-Rh-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl), respectivement le donneur fluorescent et l'accepteur (Struck et al., 1981). Le couple de sondes est habituellement incorporé à 0.2 mol% (0,2 moles pour 100 moles) chacun dans les vésicules. Lorsque ces vésicules sont mises en présence de la longueur d'onde excitatrice de la sonde

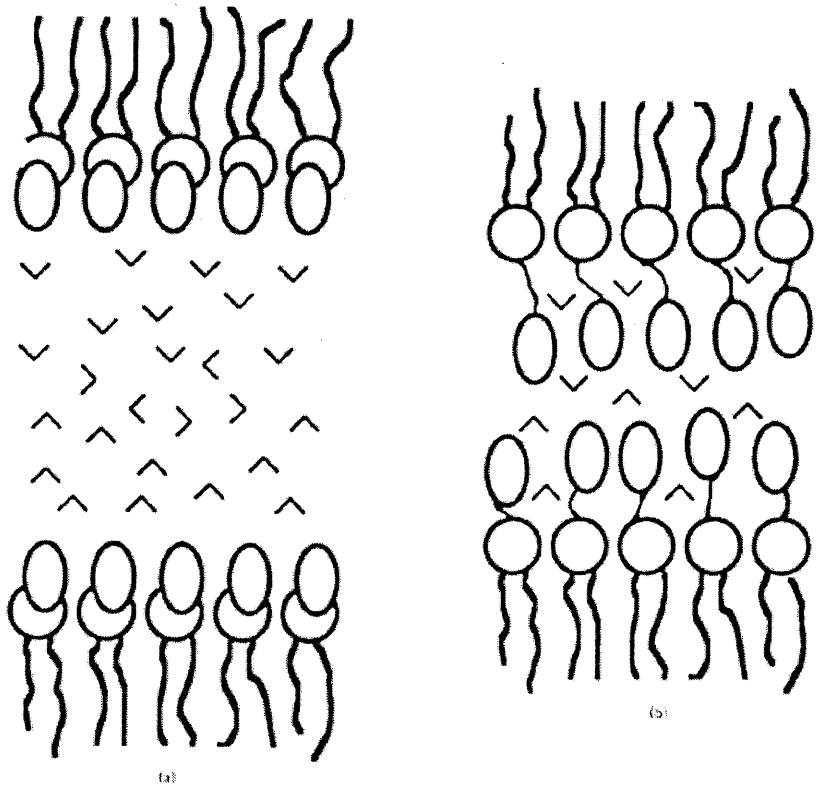

**Figure 5.** Illustration du déplacement des molécules d'eau lors du rapprochement de deux membranes (Wilschut, 1991)

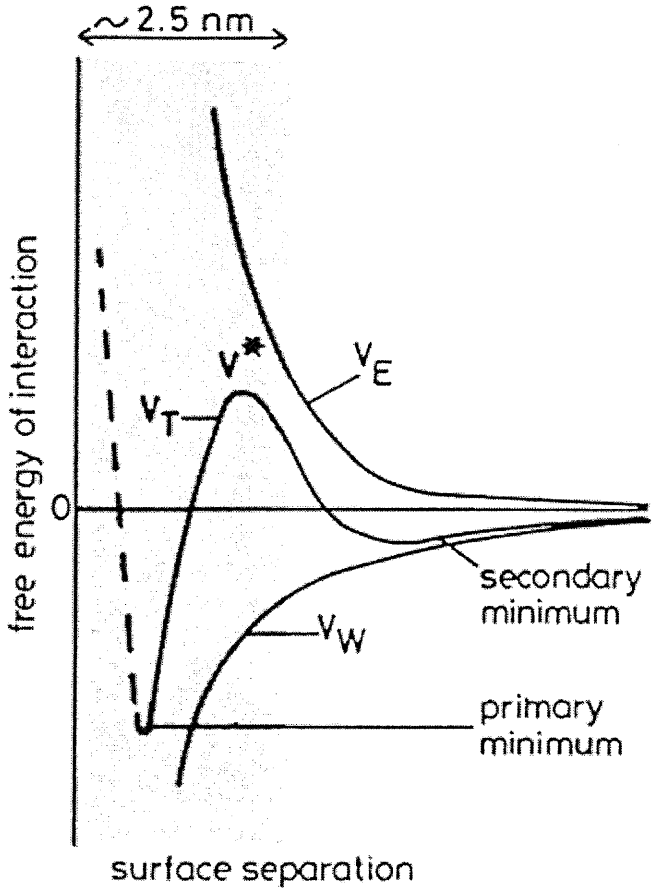


**Figure 6.** Forces en jeux lors de l'interaction entre deux vésicules lipidiques, de même charge de surface, en fonction de la distance les séparant et en ignorant les forces d'hydratation répulsives. Le total des potentiels d'énergie, $V_T$, correspond à la somme des répulsions électrostatiques, $V_E$ (Énergie positive qui diminue avec la distance), et des attractions de Van der Waals, $V_W$ (Énergie négative qui diminue avec la distance). La portée de la force répulsive d'hydratation est de 2,5 nm. Au delà de cette distance, les forces d'interactions entre les vésicules ne sont plus présentes. (Wilschut, 1991)

N-NBD-PE (475 nm), la fluorescence émise par cette dernière (520 nm) est directement absorbée par une molécule de N-Rh-PE qui émet alors sa propre fluorescence (590 nm). Ce transfert d'énergie est proportionnel à la distance entre les deux sondes. Lorsque ces vésicules fusionnent avec des liposomes ou des cellules non marquées, les sondes vont s'éloigner l'une de l'autre par dilution et l'efficacité du TEFR va diminuer. Ceci va se refléter par une augmentation de l'énergie de fluorescence du N-NBD-PE (520 nm) (moins absorbée par l'accepteur) et une diminution de l'intensité de fluorescence du N-Rh-PE (590 nm) proportionnellement aux événements de fusion.

### 1.4.3 Facteurs affectant le processus de fusion

Les différents paramètres externes ayant un effet sur le processus de fusion sont : les cations divalents, le pH, et la température. Le calcium est le principal cation divalent étudié lors de l'interaction des vésicules composées de phospholipides chargés négativements (Wilschut et al., 1981; Wilschut et al., 1985). Le calcium possède notamment la capacité de déshydrater les têtes polaires des phospholipides, tout en créant également un pont ionique entre deux phospholipides chargés négativement (Borovjagin et al., 1982).

## 1.5 Les agents perméabilisants

### 1.5.1 Introduction

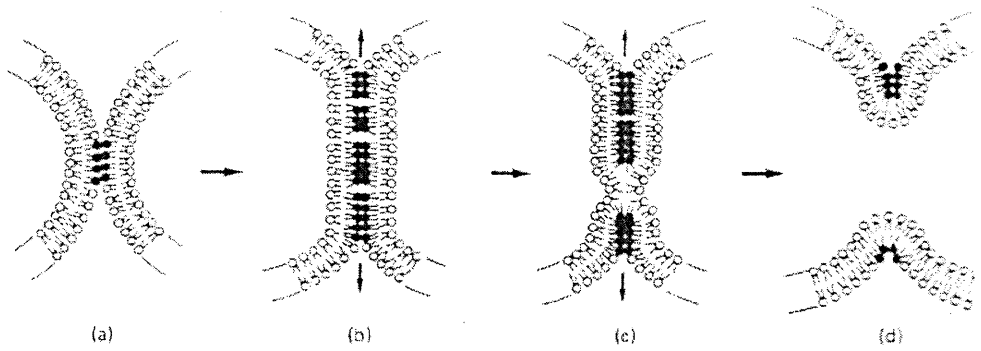


**Figure 7.** Mécanisme hypothétique représentant la fusion induite par le calcium entre deux vésicules chargées négativements. (a) Agregation des vésicules et formation d'une surface de contact déshydratée. (b) Expansion de la surface de contact générant une tension sur la bicouche lipidique. (c) Désorganisation de la bicouche et formation d'un diaphragme unilamellaire. (d) Rupture du diaphragme et fusion des membranes. (Wilschut, 1991)

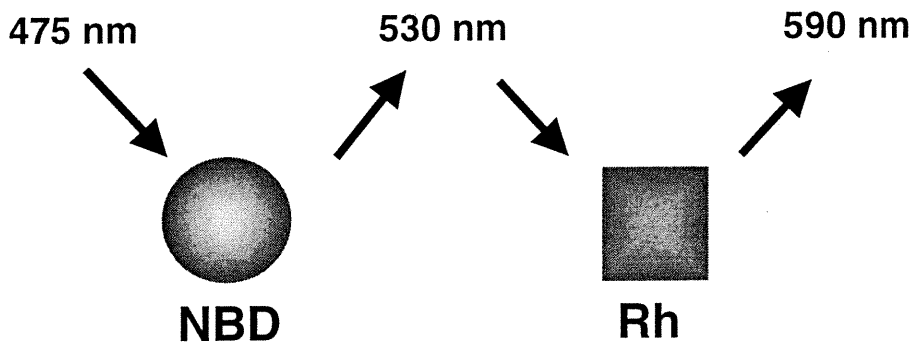


**Figure 8.** Représentation du transfert d'énergie de fluorescence entre le NBD et la Rhodamine.

La base moléculaire de l'intégrité de la ME repose sur les LPS. Ces derniers ont la capacité de fixer des cations grâce aux nombreuses charges négatives du lipide A et de la partie interne du « core ». De ce fait, la membrane externe est une remarquable barrière et les sites de fixation des cations sont essentiels à son intégrité. Cependant ces sites sont également le « talon d'achille » de la ME.

### 1.5.2 Les agents cationiques

Il est connu depuis longtemps que les antibiotiques naturels polycationiques, du groupe des polymixines, forment avidement un complexe avec les LPS désorganisant toute la ME (Nikaido and Vaara, 1985; Hancock, 1984; Storm et al., 1977). Ceci perméabilise la membrane permettant à l'antibiotique d'atteindre la membrane cytoplasmique qu'il désorganisera également causant la mort bactérienne.

Des dérivés de polymixine, dont le plus connu est la polymixine B nonapeptide (PMBN), ont été développés afin d'augmenter la perméabilité de la membrane externe sans affecter celle de la membrane cytoplasmique (Vaara and Vaara, 1983). L'utilisation de la PMBN permet d'augmenter la sensibilité de nombreuses bactéries Gram négatives aux antibiotiques hydrophiques(Vaara, 1992).

### 1.5.3 Les chélateurs d'ions

Un autre moyen de déstabiliser la ME consiste à retirer les cations divalents qui permettent une jonction serrée des LPS. C'est le cas notamment de l'EDTA, un chelateur de cations divalents qui augmente considérablement la perméabilité de bactéries Gram négatives entériques tel *Pseudomonas aeruginosa* (Nikaido and Vaara, 1985; Hancock, 1984).

### 1.5.4 Polyethylenimine

#### 1.5.4.1 Description

Le polyethylenimine (PEI) est un polymère aliphatique, faiblement basique, qui est polycationique dû à la présence de groupements amines primaires, secondaires et tertiaires. Ce n'est ni un peptide, ni une protéine, et par conséquent il ne possède pas de potentiel immunogénique. Le PEI est un produit synthétique disponible dans différents poids moléculaires et formes. Il est principalement utilisé pour précipiter des contaminant cellulaires tels que les acides nucléiques, les lipides et des débris de lysats cellulaires afin de faciliter la purification de protéines solubles (Cordes et al., 1990; Kirk and Cowan, 1995; Milburn et al., 1990). Le PEI est un ingrédient souvent retrouvé dans une variété de formulations allant du savon au matériel d'emballage. Dû à son caractère polycationique, le PEI est principalement utilisé pour compacter l'ADN et les oligonucléotides. En effet de nombreuses recherches sont en cours afin d'augmenter le caractère

transformant des nanoparticules de PEI/ADN. Ces nanocomplexes ont notamment été utilisés comme vecteur de transfection in-vivo au niveau pulmonaire (Gautam et al., 2000) mais également au niveau du système nerveux central (Goula et al., 1998). Ce n'est que récemment que le PEI a été étudié comme agent perméabilisant des bactéries Gram négatives (Helander et al., 1998; Helander et al., 1997).

#### 1.5.4.2 Rôle d'agent perméabilisant

Helander et collaborateurs ont récemment démontré que le PEI, ce polyamine polycationique, possède toute les caractéristiques d'un agent perméabilisant tel que décrit par Vaara M. (Vaara, 1992). Ils ont notamment mis en évidence que le PEI (50 Kda) : 1) augmente l'entrée d'une sonde hydrophobique, le 1-N-phenylnaphtylamine, dans des bactéries Gram négatives; 2) les sensibilise à des antibiotiques hydrophobes dans des tests de diffusion en agar; 3) et augmente leur susceptibilités à la lysozyme et au détergent non-ionique, le Triton X-100. Cependant contrairement aux autres perméabilisants connus, tels que les protamines et polylysines, le PEI n'induit pas la libération de LPS de la membrane externe. Cette propriété à également été observée dans le cas de la polymixine B nonapeptide (Nakae, 1986)

## 1.6 Objectifs du présent travail et stratégie expérimentale

Les travaux de recherche du présent travail étaient divisés en deux volets gravitant autour de la question de l'imperméabilité membranaire des bactéries Gram négatives. Le premier avait pour but de mettre en évidence, par la technique du TEFR, un processus de fusion entre les liposomes fluides et les bactéries Gram négatives ainsi que d'identifier les différents paramètres en jeu dans ce phénomène. Le second volet consistait à mettre en évidence le caractère synergique du PEI en le combinant avec une large gamme d'antibiotiques hydrophobiques et hydrophiliques.

A l'aide de la technique du transfert d'énergie de fluorescence par résonance, nous avons tenté de répondre au premier volet de l'étude. Cette technique a permis de confirmer les résultats obtenus précédemment au sein de notre équipe par les techniques de microscopie électronique, d'immunocytochimie et de cytométrie en flux. Les résultats de cette étude sont présentés au chapitre 4 (Sachetelli et al., 2000).

Pour mettre en évidence les différents paramètres en jeu dans le processus de fusion entre les liposomes fluides et les bactéries Gram négatives, nous nous sommes également servis de la technique du TEFR. Nous avons mesuré le niveau de fusion en faisant varier différents paramètres : 1) la fluidité des liposomes; 2) la concentration en calcium du milieu; 3) l'addition de différents cations divalents; 4) le changement de pH; 5) et finalement la température. Les résultats de ces études sont présentés au chapitre 2 (Khalil et al., 2000)

Comme il a été mentionné précédemment, un autre moyen de vaincre la perméabilité de la ME des bactéries Gram négatives consiste à employer des agents perméabilisants. Nous avons tout d'abord mis en évidence, pour la première fois, le caractère synergique du PEI en combinaison avec des antibiotiques de la famille des β-lactamines. Des cinétiques de croissance ainsi que des mesures de la concentration minimale inhibitrice ont été effectuées chez des souches cliniques résistantes de *P. aeruginosa,* en présence ou non de PEI. Les résultats de ces études sont présentés au chapitre 5 (Chen et al., 2000).

Finalement nous avons évalué le spectre d'action du PEI sur une vaste gamme d'antibiotiques couramment utilisés. Nous avons quantifié l'effet du PEI en mesurant des concentrations minimales inhibitrices (CMI), des concentrations fractionnelles inhibitrices (CFI) et des courbes de mortalités. Cette étude nous a permis de distinguer l'effet agoniste et synergique du PEI en fonction de l'antibiotique utilisé. Les résultats de cette étude sont présentés au chapitre 3.

# CHAPITRE 2- ARTICLE

## Fusion between fluid liposomes and intact bacteria:

## I. Study of driving parameters

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**Résumé**

Dans une étude antérieure nous avons démontré à l'aide de la microscopie électronique, de la cytométrie en flux et par la technique de « lipid mixin assay » (9) la présence d'un mécanisme de fusion entre les liposomes fluides (DPPC/DMPG 9 :1) et la membrane de bactéries intactes. Suite à cette étude, nous avons décidé de mettre en évidence les facteurs physico-chimiques en jeux lors de l'interaction entre les liposomes fluides et *Pseudomonas aeruginosa*. Nous avons ainsi démontré que le mécanisme de fusion dépend de plusieurs facteurs clefs. La perturbation de la fluidité des liposomes par l'addition de seulement 10% de cholestérol diminue le niveau de fusion avec *Pseudomonas aeruginosa* de 44% à 5%. Le degré de fusion entre les liposomes fluides et *Escherichia coli* (66 ± 7 %) ou *Pseudomonas aeruginosa* (45 ± 6%) semble corréler avec le pourcentage de phosphatidylethanolamine au niveau de la membrane externe (respectivement 91% et 71%). Les cations divalents augmentent la fusion selon la séquence $Fe^{2+}$>$Mg^{2+}$>$Ca^{2+}$>$Ba^{2+}$ alors qu'une température plus basse que la température de transition de phase des liposomes réduit leur capacité de fusion. Finalement une augmentation de la fusion est également observée à des pH acides et basiques (respectivement 54% et 45%) comparativement au pH neutre (35%). A la lumière de cette étude, un mécanisme de fusion impliquant la formation d'un pont cationique entre les LPS bactériens chargés négativement et le DMPG des liposomes fluides est proposé.

# Fusion between fluid liposomes and intact bacteria:

# I. Study of driving parameters

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Running title: Study of driving parametrers

Key words: Liposomes, Fusion, Bacteria, *Pseudomonas aeruginosa*, lipid-mixing assay, cations, pH

*. corresponding author [redacted]

**Abstract**

We have previously demonstrated a mechanism of fusion between liposomes (DPPC/DMPG 9:1) in a fluid state, and intact bacterial cells, by electron microscopy, fluorescence activated cell sorter and by lipid mixing assay (9). As a continuation of this research, the factors involved in fluid liposome interaction with *Pseudomonas aeruginosa*, a clinically significant bacteria, has been investigated. The observed fusion process is shown to be mainly dependent on several key factors. Pertubation of liposome fluidity by addition of only 10% cholesterol dramatically decreased the degree of fusion with *Pseudomonas aeruginosa* from 44% to 5%. The level of fusion observed when fluid liposomes were mixed with *Escherichia coli* (66 ± 7 %) or *Pseudomonas aeruginosa* (45 ± 6 %) seems to be correlated to their outer membrane phosphatidylethanolamine phospholipid composition (respectively 91% and 71%). Divalent cations increased the degree of fusion in the sequence $Fe^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$ whereas temperatures lower than the Tc of DPPC/DMPG (9:1) vesicles decreased their fusion capacity. Acid as well as basic pH's conferred higher degree of fusion (respectively 54% and 45%) when compared to neutral pH (35%). Based on the results of this study, a possible mechanism involving cationic bridging between bacterial negatively charged LPS and fluid liposomes DMPG phospholipids was outlined.

## 1- Introduction

*Pseudomonas aeruginosa* is a remarkably adaptable pathogen. It has assumed an important role in the infections of patients with various impairments of host defences. In cystic fibrosis, chronic pulmonary infections with *Pseudomonas aeruginosa* and other related strains are considered the most important factors determining the prognosis of these patients (1-2) In fact, *Pseudomonas* causes rapid, extensive and fatal diseases in the compromised host and claims the highest crude mortality of any Gram-negative pathogen causing bacteremia (3). *Pseudomonas aeruginosa* represents a good model of antibiotic resistance. These organisms have an outer membrane with a low level of permeability to drugs that is often combined with multidrug efflux pumps, enzymatic inactivation of the drug or alteration of its molecular target (4-5). The acute and growing problem of antibiotic resistance of *Pseudomonas* to conventional antibiotics made it imperative to develop new approaches to overcome these mechanisms.

To increase the bactericidal efficacy of antibiotics, different liposomal formulations for pulmonary administration were developed in our laboratory with the aim of promoting effective interactions between bacteria and encapsulated drugs, increasing the resident time of the encapsulated antibiotics in the lungs and reducing systemic drug absorption (6). Tobramycin encapsulated in a negatively charged liposomal formulation presenting a low gel-liquid crystalline transition temperature ($T_C$) $\leq 37^{\circ}C$ succeeded, for the first time, in eradicating mucoid *P. aeruginosa* in an animal model of chronic pulmonary infection (7). This fluid

liposomal encapsulated tobramycin, named Fluidosomes™, was later shown to be effective against other bacterial strains: *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Escherichia coli* and *Staphylococcus aureus* (8). It was found in our previous study (9) that this enhanced bactericidal activity is due to a potential mechanism of fusion between the fluid liposomes and the bacterial membranes. In the present study, we systematically evaluated major driving forces behind such fusion process.

The fusion process between two vesicles occurs in three distinct steps. First, the vesicles have to adhere or aggregate; this approach is subject to a number of different interaction forces. These forces comprise: 1. electrostatic interactions; 2. attractive Van der Waals interactions; 3. and hydration forces. Second, a local perturbation of the packing of the lipid bilayers at the site of contact seems required, initiating the merging of the outer monolayers of the two bilayer membranes. Finally, the aqueous vesicle interiors have to coalesce with the concomitant mixing of the inner-monolayer lipids (24).

A number of different fusogens have now been established as agents which accelerate the process of fusion. They range in structure from charged-organic compounds, such as lecithin, to inert biological substances, such as metal ions (10-12). The divalent cations that induce interaction between negatively charged phospholipid vesicles have been studied extensively (13-15).

Negatively charged phospholipid vesicles do not naturally fuse or aggregate due to the long–range electrostatic repulsion. Divalent cations, by binding to negatively charged vesicles reduce electrostatic repulsion, inducing aggregation shortly followed by fusion. This is due to structural changes in the vesicle bilayers resulting from the disruption of the strong repulsive hydration forces that prevents hydrophobic interaction between phospholipid bilayers at short distances of separation (13-15). To understand further how these liposomes interact with bacteria, we investigated the factors involved in this process.

## 2- Materials and methods

### *2.1 Bacterial strains and mediums*

Two reference strains, *Pseudomonas aeruginosa* ATCC 25619 obtained from ATCC (American Type Culture Collection, Rockville, Md) and *Escherichia coli* K12 were used in this study. All other strains, *Burkholderia cepacia* 1368, *Streptococcus agalactiae* 910121 and *Staphylococcus aureus* 91131 are clinical isolates. For the experiments, overnight cultures in Mueller Hinton broth (MH) (Difco Laboratories, Detroit, Michigan) were prepared. The following day, 30 µl of these cultures were used to innoculate 20 ml of fresh MH Broth under agitation at $37^0$C. Experiments were always carried out when the culture reached an $O.D_{660nm}$ of 0,6. MH Broth was used because it has the particularity of lacking all known divalent cations (trace amounts).

*2.2 Fluid liposomes*

Fluid liposomes are composed of dipalmitoyl phosphatidylcholine (DPPC) and dimiristoyl phosphatidylglycerol (DMPG) (9:1, mol/mol) (Avanti polar lipids, Alasbaster, AL) which have an overall low gel-liquid crystalline transition temperature ($T_C < 37^oC$ ). They were prepared by a hydration-extrusion method previously described (7,9) . Appropriate amounts of lipid mixtures were dissolved with a solution of methanol/chloroform (1:2). The fluorescence-labeled lipid marker solution (NBD-PE and Rh-PE, 0.5mol% each of the total lipids) was added and the solvents were evaporated under nitrogen stream with a warm water bath until a homogeneous lipid film was produced. The resultant film was dried under vacuum overnight, hydrated with HBS buffer (25mM HEPES and 150mM NaCl, pH 7.5) by vigorous mixing, and followed by five times freeze-and-thaw (freeze with dry-ice acetone, and thaw in hot water 65°C). The sample was then extruded though 2 stacked polycarbonate filters (Nuclepore) with designed size, ten times using an extrusion device (Lipex Biomembranes, Inc., Vancouver, BC, Canada) as previously described (7).

*2.3 Bacteria and liposome fusion*

Liposomal 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.* (16) . All fluorescence measurements were carried out with a Perkin-Elmer LS-50B fluorescence spectrophotometer. In a typical experiment, the NBD/Rh-labeled liposomes were prepared as described previously containing both NBD-PE and Rh-PE at 0.2 mol% each (9). The NBD/Rh labeled vesicles (10 μg/ml final concentration) were mixed with 1,9 ml of bacteria ($OD_{660}$ = 0,6) and incubated at 37°C under constant stirring. RET efficiency was monitored by measuring time-drives of the reaction mixture fluorescence in quartz cuvettes. The fusion of NBD/Rh labeled liposomes 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. Continuous monitoring of rhodamine fluorescence (590nm) was done at an interval of 1 min under steady-state excitation at 475 nm. The final fluorescence intensity ($\mathbf{F_{max}}$), which represents maximal fluorescent lipid probe dilution in each sample, was determined following the solubilization of vesicles with Triton X-100 detergent (0.2% volume). The percentage of fusion (or lipid dilution) was calculated using the following equation:

$$\% \text{ Fusion} = \frac{F_t - F_o}{F_{max} - F_o} \times 100 \qquad (1)$$

where $\mathbf{F_t}$ is the fluorescence intensity at given time point; $\mathbf{F_o}$ is the initial fluorescence intensity. Each experiment for a given conditions was repeated at

least three times. Rhodamine fluorescence intensity was monitored in order to avoid any artifact caused by *P. aeruginosa* natural green fluorescence.

*2.4 Effect of calcium concentration on fusion*

A sample of 1,9 ml of bacteria grown in MH broth($OD_{660}$ = 0,6) was supplemented with the proper amount of $CaCl_2$ from a stock solution of 1M to reach the desired concentration. Volumes were adjusted to avoid any dilution artifacts. After 5 min of equilibration time, labeled liposomes were added to give a final concentration of 10 µg/ml as described before and measurements started immediately.

*2.5 Effect of different divalent cations on liposomal fusion with intact bacteria*

Stock solutions of each divalent cations salt ($CaCl_2$, $BaCl_2$, $FeSO_4$, $MgCl_2$) were prepared at a concentration of 1M. For each measurement, divalent cations were added to 1,9 ml of bacteria grown in MH Broth ($OD_{660}$ = 0,6) to give a final concentration of 1mM. Following 5 min of equilibration time, labeled liposomes were added (final concentration of 10 µg/ml) as described before and measurements started immediately.

*2.6 Effect of pH on liposomal fusion*

1,9 ml sample of bacteria grown in MH Broth ($OD_{660}$ = 0,6) was taken and the medium pH adjusted to the proper value. Following 5 min of equilibration time, labeled liposomes were added as described before (final concentration of 10 μg/ml) and measurements started immediately.

*2.7 Effect of temperature on liposomal fusion*

Labeled liposomes were added at a final concentration of 10 μg/ml to 1,9 ml of bacteria grown in MH Broth ($OD_{660}$ = 0,6). Fusion measurements were carried at three different temperatures ($4^0$C, $22^0$C and $37^0$C) with two Gram-negative bacteria, *P.aeruginosa* ATCC 25619 and *E.coli* K12 (wild type) for 30 minutes.

## 3. Results

*3.1 Monitoring and quantification of fusion by lipid-mixing assay*

Previously, our group demonstrated a fusion process between fluid liposomes and bacteria by several techniques: 1) negative staining, 2) fluorescence activated cell sorter, 3) Immunoelectron microscopy and 4) lipid-mixing (9). Therefore, in the present study we investigated the driving parameters behind liposome fusion with bacteria by lipid-mixing assay with Rh-PE and NBD-PE as fluorescent probes. 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, and not to vesicular aggregation (16-18) . This assay has been widely used for study of membrane fusion (16-22). Figure 1a. represents a typical fluorescence emission scan profile of liposome-bacterium fusions monitored by a spectrofluorometer with a constant excitation at 475 nm (excitation peak of NBD). As time progresses, fluorescence intensity of Rh (590 nm) decreases and NBD signal (520 nm) increases. The decrease in resonance energy transfer efficiency indicates that a significant fusion occurred when fluid liposomes were mixed with bacteria and incubated at 37°C.

Figure 1b. is a time-drive representation of Rhodamine fluorescence intensity decrease after liposomal fusion with *Pseudomonas aeruginosa*. A rapid accelerating phase can be observed in the first five minutes followed by a slower phase. Addition of Triton X-100 leads to a maximal dilution of the probes allowing to calculate the extent of fusion using equation 1 (see material and methods).

In control experiments, a mixture of NBD/Rh labeled liposomes and non-labeled liposomes did not produce any decrease in Rh fluorescence indicating that these vesicles do not fuse together. Controls were also made by incubating vesicles made of DPPC/DMPG/Chol (8:1:1 molar ratio) with intact bacteria under the same experimental conditions. This latter control, where only 10% of cholesterol is added to the phospholipids, clearly demonstrates that the decrease in Rh fluorescence is not due to lipid exchange (Figure 1).

*3.2 Effect of calcium concentration on fusion*

It has been reported that calcium cations increased the interaction and fusion of vesicles prepared from acidic phospholipid membranes (22-23). To verify whether the interaction of liposomes with bacteria can be enhanced by calcium, we added increasing concentrations of calcium to the medium prior to fusion measurements.

Fusion of liposomes with intact cells of *Pseudomonas aeruginosa* was greatly enhanced by increasing concentration of calcium in the medium (Figure 2). Concentrations of calcium ranging from 1 mM to 7 mM were used in a medium deficient in calcium and magnesium (MH Broth). Calcium mainly affects the rapid accelerating phase in the first 5 minutes of the reaction. Concentration above 7 mM could not be used due to bacterial precipitation. Controls in presence of calcium, where labeled liposomes were mixed with unlabeled ones, or DPPC/DMPG/Chol (8:1:1) vesicles were mixed with bacteria, showed no decrease or significantly lower decrease in RET (fig.1b). DPPC/DMPG/Chol have roughly the same surface charge ratio as liposomes but are less fluid due to the addition of cholesterol. Fluorescence emissions of labeled liposomes were not affected by calcium neither natural fluorescence of bacteria (Data not shown).

*3.3 Effect of various divalent cations on the degree of fusion*

Figure 3 shows the degree of fusion calculated with equation 1 between liposomes and bacteria when the medium is supplemented with 1 mM of each appropriate cation. The highest degree of fusion is observed with iron and decreases in the sequence $Fe^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$. The extent of fusion seems to be inversely proportional to the atomic radius of the ions suggesting a possible steric crowding effect.

*3.4 Effect of pH on the degree of fusion*

Previous studies have shown that pH could affect the degree of fusion of negatively charged liposomes (24). We therefore studied the effect of pH on the degree of fusion of liposomes and *Pseudomonas aeruginosa* in a medium containing calcium. Figure 4 shows that almost sixty percent of fusion can be achieved at a low pH of 5.5 in presence of 3 mM $Ca^{2+}$. In fact, significant increase in the degree of fusion is observed either at low pH or high pH. All controls were not affected by the different pH's.

*3.6 Effect of temperature on fluid liposome fusion*

The extent of fusion between two different Gram-negative bacteria, *P. aeruginosa* 25619 and *E.coli* K12, and liposomes was measured at three different temperatures (Figure 5). At 4°C no decrease in RET or fusion could be observed for both bacterial strains. The highest percentage of fusion was obtained at 37°C

(near the Tc of fluid liposomes) whereas an intermediate level was measured at 22°C.

## 4. Discussion

Membrane fusion between liposome-liposome and liposome-biological membrane has been extensively investigated (25-30), but few of these studies are related to prokaryotes. Liposomes have been successfully used in delivery of bio-active proteins into flagellated *Escherichia coli* envelope (31) and to transfect competent *E. coli* and *Mycoplasma capricolum* cells with encapsulated plasmid DNA (32-33). By using liposomes made of lipid extracts from the whole bacterial membranes of *Salmonella minnesota* Re595 (the composition and lipids were not identified), Tomlinson and his co-workers (34) successfully incorporated phospholipids and proteins into Gram-negative bacteria and confirmed that a fusion process was involved in this extensive phospholipid transfer. There is still, however, no studies other than ours reporting liposome-bacteria fusion with, 1) an artificial liposomes of known lipid composition and 2) intact bacterial cells (both Gram-positive and Gram-negative). Liposomes were developed in order to treat bacterial infections in cystic fibrosis with regard to the enhanced bactericidal activity of entrapped antibiotics released through their fusion with bacterial membranes. Considering this, it is desirable to understand liposome-bacteria fusion to further optimize this fusion potential of liposomes. We have previously demonstrated a fusion process between fluid liposomes and bacteria by several

techniques (9), but the main factors which drive or control this fusion process are still not completely clear. We therefore used the lipid-mixing method in the present study to systematically identify and investigate such parameters. Several controls were added in our study to confirm our measurements. When DPPC/DMPG (9:1 molar ratio) vesicles where mixed with bacteria in presence of calcium (5mM), a significant decrease in Rh fluorescence intensity (590nm) and an increase in NBD signal (520nm) was observed when time progressed (Fig.1a.) This decrease (fig.1b.) cannot be attributed to lipid exchange, light scattering, or aggregation because when vesicles composed of DPPC/DMPG/Chol (8:1:1) are mixed with bacteria in the same conditions as previously described, a significantly lower decrease (fig.1b. grey line) in Rh fluorescence intensity was observed. Cholesterol effect on liposome fluidity has been well documented since 25 years. A recent report has recently shown that cholesterol but also flavonoids and isoflavonoids, partition into the hydrophobic core of membranes and cause a dramatic decrease in lipid fluidity of the membrane (44).

With few exceptions, all membrane fusion processes are regulated by three parameters: the nature of attacking vesicles, the property of the recipient membrane and the potential fusogens which facilitate or regulate the fusion process. We will discuss these three parts in regard to the fusion between fluid liposomes and bacterial membranes in the following sections.

**1. Nature of attacking vesicles**

Liposomes fluid state has been shown to be an essential prerequisite to enhance the bactericidal activity of encapsulated antibiotics both *in vivo* and *in vitro* (6,7,8). DSPC/DMPG vesicles containing Tobramycin were shown to lack the bactericidal enhancement capacity conferred by DPPC/DMPG vesicles. This requirement of fluidity was further confirmed in the present study by lipid-mixing assay. Figure 5 shows that the percentage of fusion, between two distinct gram negative bacteria and liposomes, is temperature dependant. When temperatures lower than the Tc of fluid liposomes (arround 35°C) are used, the rate of fusion decrease dramatically. When cholesterol is added to liposomes, at concentrations as low as 10%, the degree of fusion is dramatically decreased (Fig.1b).

2. **Property of recipient membrane**

Regardless of the fluidity of the liposomes, vesicles composed of DPPC/DMPG are not fusogenic by themselve. Such non-fusogenic property of DPPC vesicles was well documented by Sanchez-Migallion (25). In our study, similar observation was obtained by a lipid-mixing assay with a mixture of fluorescent-labeled and unlabeled liposomes as shown in Figure 1b. Upon mixing fluid liposomes with intact bacteria, a dramatic fusion was observed. Since it seems that the main driving force of such liposome-bacteria fusion is not due to the DPPC vesicles, it must come from the bacteria.

We suggest that the main driving force for the liposome-bacterial fusion is due to the nature of bacterial membranes. As indicated in Table 1, fluid liposomes fused more with Gram-negative bacteria than with Gram-positive. It is well known that

two of the most significant differences between Gram-positive and Gram-negative bacteria are an outer-leaflet layer of lipopolysaccharide (LPS) in the outer-membrane and a high amount of phosphatidylethanolamine (PE) in the membranes of Gram-negative bacteria. It is believed that the mechanisms involved in liposome-bacterial fusion may be different for Gram-negative and Gram-positive bacteria.

In Gram-negative bacteria, the LPS forms protruding chains of polysaccharides, which are highly negatively charged, around the bacteria. In order to fuse with Gram-negative bacteria, liposomes must therefore overcome this natural barrier. Only a few studies have been published on the interaction of liposomes with prokaryotic cells (32-33). Our result nonetheless seems to corroborate with these groups and we therefore propose a possible mechanism to explain the fusion process between fluid liposomes and Gram-negative bacteria. They both possess negatively charged membranes respectively due to phosphatidylglycerol and LPS. Long-range electrostatic repulsion usually prevents negatively charged vesicles from aggregation and fusion. However aggregation can occur upon addition of calcium by bridging liposomes with bacteria. This would lead to neutralisation of negative surface charges and dehydration of phospholipids head groups, which would induce a local defect in membranes and fusion. Vesicle fusion induced by divalent cations requires that the lipids of the interacting membranes are in a 'Fluid' state ($T \geq Tc$). This has been previously reported by different groups in the case of artificial negatively charged vesicles (17, 21-23). It is suggested that under

these conditions membranes become transiently susceptible to fusion as a result of changes in molecular packing and creation of new phase boundaries induced by $Ca^{2+}$. This requirement would further explain the importance of fluidity for liposomes in order to achieve bactericidal enhancement and fusion. Following this stage of altered membranes, LPS are displaced by the divalent cations and fusion can occur with the inner membrane, which is mainly composed of PE, a fusogenic phospholipid. It is interesting to note that there is a correlation between the degree of liposome-bacterium fusion and the percentage of PE in their membrane: the inner layer of the outer membrane of *P. aeruginosa*, *B.cepacia* and *E.coli* contains respectively 71%, 87% and 91% PE phospholipids (35). Whereas the outer layer of Gram-negative bacteria are composed predominantly of LPS, Boshoff *et al.* (1992) (36) reported that the LPS can be fusion inducers. This observation would be most likely one of the reasons for the higher degree of fusion observed in Gram-negative bacteria when compared to Gram-positive bacteria. The observed fusion difference may also be due to lack of PE in the membrane of the two Gram-positive bacterial strains used in this study.

In the case of Gram-positive bacteria, higher degree of the liposome-bacterial fusion was also observed and correlated well to their membrane phosphatidylglycerol (PG) content. 45% fusion between liposomes and *Staphylococcus aureus* (with 57% PG content) was observed while only 23% fusion for *Streptococcus agalactiae* (23% PG) (37). Nevertheless our suggested mechanism cannot fully explain the extent of fusion observed with Gram-positive

bacteria (Table 1), which lacks PE and LPS. Presence of high percentage of PG in Gram-positive bacterial membranes results in an overall highly negatively charged surface. Recently the group of Wilschut (27) observed membrane fusion and lamellar-to-inverted-hexagonal phase transition between negatively charged vesicles composed of phospholipids similar to PG when calcium was added. Further studies with artificial liposomes composed with a high content of PG and Gram-positive bacterial ghost vesicles are needed in order to identify the main inducers and understand their involvement.

**3. Potential fusogens**

Besides the involvement of liposome fluidity and bacterial membrane characteristics, other fusogens or environemental factors such as divalent cations, pH and temperature are known to affect membrane fusion. It is well known that calcium induces membrane fusion in several models of liposomal vesicles. We demonstrated in this study that liposome-bacteria fusion is directly proportional to the amount of calcium in the medium (Figure 2). It is thought that calcium neutralizes the negative surfaces charges of membranes resulting in dehydration of cell surfaces (36). It has been suggested that hexagonal II phase lipids are important for membrane fusion (38) and that calcium is able to trigger a bilayer-hexagonal II phase transition of some phospholipids. Phosphatidylethanolamine, the major phospholipid of Gram-negative bacteria, can also adopt both bilayer and hexagonal II arrangements. This would suggest that fusion between liposomes and Gram-negative bacteria is dependent on the presence of phospholipid in the

outer leaflet of the outer-membrane although it is thought that the OM outer leaflet of Gram-negative bacteria is completely deficient in phospholipids (39). This may in fact be due to the effect of calcium which have been reported to induce a loss of OM proteins (40) directly leading to redistribution of lipids and increased levels of phospholipids in the OM.

Two different mechanisms of calcium induced fusion have been reported in the literature. Fusion can be initialized by a calcium triggered enzymatic reaction (41) or simply as a bridge which induces aggregation and destabilization (36). At the present, all of the evidences suggest that calcium with its divalent cationic charges plays a major role as a bridge rather than activation of phospholipase activity in liposome-bacterial fusion. First, calcium induced enzymatic reaction is ion-specific, but we observed similar fusion increase for other divalent cations as indicated in Figure 3. This is also supported by the fact that only liposome in the fluid state are able to fuse with bacterial membranes. Second, fusion is triggered by different divalent cations increased in the sequence of $Fe^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$. This difference between the different cations could be due to their different capacity to cause aggregation and fusion. The degree of aggregation between liposomes and bacteria would increase according to two parameters. First, each cation has an electronegative affinity to phosphatidylcholine decreasing in the sequence $Mg^{2+} > Ca^{2+} > Ba^{2+}$as demonstrated by McLaughlin *et al.* (1978) (42). Second, fusion could be dependent on the atomic radius of the ions. Figure 3 shows that the extent of fusion is inversely proportional to the atomic radius. The

smaller the ions, the closer liposomes interact with the bacterial membrane, and the higher the fusion is induced between them. To our knowledge, this is the first time a study demonstrates the importance of cations in liposome-bacterium fusion.

The pH of the reaction solution has also influenced liposome-bacterial fusion as shown in Figure 4. Acidic pH and basic pH induced respectively 19% and 10% more fusion than neutral pH. One hypothesis is provided by the group of Coughlin *et al.* (1985) (43) which reported that the lamellar structure of lipopolysaccharide is stabilized at neutral pH both by ionic interactions within the LPS, and by divalent cation bridges. Hydrogen bonds would be lost at basic pH resulting in an increase of hydration and an increase of negative charge of the head groups. This would cause the formation of micellar structure or instable outer membrane. In the acidic range, there would be reduction of the repulsion by charges and hydration due to increase in hydrogen bonds. This would lead to insoluble complexes also responsible for an instable membrane. These altered membranes will therefore be more susceptible to fuse easily with liposomes.

In summary, the nature of bacterial membrane and fluidity of liposomes are identified as the most important driving forces in liposome-bacterial fusion. However, divalent cations, pH, and temperature have an important effect on the rate and extent of fusion.

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Fig. 1. Fluid liposome fusion with *Pseudomonas aeruginosa* 25619 monitored by lipid mixing assay.

2 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then mixed with a final concentration of 10 μg/ml of labeled fluid liposomes and 5 mM of $CaCl_2$ at 37°C. A) Fluorescence emission scans of a mixture of labeled liposomes and intact bacteria. B) Time drive of Rhodamine fluorescence intensity (590 nm) when liposomes were mixed with intact bacteria. Dashed lines represent a control experiment of labeled liposomes with unlabeled one. Grey lines represent Rh fluorescence decrease when DPPC/DMPG/Chol (8:1:1) are mixed with intact bacteria under the same conditions as described. Arrows indicates addition of triton X-100 to a final concentration of 0,1%.

Fig. 2. Effect of calcium concentration on degree of fusion between liposomes and intact *Pseudomonas aeruginosa* 25619.

2 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then mixed with a final concentration of 10μg/ml of the labeled liposomes and appropriate calcium concentrations (A:0 mM, B:1 mM, C:2 mM, D:3 mM, E:5 mM, F:6mM, G:7mM ) . Fusion was monitored by rhodamine fluorescence decrease at 590 nm after 30 min resulting from resonance energy transfer efficiency decrease (see text).

Fig. 3. Effect of divalent cations on percentage of fusion between liposomes and intact *Pseudomonas aeruginosa* 25619 correlated to atomic radius.

2 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then mixed with a final concentration of 10 μg/ml of the labeled liposomes and 1mM of each appropriate ion. Fusion was monitored by rhodamine fluorescence decrease at 590 nm after 30 min resulting from resonance energy transfer efficiency decrease (see text).

Fig. 4. Effect of pH on degree of fusion between fluid liposomes and *Pseudomonas aeruginosa* 25619 after 30 min. at 37°C.

2 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then supplemented with 3 mM calcium and adjusted to the proper pH. Fusion was monitored by rhodamine fluorescence decrease at 590 nm after 30 min. resulting from resonance energy transfer efficiency decrease (see text).

Fig. 5. Effect of temperature on the degree of fusion between fluid liposomes and two Gram-negative strains, *Escherichia coli* K-12 and *Pseudomonas aeruginosa* 25619 after 30 min at $37^0$C.1,9 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then mixed with a final concentration of 10 µg/ml of labeled liposomes and supplemented with 5 mM calcium. Fusion was monitored by rhodamine fluorescence decrease at 590 nm after 30 min resulting from resonance energy transfer efficiency decrease at three different temperatures.(see text).

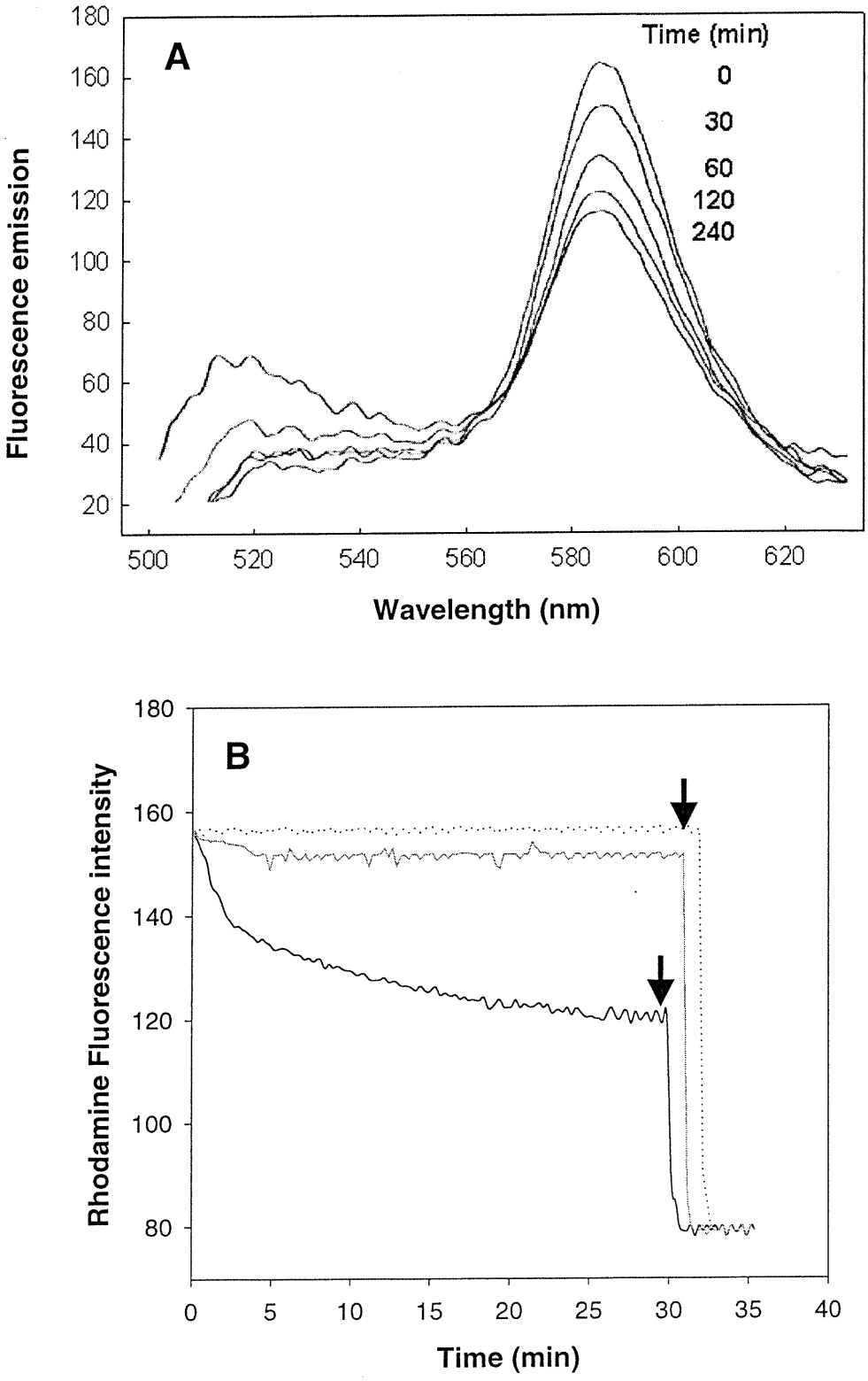
A
Time (min)
0
30
60
120
240
Fluorescence emission
Wavelength (nm)
180
160
140
120
100
80
60
40
20
500
520
540
560
580
600
620
B
Rhodamine Fluorescence intensity
Time (min)
180
160
140
120
100
80
0
5
10
15
20
25
30
35
40

Figure 1

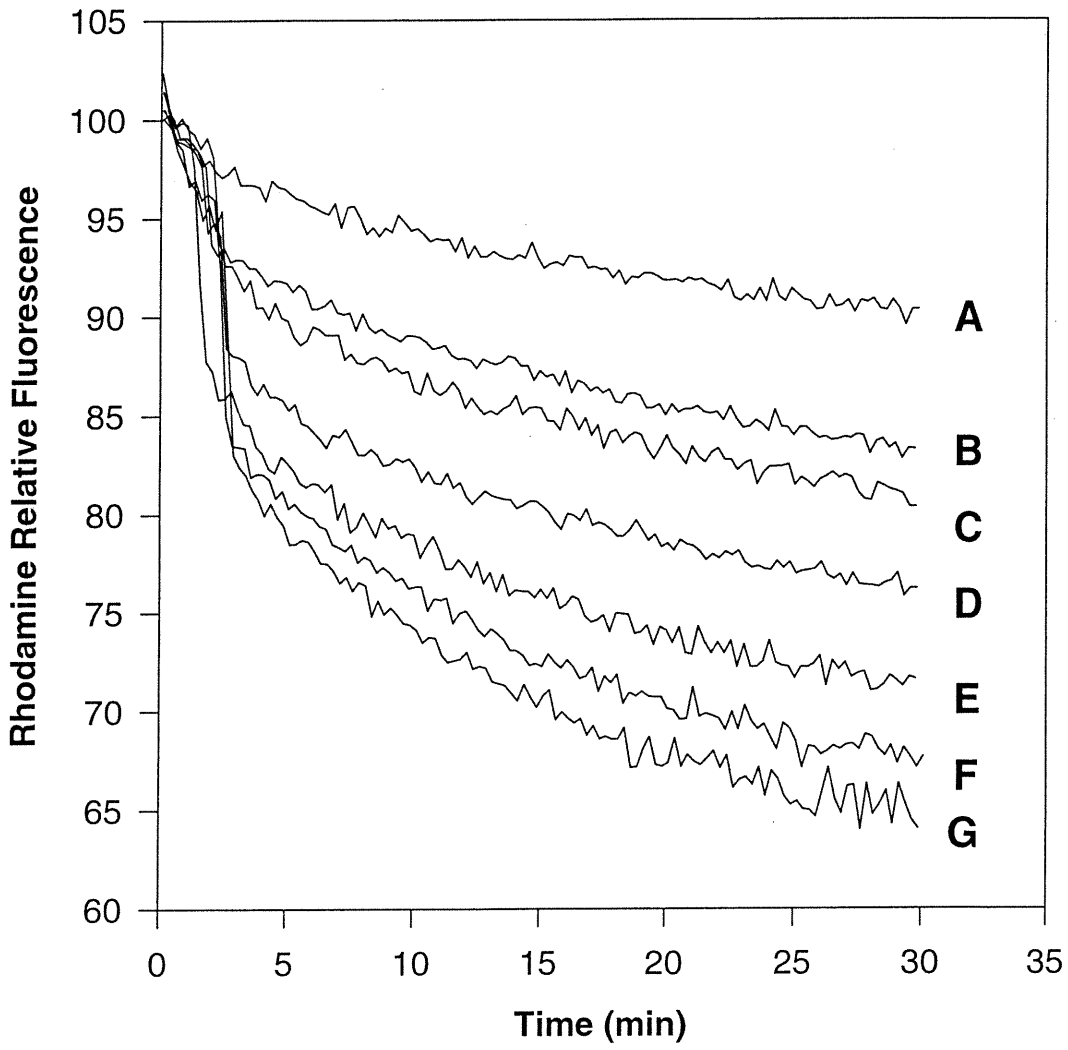
105
100
95
90
85
80
75
70
65
60
Rhodamine Relative Fluorescence
0
5
10
15
20
25
30
35
Time (min)
A
B
C
D
E
F
G

Figure 2

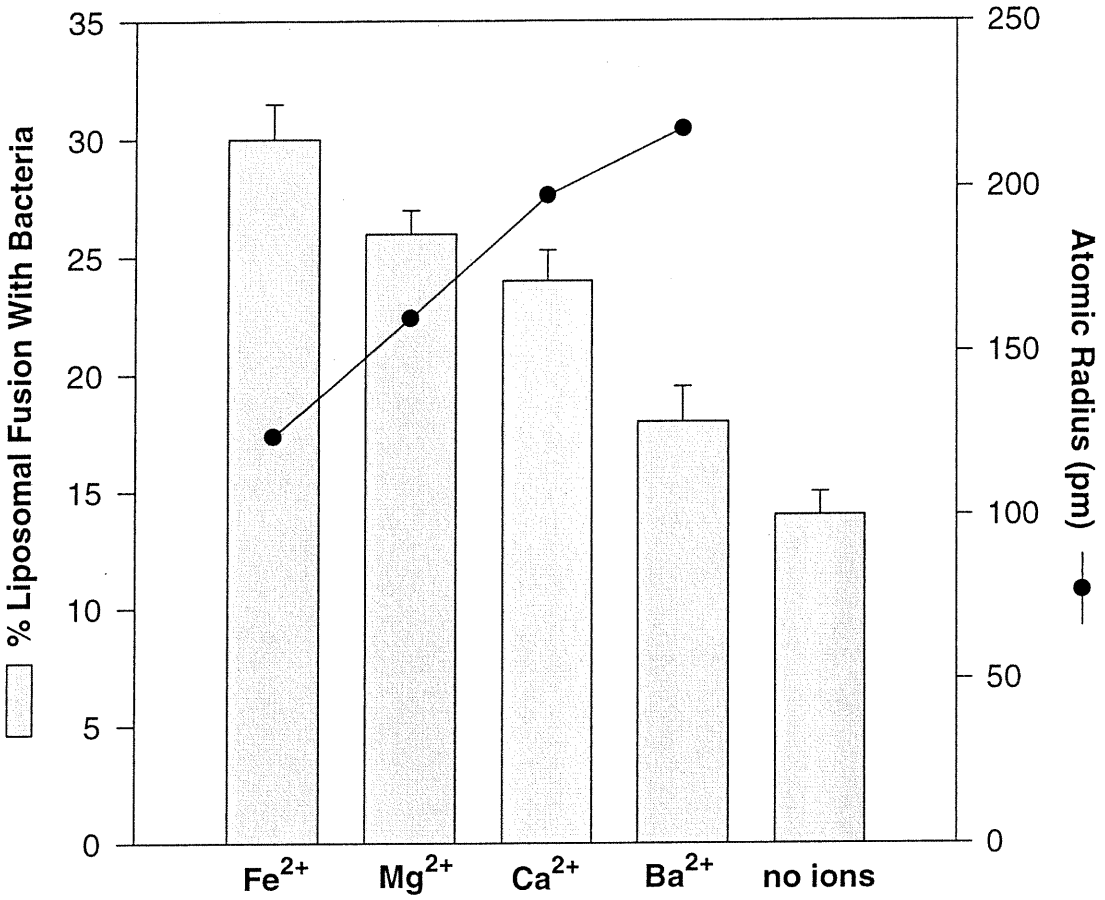
% Liposomal Fusion With Bacteria
Atomic Radius (pm)
35
30
25
20
15
10
5
0
250
200
150
100
50
0
Fe2+
Mg2+
Ca2+
Ba2+
no ions

Figure 3

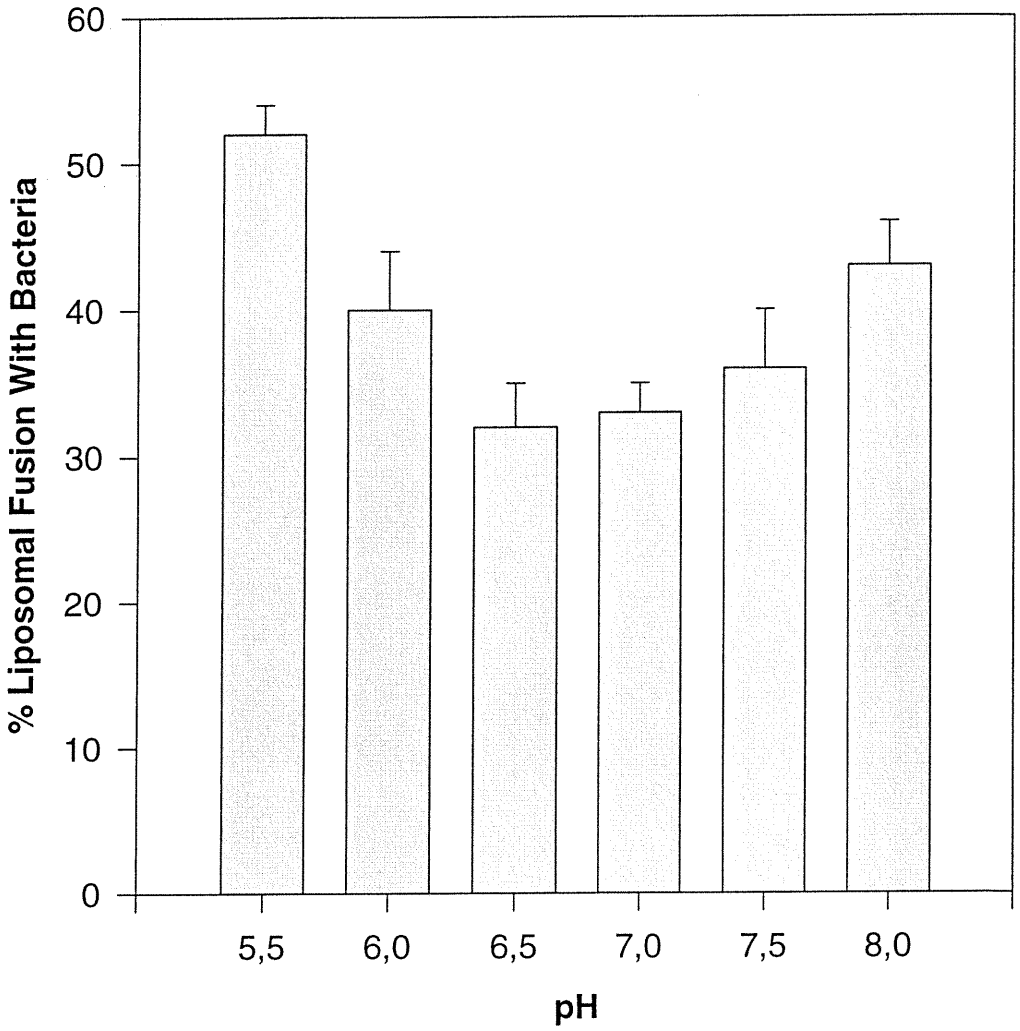
60
50
40
30
20
10
0
% Liposomal Fusion With Bacteria
5,5
6,0
6,5
7,0
7,5
8,0
pH

Figure 4

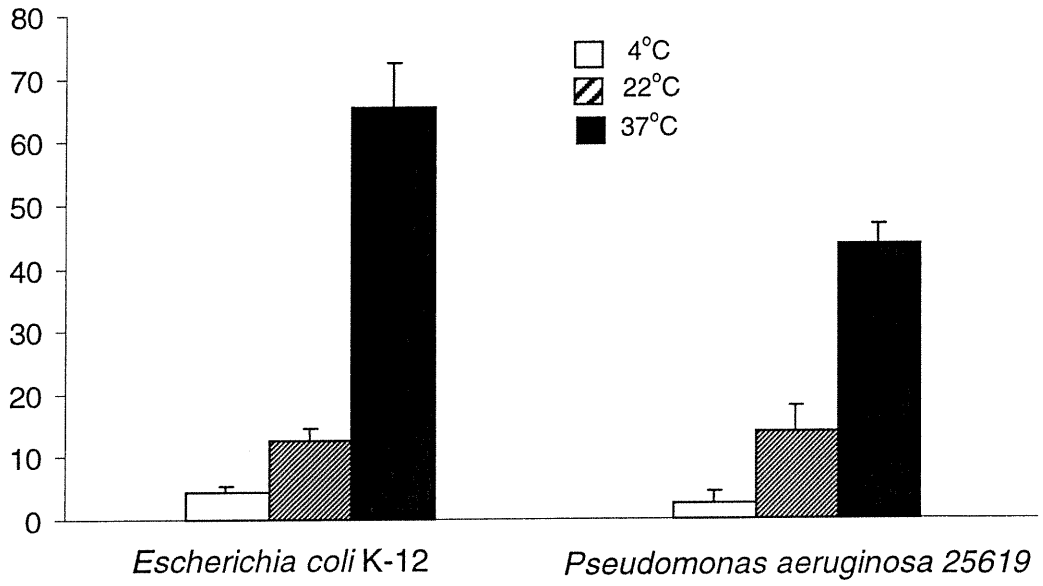
80
70
60
50
40
30
20
10
0
4°C
22°C
37°C
Escherichia coli K-12
Pseudomonas aeruginosa 25619

Figure 5

Table1
Comparaison of the percentage of fusion between fluid liposomes and Gram-negative bacteria

| Gram negative strains | % fusion [a] | Gram positive strains | % fusion [a] |
|---|---|---|---|
| *Pseudomonas aeruginosa* 25619 | 43,67 ± 3 | *Streptococcus agalactiae* 910121 | 22,98 ± 3 |
| *Burkholderia cepacia* 1368 | 51,50 ± 2 | *Staphylococcus aureus* 91311 | 45,00 ± 6 |
| *Escherichia coli* K12 | 65,53 ± 7 | | |

[a] 2 ml of bacteria grown to an $O.D_{660nm}$ of 0,6 in Mueller Hinton broth where then mixed with a final concentration of 10µg/ml of the labeled liposomes and 5mM of CaCl2. Fusion was monitored by rhodamine fluorescence decrease at 590 nm after 30 min resulting from resonance energy transfer efficiency decrease (see text).

# CHAPITRE 3- ARTICLE

## Synergy Between Polyethylenimine and Different Families of Antibiotics against a Resistant Clinical Isolate of *Pseudomonas aeruginosa*

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**Résumé**

L'activité bactéricide *in-vitro* de 10 familles d'antibiotiques seules et en combinaison avec un polymère polycationique synthétique, le polyéthylenimine (PEI) à été évalué contre une souche clinique résistante de *Pseudomonas aeruginosa*. Cette évaluation du niveau de résistance à été établie par mesure de la CMI, par des tests en échiquier et des courbes de mortalité. A une concentration de 250 nM, le PEI (10 kDa) n'était ni directement bactéricide ni bactériostatique. Cependant lorsque utilisé en combinaison avec la novobiocine, la ceftazidime, l'ampicilline, la ticarcilline, la carbenicilline, la piperacilline, la cefotaxime, le chloramphenicol, le rifampin ou la norfloxacine, il diminuait la CMI de ces antibiotiques de 1,5 à 50 fois. En revanche, la CMI des aminoglycosides, des polymixines et de la vancomycine sont augmentées de 1 à 5 fois et celles de la tetracycline, l'érythromycine, la ciprofloxacine et l'ofloxacine ne sont pas affectées. Dans les études de courbe de mortalité la combinaison du PEI avec la novobiocine, la ceftazidime, le chloramphenicol et le rifampin réduit de 5 à 10-$\log_{10}$ le nombre de CFU/ml lorsque 25% de la CMI de chaque antibiotique est utilisé. Ces résultats suggèrent que les infections causées par des souches résistantes de *Pseudomonas aeruginosa* puisse être potentiellement traitées par une combinaison de polytethylenimine et d'antibiotiques.

# Synergy Between Polyethylenimine and Different Families of Antibiotics against a Resistant Clinical Isolate of *Pseudomonas aeruginosa*

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Running title: Synergistic effect of polyethylenimine

Key words: Polyethylenimine, Bacteria, *Pseudomonas aeruginosa*, Antibiotics, Synergy, Permeability

*. corresponding author [redacted]

**Abstract**

The in-vitro activity of 10 families of antimicrobial agents alone and in combination with a synthetic polycationic polymer, polyethylenimine (PEI) was investigated against a resistant clinical isolate of *Pseudomonas aeruginosa* by MIC, checkerboard testing and killing curve studies. At a concentration of 250 nM, PEI (10 kDa) was not directly bactericidal nor bacteriostatic but when used in combination with novobiocin, ceftazidime, ampicillin, ticarcillin, carbenicillin, piperacillin, cefotaxime, chloramphenicol, rifampin or norfloxacin it significantly reduced the MICs of these antibiotics by 1,5- to 50-fold. However MICs of aminoglycosides, polymyxins and vancomycins were increased by 1- to 5-fold and finally tetracycline, erythromycin, ciprofloxacin and ofloxacin MICs were not affected when combined with polyethylenimine. In killing curve studies, combination of PEI with novobiocin, ceftazidime, chloramphenicol and rifampin resulted in 5- to 8-$\log_{10}$ CFU/ml decrease in bacterial counts when 25% of the MIC of each antibiotics were used. These results suggest that infections due to resistant *Pseudomonas* strains could be potentially treated by a synergistic combination of polyethylenimine and antimicrobial drugs.

## 1. Introduction

Over the past two decades, *Pseudomonas aeruginosa* has attracted attention as an opportunistic pathogen in hospitalized, immunocompromised and cystic fibrosis patients (1-3). Despite aggressive antibiotic therapy, *P. aeruginosa* is rarely eradicated due to high intrinsic resistance to many drugs (4-6). This results from the effective permeability function of Gram-negative bacteria outer membrane (OM) to both hydrophobic antibiotics and high molecular weight hydrophilic drugs (for reviews, see references 7-10). Unlike most type of cells, Gram-negative bacteria surround themselves with a double membrane. The outermost of these two membranes is asymmetric, with the inner leaflet composed of glycerophospholipids whereas the outer predominantly constituted of lipopolysaccharide (LPS). Tight interactions between the highly negatively charged LPS are believed to form a very effective barrier against hydrophobic compounds. In order for these polyanionic molecules to form a stable "tiled roof" on the surface of the OM, adjacent LPS molecules are linked electrostatically by divalent cations ($Ca^{2+}$, $Mg^{2+}$) available in the OM.

Polyethylenimine (PEI) is a weakly basic, aliphatic, non-toxic, polymer which is polycationic due to the presence of primary, secondary and tertiary amino groups. It is well known that certain polycationic agents such as polymyxin and its derivatives, polylisines and protamine are able to make the Gram-negative bacterial OM permeable to solutes that normally are unable to penetrate (10). Helander et al. observed that PEI possesses strong permeabilizing activity on

Gram-negative bacteria without having any bactericidal effect when applied alone (11). *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* were effectively sensitized to hydrophobic antibiotics (Erythromycin, Novobiocin, Rifampicin, Clindamycin and Fucidin) by PEI in an agar diffusion assay (11). In the present study we have investigated whether such permeabilizer compound could be used in combination with a large spectrum of antibiotics against a resistant clinical strain of *Pseudomonas aeruginosa*. To ascertain a synergestic effect of PEI on various antibiotics, three currently used methods were applied in this study (12). We evaluated the minimal inhibitory concentration (MIC) of each antibiotic with or without PEI. The synergism was also assessed by measuring the fractional inhibitory concentration indices (FIC) of each combination by checkerboard testing (13). Finally, synergism was measured by killing curve test. Our results indicate that polyethylenimine enhances the bactericidal efficacy of both hydrophilic and hydrophobic antibiotics with some exceptions. Polyethylenimine decreases the uptake of polycationic antibiotics of the polymyxin group and aminoglycosides probably by competition for the cation binding sites of bacterial LPS. All the line of evidence suggest that the enhanced bactericidal activity of antibiotics by PEI results from a strong binding to LPS, disorganizing the whole bacterial OM and thus facilitating antibiotic penetration.

## 2. Material and Methods

### *2.1 Reagents*

PEI (mean molecular weight 10 kDa) was purchased from Polysciences Inc. (Warrington, PA). A stock solution of PEI, pH adjusted to 7.4, was freshly prepared and filter sterilized through a 0.22 μm membrane. Novobiocin, Tobramycin, Kanamycin, Gentamicin, Tetracycline, Cefotaxime, Chloramphenicol, Erythromycin, Polymyxin B, Polymyxin E, Rifampicin, Vancomycin, Ofloxacin and Norfloxacin were obtained from Sigma (Oakville, Ontario, Canada). Ciprofloxacin was from Bayer (Kankakee, IL) and Ceftazidime from Glaxo Canada Inc. (Montréal, Quebec, Canada).

*2.2 Bacterial strains and growth conditions*

The resistant clinical strain used for this experiment was *Pseudomonas aeruginosa* 100609, isolated from the sputum of a cystic fibrosis patient. Microorganisms were stored at $-70^0$C in Brain Heart Infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% glycerol. The growth medium in all liquid cultures was Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, Mich.)

*2.3 MIC determinations*

The minimal inhibitory concentration were determined by broth microdilution assay in accordance with the procedures recommended by the National Committee for Clinical Laboratory Standards [13]. Briefly, a bacterial inoculum of 50 μl corresponding to $5 \times 10^5$ colony forming units (CFU) / ml (0.02 O.D., 660 nm) with or without PEI (250 nM final concentration), were added to 50 μl of serial two-fold dilutions of antibiotics into the wells of microtiter plates (Sarstedt,

Quebec, Canada). The plates were incubated under agitation at 37°C for 20 h. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth. MICs were determined three times in triplicate.

*2.4 Checkerboard Testing*

Checkerboard tests were performed in triplicate for all combinations as described previously (13). TSB with an inoculum of approximately 5 x $10^5$ CFU/ml was used for all checkerboard experiments. Synergy was defined as a fractional inhibitory concentration (FIC), which is the MIC ratio between a drug in combination and the drug alone . For two interacting compounds A and B, the sum of the FIC's ($\sum FIC = FIC_A + FIC_B$) expresses the extent of the interaction. When the $\sum FIC \leq 0.5$, there is a synergistic effect. $\sum FIC \geq 0.5$ to $\leq 2.0$ is defined as indifference and antagonism has a $\sum FIC \geq 2.0$.

*2.4 Killing curves*

In-vitro killing curves were performed as previously described (14-15) with modifications. Briefly, TSB were inoculated with a 17 h pre-culture of *P.aeruginosa* 100609 giving a stock preparation containing a final inoculum of 5 x $10^7$ cfu/ml, as evaluated by O.D. at 660 nm. This value allowed control growth to reach a density of $10^8$ to $10^{10}$ cfu/ml. PEI was added to a final concentration of 250 nM. Studies were performed in the presence of sub-MIC concentrations (1/4 × MIC) of each antibiotics and controls made of PEI alone, antibiotics alone or only bacteria. After 0, 1.5, 3, 6 and 9 hours samples were diluted serially and plated on

to LB agar plates to obtain viable colonies counts. Experiments were performed in triplicate. Synergy by time-concentration-kill curve was defined as a reduction of $\geq 2.0$ in mean $\log_{10}$ CFU/ml in bacterial counts at any time point during 9-hour experiment when comparing time-concentration-kill curves generated from the combination of antibiotic and PEI as opposed to antibiotic alone. Indifference or antagonism in time-concentration-kill curves was defined as a $\geq 0$ to $\leq 2$ or $\leq 0$ reduction in mean $\log_{10}$ CFU/ml in bacterial counts at any time point during the 9-hour experiment, respectively.

## 3. Results

### *3.1 Minimal inhibitory concentration and checkerboard synergy*

Table 1 shows MIC values obtained with *Pseudomonas aeruginosa* 100609 grown in TSB for 16 antibiotics without PEI and in the presence of 250 nM PEI. This PEI concentration is subinhibitory, indeed the minimal inhibitory concentration for PEI against *Pseudomonas aeruginosa* 100609 was 1μM. PEI at a final concentration of 250 nM decreased by 1,5- to 40-fold the MIC of Novobiocin, Ceftazidime, Cefotaxime, Chloramphenicol, Rifampicin and Norfloxacin. All these antibiotics had checkerboard synergy effect (FIC $\leq 0.5$) except Norfloxacin. For Aminoglycosides, Polymyxins and Vancomycin, in contrast, 250 nM PEI increased the MIC. Among these only Tobramycin and Vancomycin had an FIC $\geq$ 2.0 (antagonism), the others had an indifference checkerboard effect. As for

Tetracycline, Erythromycin, Ciprofloxacin and Ofloxacin, PEI had no effect on the MIC.

*3.2 In vitro determination of the antibacterial action at intervals in time*

Eight antibiotics from different class were chosen for time kill curves, from which four had a decrease in MIC in combination with PEI (Fig.2) and the others had either an increase or no change in MIC (Fig.3). In all killing curves, PEI alone (250 nM) had no effect on bacterial growth when compared to control growth. Fig.2a shows the effect of PEI on the bactericidal activity of Novobiocin (1/4 × MIC). When used in combination, after 9 hours bacterial growth was reduced by more than $10^8$ times whereas both controls and novobiocin alone had no significant difference. Combination of PEI with Ceftazidime (1/4 × MIC) (Fig.2b), Chloramphenicol (1/4 × MIC) (Fig.2c) and Rifampicin (1/4 × MIC) (Fig.2d) decreased the number of *P. aeruginosa* CFUs $10^5$ times more than each antibiotic alone in all three combinations after 9 hours.

In contrast, PEI decreased the bactericidal efficacy of polymyxin B and Vancomycin. Bacterial growth was increased by 2 $\log_{10}$ after 9 hours when PEI was combined to these antibiotics (Fig.3a., 3b.). Finally when PEI was combined to Tobramycin and Ciprofloxacin, no significant difference was observed between the combination, the antibiotic alone and the controls (Fig.3c., 3d.)

## 4. Discussion

Several agents have been used in attempts to alter the OM permeability of Gram negative bacteria (9); these include EDTA and lysozyme (26,27), sodium hexametasulphonate (28), detergents like SDS (29) and deoxycholate as well as permeabilizing portions of antibiotics like polymyxin B (30,31) and other compounds containing positively charged amino groups such as spermidine and lysine (32), DAA and methylglyoxal bis-guanylhydrazone (33).

In the present study we have measured the effect of polyethylenimine on the bactericidal activity of 16 antibiotics divided from 10 different classes. PEI decreased the MIC of either hydrophilic or hydrophobic compounds (with some exceptions) 1,5 to 40 fold (Table 2) and increased their bactericidal effect in killing curves experiments (Fig.2, Fig.3). We had previously demonstrated that PEI also decreased the MIC of four β-lactams by 5 to 50 fold (25).

Intact OM of Gram-negative bacteria generally prevents the diffusion of hydrophobic solutes, because its outer leaflet contains no glycerophospholipids but only highly ordered, quasicrystalline LPS (10,14-16). PEI has recently been reported to make Gram-negative bacteria permeable to hydrophobic antibiotics (11). This polycationic polyamine has all the characteristics of a permeabilizer as described by Vaara M. (1992). Helander I.M. et al (1997) demonstrated that PEI (50 kDa) increased the bacterial uptake of 1-N-phenylnaphtylamine, a hydrophobic probe, sensitized to hydrophobic antibiotics in agar diffusion assay

and increased the susceptibility to lysozyme and the nonionic detergent Triton X-100. But in contrast to other permeabilizer such as protamine and polylysines, PEI does not induce LPS release from the OM. This property was also observed for polymixin B nonapetide (PMBN) (17).

Our results show that depending on the chemical structures of the antibiotics tested, PEI can be either synergistic, antagonist or have no effect when used in combinations. Synergistic effect were observed in both hydrophobic and hydrophilic antibiotics. Table 2. shows that a PEI concentration as low as 250 nM decreased the MIC of hydrophobic antibiotics such as chloramphenicol, novobiocin and rifampicin by 2 to 12 fold. This synergistic effect was also confirmed by checkerboard testing and killing curves studies (Fig.2). All three hydrophobic antibiotics had an FIC index $< 0.5$ indicating a synergy between PEI and the antibiotic. The molecular mechanism by which PEI increases the permeability of the OM has not been elucidated, but it is not due to any marked LPS release (24). The enhanced antibacterial activity is probably due to a redistribution of phospholipids from the inner to the outer layer of the outer membrane by disruption with PEI. It would increase the bacterial membrane permeability and cause partitioning and therefore entry of hydrophobic antibacterial agents.

Two exceptions were observed among the hydrophobic drugs tested. Norfloxacin bactericidal efficacy was only slightly enhanced by PEI, which was not sufficient to decrease the FIC index below 0,5. This is attributable to a gyrase mutation which results in fluoroquinolones resistance (Woods, D.E. Personal communication), therefore an increase in the uptake of Norfloxacin will not affect bacterial growth significantly. The second exception we observed was the case of Erythromycin, PEI had no effect on its bactericidal efficacy. This hydrophobic antibiotic is often used in experiments involving permeabilizing effect of *Escherichia coli* but not *Pseudomonas aeruginosa* membranes. This is because Erythromycin has no effect on *Pseudomonas aeruginosa* proliferation in vitro but only inhibits the production of virulence factors (35). These results are in accordance with those obtained by Helander et al. (11). Further studies with different bacterial strains will help us demonstrate that PEI could have the same effect on erythromycin as PMBN, which increase by 8-30 fold the sensitivity of *Escherichia coli* cells (10).

Among the hydrophilic drugs tested, we observed a decrease of 5 to 56 fold in MIC when ceftazidime, cefotaxime, ampicillin, carbenicillin, piperacillin and ticarcilline were tested in combination with PEI. Hydrophilic compounds usually cross the outer membrane via porine channels (16). In contrast to PMBN, PEI have an effect on the MICs of antibiotics which are believed to traverse the OM through porin pores (10).

On the other hand, PEI decreased the bactericidal activity of polymyxins and aminoglycosides (Table 2.). These antibiotics acts first through their fixation to the outer membrane of the bacteria, the site which is probably located in the deep core region and includes part of the lipid A of the lipopolysaccharides (17,37). The interaction between PEI and these positively charged hydrophilic antibiotics seems to be a competition for the same site of fixation, so the affinity between PEI and the outer membrane should be greater than for the antibiotics.

A criticism that could be levelled at the procedure used here is that the permeabilizer might sequester ions from the medium which could result in either, or both, the production of weakened or nutritionally-defective cells. However this would not explain the effect of PEI on Polymyxins and aminoglycosides, neither the increased uptake of NPN observed by Helander et al. (11).

*P.aeruginosa* infections are difficult to overcome due to their high intrinsic resistance to antibiotics. The primary cause of such resistance is attributed to reduced outer membrane permeability (18-20). It has been estimated that the rate of permeation of β-lactam antibiotics across the *P.aeruginosa* outer membrane is about 12-fold less than that across *E.coli* outer membranes (21). One possible therapeutic strategy against *P.aeruginosa*, therefore, might be to attempt to reduce the effect of lower outer membrane permeability by cotreatement with permeabilizers such as polyethylenimine, thus allowing increased uptake of antibiotics across the outer membrane. Further work is required to investigate the

ability of polyethylenimine to increase susceptibility to antibacterials in other strains such as *Burkholderia cepacia* and *Escherichia coli*.

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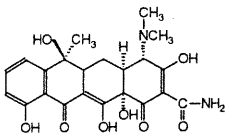
Fig.1. Chemical structure of the hydrophilic (A) and Hydrophobic (B) drugs tested in this study.

Fig.2. In-vitro growth and killing curves of *Pseudomonas aeruginosa* 100609 exposed to 4 hydrophobic antibiotics synergistic with PEI. (●) Control growth curve of the strain in absence of antibiotics and PEI, (■) with PEI (250 μg/ml), (▲) with antibiotic (1/4 x MIC), ( ◇ ) with antibiotic (1/4 x MIC) and PEI (250 μg/ml). Data are means ± SD of an experiment performed in triplicate.

Fig.3. In-vitro growth and killing curves of *Pseudomonas aeruginosa* 100609 exposed to 3 antibiotics antagonized or not affected by PEI. (●) Control growth curve of the strain in absence of antibiotics and PEI, (■) with PEI (250 μg/ml), (▲) with antibiotic (1/4 x MIC), ( ◇ ) with antibiotic (1/4 x MIC) and PEI (250 μg/ml). Data are means ± SD of an experiment performed in triplicate.



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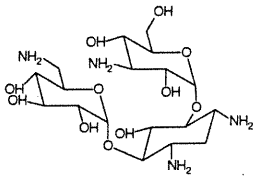
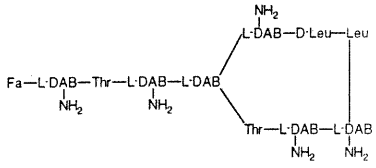
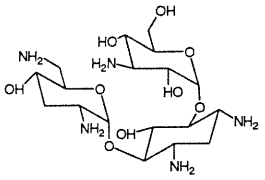


Kanamycin A



Polymyxin E



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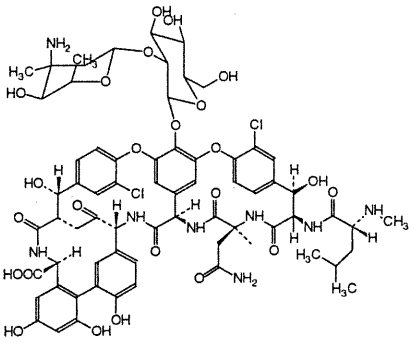

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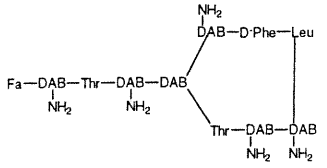
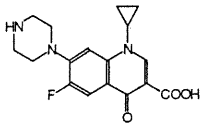
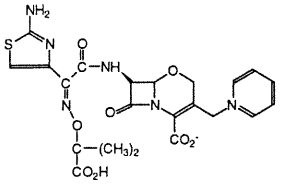


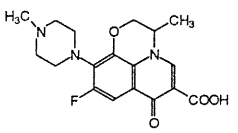
Polymyxin B



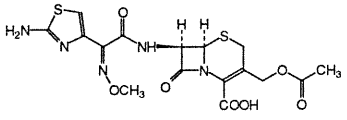
Ciprofloxacin



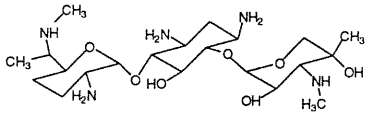
Ceftazidime



Ofloxacin



Cefotaxime



Gentamycin

**A.** Hydrophilic drugs

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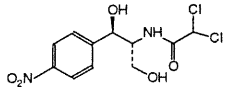
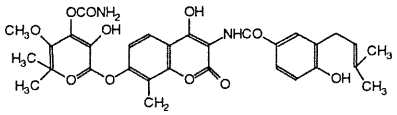
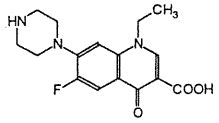


Chloramphenicol



Novobiocin



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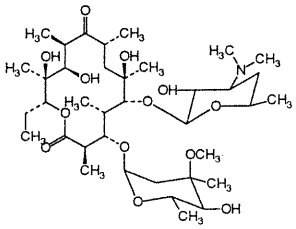
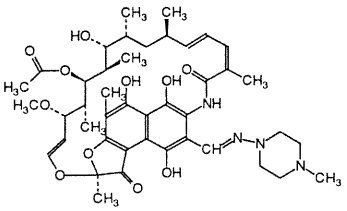


Erythromycin



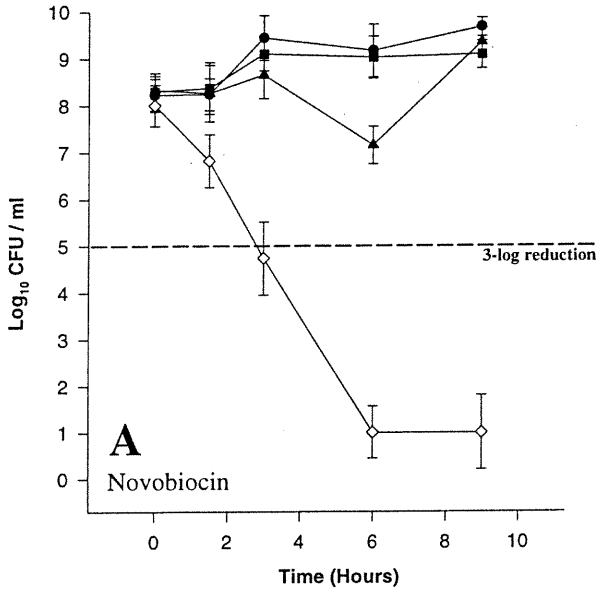
Rifampicin

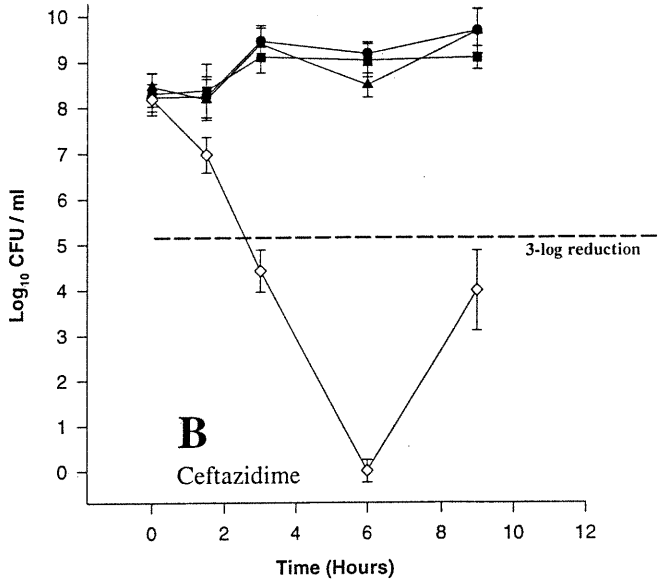
**B.** Hydrophobic drugs

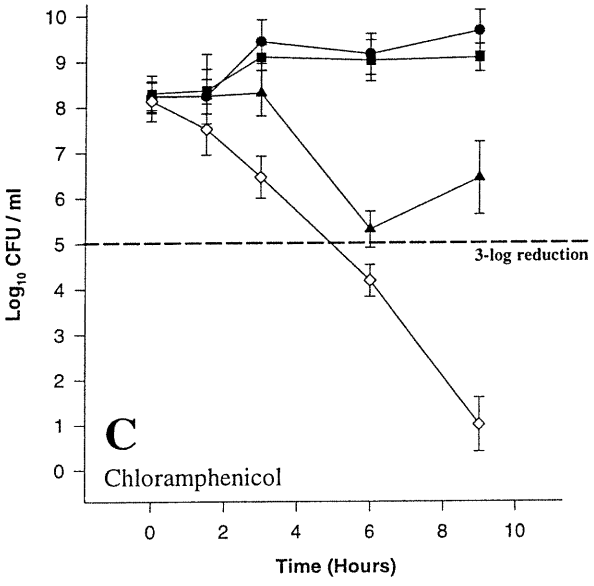
**Table 2.** Effect of PEI on the efficacy different families of antibiotics against *P. aeruginosa* 100609

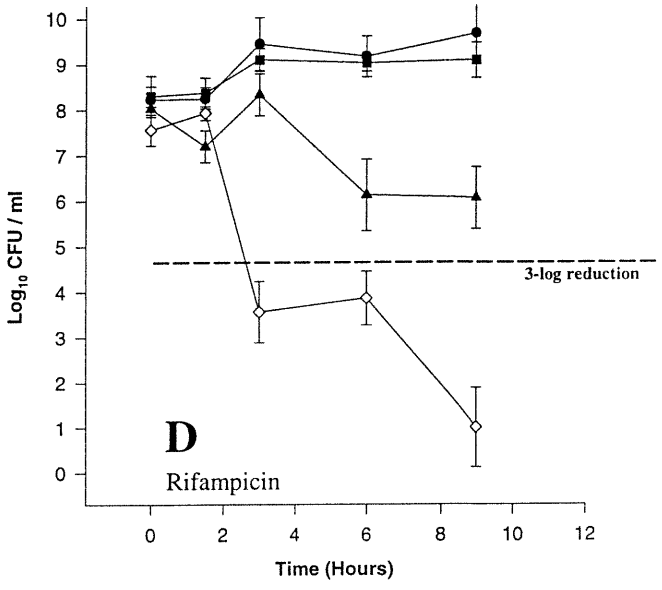
| Class | Antibiotic | MIC (µg/ml) | | Checkerboard FIC | Checkerboard effect | Killing curve effect |
|---|---|---|---|---|---|---|
| | | Alone | + PEI (250 µg/ml) | | | |
| **Novobiocin** | Novobiocin | 1600 | 140 | 0.10 | synergy | synergy |
| **Aminoglycosides** | Tobramycin | 50 | 240 | 4.81 | antagonism | indifference |
| | Kanamycin A | 1600 | 1920 | 1.21 | indifference | ND |
| | Gentamycin | 1120 | 1920 | 1.72 | indifference | ND |
| **Tetracyclines** | Tetracycline | 20 | 20 | 1.00 | indifference | ND |
| **Cephalosporines** | Ceftazidime | 400 | 10 | 0.04 | synergy | synergy |
| | Cefotaxime | 960 | 120 | 0.14 | synergy | ND |
| **Chloramphenicol** | Chloramphenicol | 100 | 40 | 0.41 | synergy | synergy |
| **Macrolides** | Erythromycin | 140 | 140 | 1.00 | indifference | ND |
| **Polymyxins** | Polymyxin E | 5 | 10 | 2.01 | antagonism | ND |
| | Polymyxin B | 5 | 10 | 2.01 | antagonism | Antagonism |
| **Rifamycins** | Rifampicin | 20 | 10 | 0.05 | synergy | synergy |
| **Cationic glycopeptides** | Vancomycin | 1120 | 3800 | 3.40 | antagonism | antagonism |
| **Fluoroquinolones** | Ciprofloxacin | 30 | 30 | 1.00 | indifference | indifference |
| | Ofloxacin | 10 | 10 | 1.00 | indifference | ND |
| | Norfloxacin | 15 | 10 | 0.68 | indifference | ND |
| **β-lactams** | Ampicillin | 2240 | 100 * | ND | ND | synergy |
| | Carbenicillin | 2240 | 60 * | ND | ND | synergy |
| | Piperacillin | 40 | 7,5 * | ND | ND | synergy |
| | Ticarcillin | 1120 | 20 * | ND | ND | synergy |

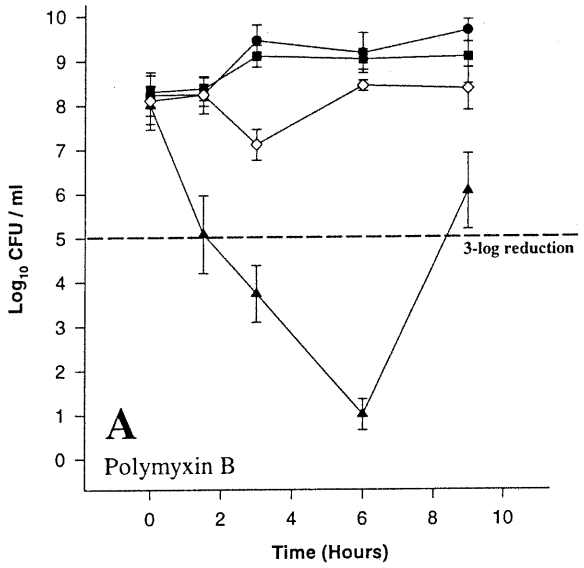
* Synergy between β-lactams and PEI were performed in a previous study in which PEI concentration was 200nM (ref. 25).

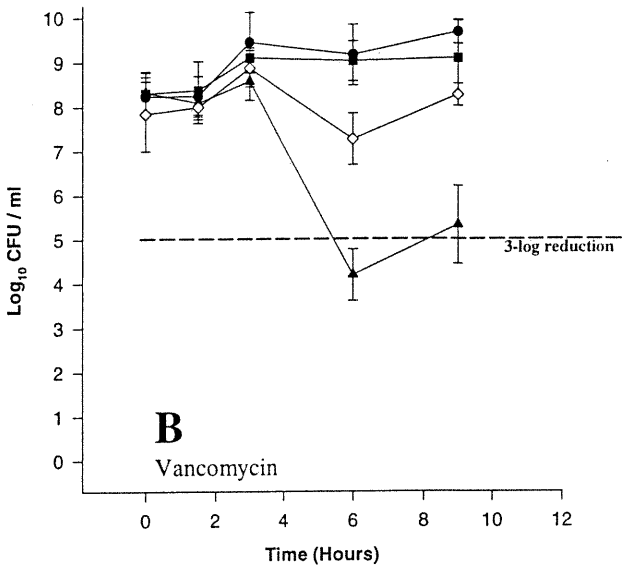

A
Novobiocin
3-log reduction
Log10 CFU / ml
Time (Hours)

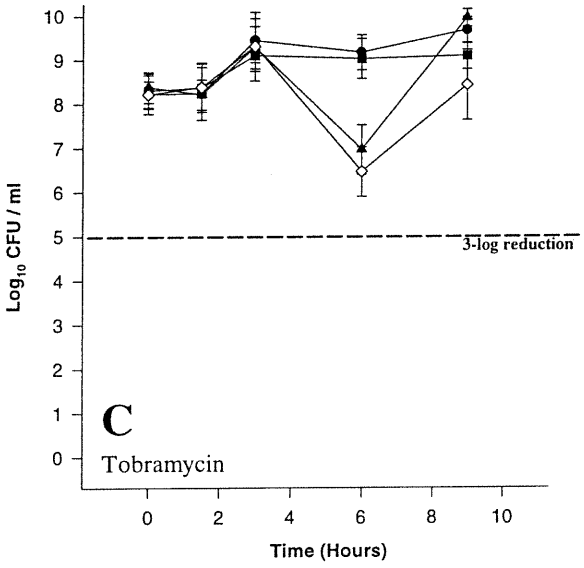

B
Ceftazidime
3-log reduction
Log10 CFU / ml
Time (Hours)

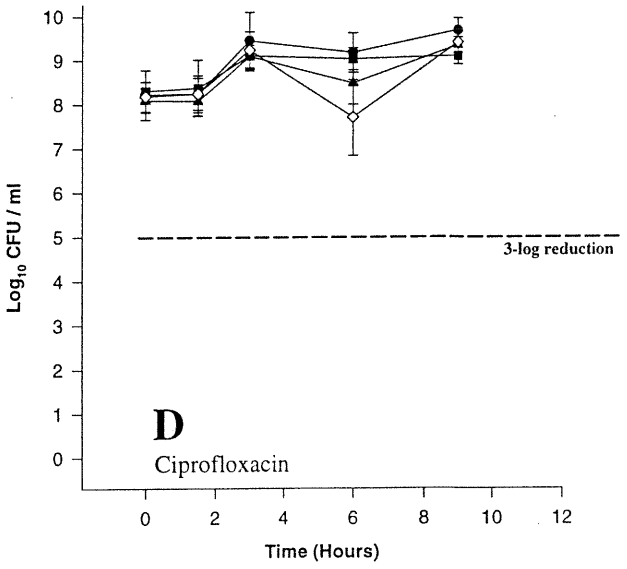

C
Chloramphenicol
3-log reduction
Log10 CFU / ml
Time (Hours)


D
Rifampicin
3-log reduction
Log10 CFU / ml
Time (Hours)


Log10 CFU / ml
3-log reduction
A
Polymyxin B
Time (Hours)


Log10 CFU / ml
3-log reduction
B
Vancomycin
Time (Hours)


Log10 CFU / ml
3-log reduction
C
Tobramycin
Time (Hours)


Log10 CFU / ml
3-log reduction
D
Ciprofloxacin
Time (Hours)

# CHAPITRE 4- ARTICLE

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

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*Biochimica et Biophysica Acta* 1463 : 254-266 (2000)

**Résumé**

Nous avons précédement démontré que de la tobramycine encapsulé dans des liposomes fluides est efficace pour éradiquer une souche de *Pseudomonas aeruginosa* muqueuse dans un modèle animal d'infection pulmonaire chronique. En revanche l'antibiotique libre était incapable de réduire le nombre de CFU au niveau pulmonaire. Nous avons également démontré que ces liposomes sont bactéricides dans des tests in vitro contre une souche résistante de *Pseudomonas aeruginosa*. Afin de définir le mode d'action de ces liposomes fluides, nous avons effectué des études de cytométrie en flux, de microscopie électronique et d'analyse de mélanges phospholipidiques. Les résultats obtenus suggèrent un mécanisme de fusion entre les liposomes et les cellules bactériennes permettant d'obtenir une augmentation du niveau de tobramycine dans le cytoplasme bactérien.

**Contribution de Hayssam Khalil :**

- Section 2.5 : *Bacteria and liposome fusion as measured by lipid-mixing assay*
- Section 3.3 : *Monitor and quantify fusion by lipid-mixing assay*
- Figure 4
- Figure 5
- Tableau 1

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

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**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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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 ($T_C$) $\leq 37°C$ 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×*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 $4 \times 10^{-6}$ 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×*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 $4 \times 10^{-6}$ 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 ≤ 50% of the MIC [12], kinetic studies with *P. aeruginosa* 429 were performed as previously described [12] to investigate

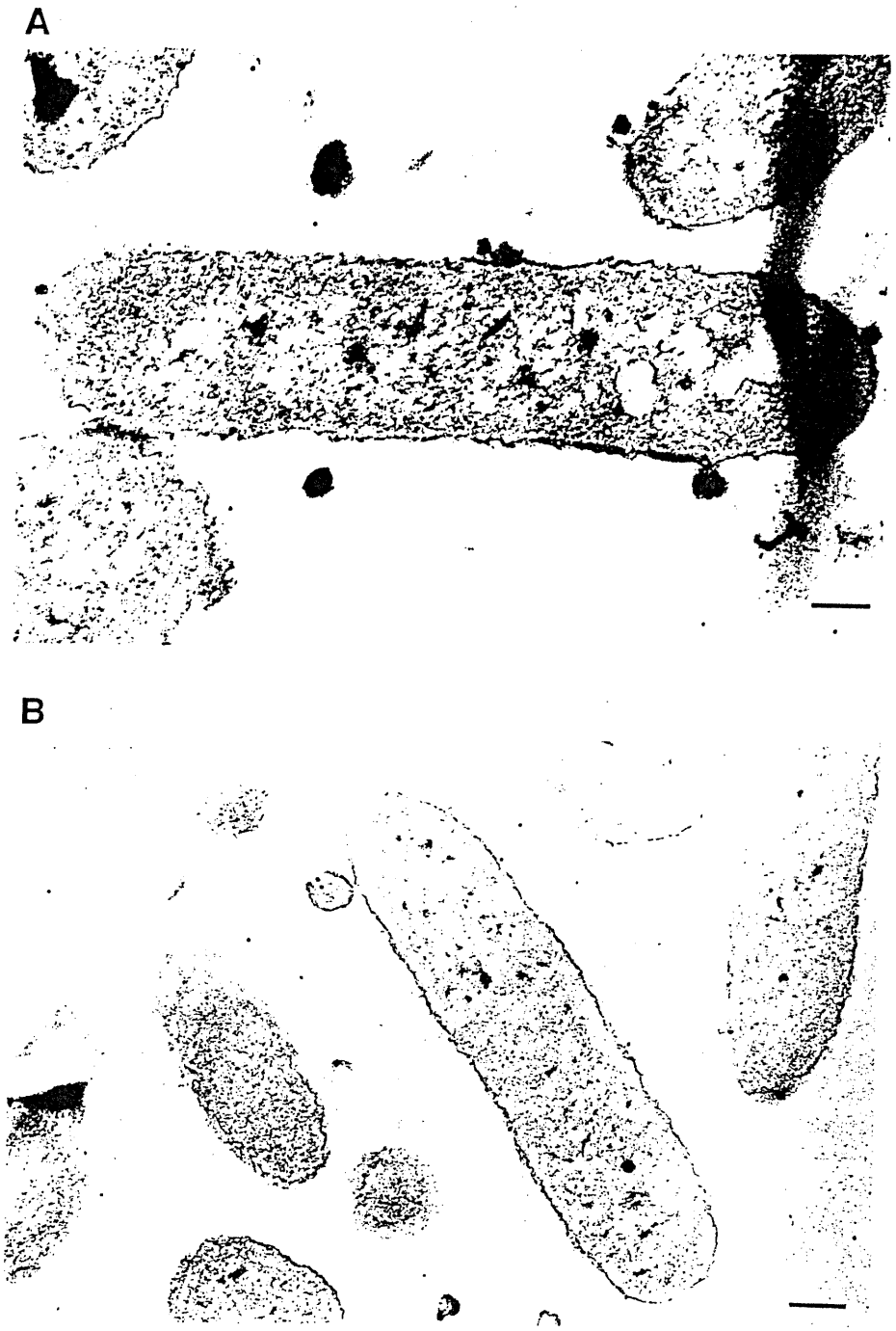


Fig. 1. Detection of tobramycin inside bacterial cells by immunogold labeling. (A) *Pseudomonas aeruginosa* 429 incubated 6 h with Fluidosomes. (B) *Pseudomonas aeruginosa* 429 incubated 6 h with free tobramycin. Magnification: A, ×41 126; B, ×36 720.

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

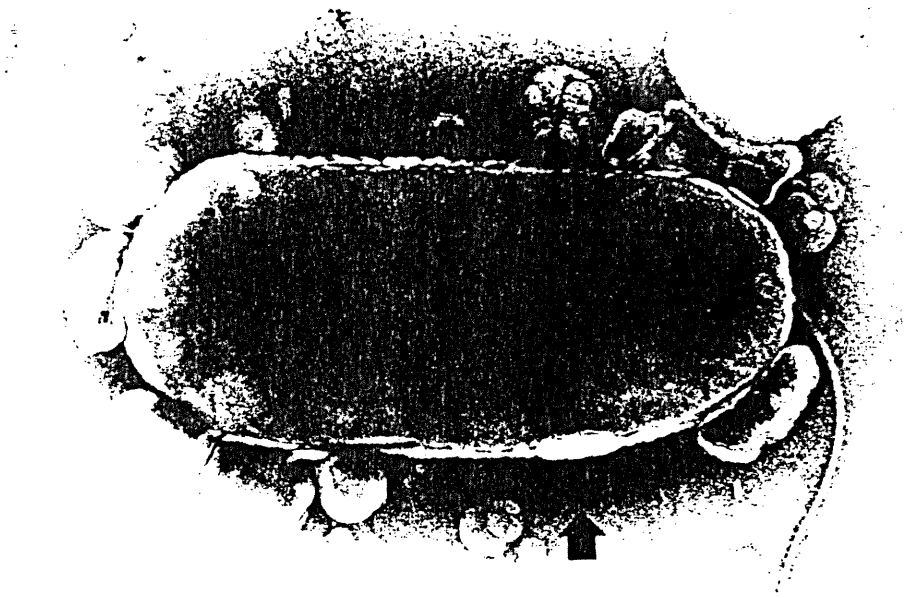

Fig. 2. Interaction of Fluidosomes with *Pseudomonas aeruginosa* 429 cells as observed by negative staining with PTA. Magnification: ×50 561.

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 $9.54 \pm 4.34$ gold particles/$\mu m^2$ in bacteria exposed to Fluidosomes, whereas only $1.20 \pm 1.22$ gold particles/$\mu m^2$ were enumerated in longitudinal sections of bacteria incubated with free tobramycin ($P < 0.001$). The PBS control gave an average labeling of $0.51 \pm 0.82$ gold particles/$\mu m^2$ 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 $1.96 \pm 0.19$, $0.25 \pm 0.12$ and $0.02 \pm 0.02$ gold particles/$\mu m^2$ 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-

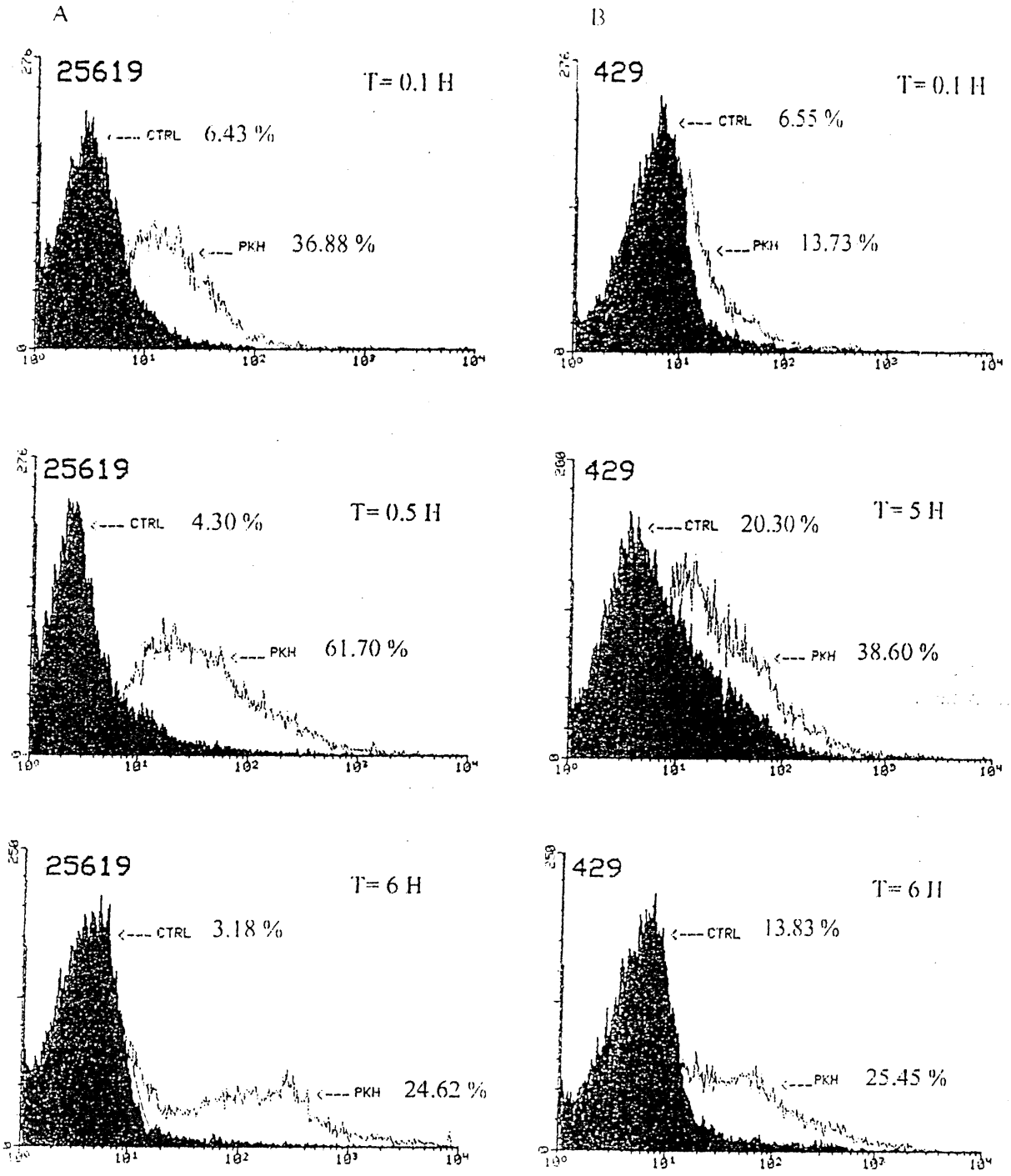


Fig. 3. Flow cytometry histograms of *Pseudomonas aeruginosa* strains ATCC 25619 (A) and 429 (B) incubated with Fluidosomes. T, time (h); CTRL, negative control; PKH, Fluidosomes labeled with PKH. The percentages of fluorescent cells for the negative control and bacteria exposed to PKH2-GL are indicated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

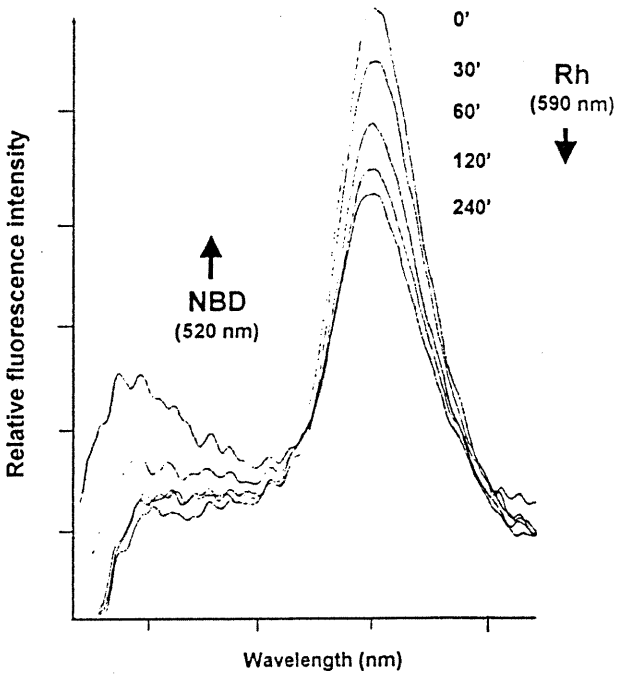


Fig. 4. Fluorescence emission scans of a mixture of Fluidosomes and intact bacteria (*P. aeruginosa* 25619) at 37°C in a wavelength range of 510–610 nm. Fusion of Fluidosomes with bacteria resulted in NBD fluorescence increase and Rh fluorescence decrease (resonance energy transfer efficiency decrease 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 |

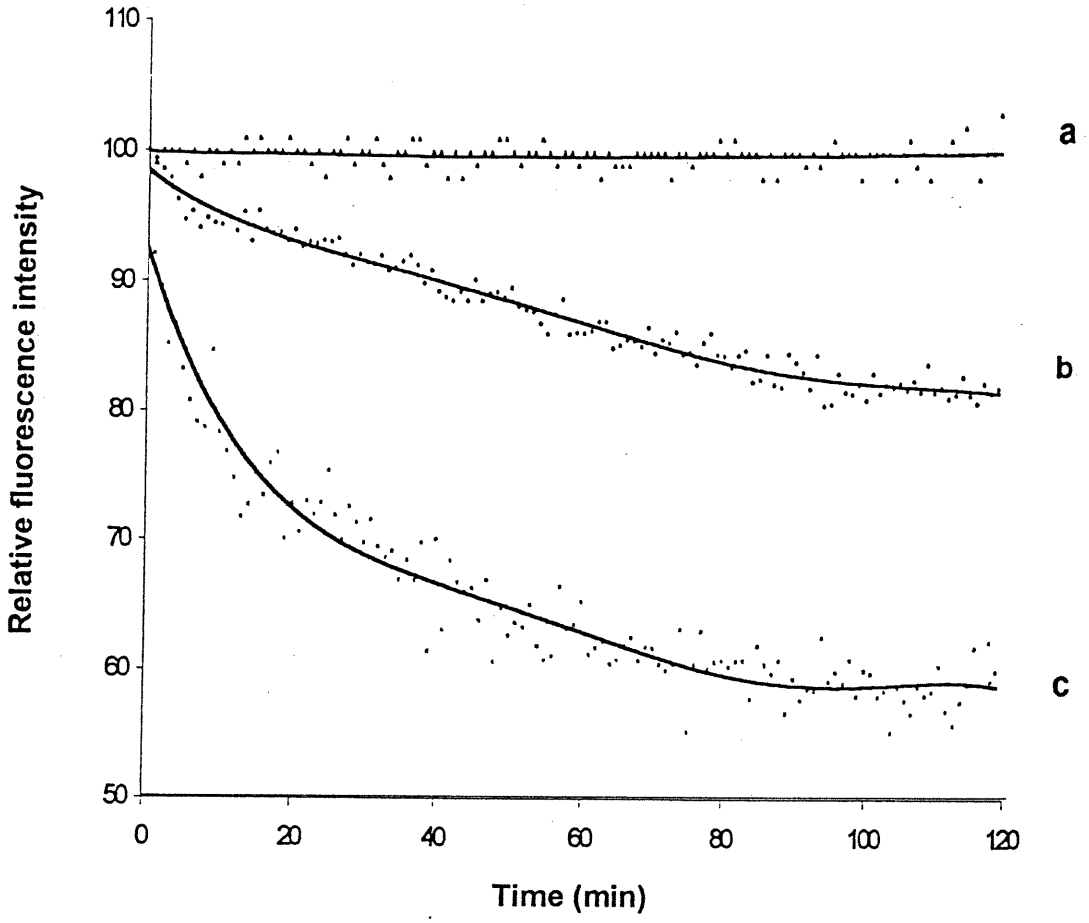


Fig. 5. Fusion of Fluidosomes with intact bacteria monitored by rhodamine fluorescence decrease at 590 nm resulting from resonance energy transfer efficiency decrease (see text). Bacteria grown to an $OD_{660}$ of 0.6 in LB medium were then mixed with 82.5 μM of NBD/Rh-labeled Fluidosomes. Rhodamine 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.

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# CHAPITRE 5- ARTICLE

## Sensitization of a Resistant Clinical Isolate of *Pseudomonas aeruginosa* to β-Lactam Antibiotics by a Synthetic Polycationic Polymer, Polyethylenimine

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**Résumé**

Nous avons étudié l'effet d'un polymère cationique synthétique, le polyéthylenimine (PEI), sur le niveau de résistance aux β-lactames in-vitro de *Pseudomonas aeruginosa*. L'activité bactéricide des β-lactames contre une souche clinique multi-résistante, *P. aeruginosa* 100609 (CMI de 1120 µg/ml pour la ticarcilline) est augmentée en présence de concentrations non-inhibitrice de PEI (CMI de 20 µg/ml pour la ticarcilline en présence de 200 µg/ml de PEI). La CMI de la ticarcilline, la carbenicilline, l'ampicilline et la piperacilline à été diminué de 5 à 50 fois en présence de 200 µg/ml de PEI. Lors d'une étude de courbes de croissance, la combinaison de la ticarcilline (1/8 x CMI) et du PEI (200 µg/ml) réduit le nombre de bactéries viables d'un ordre de 3 $\log_{10}$ lorsque comparée à la ticarcilline seule ou au PEI seul. L'augmentation de l'activité bactéricide des β-lactames par le PEI est synergique et dépendante de la concentration. A la lumière de ces résultats, l'utilisation du polyéthylenimine comme agent perméabilisant de la membrane externe de *Pseudomonas aeruginosa* est discutée.

**Contribution de Hayssam Khalil :**

- Tableau 1
- Figure 2
- Figure 3
- Figure 4

# Sensitization of a Resistant Clinical Isolate of *Pseudomonas aeruginosa* to β-Lactam Antibiotics by a Synthetic Polycationic Polymer, Polyethylenimine

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Running title: Sensitization of *Pseudomonas aeruginosa* by PEI

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**Abstract**

We have studied the influence of a synthetic polycationic polymer, polyethylenimine (PEI), on the *in-vitro* susceptibility of *Pseudomonas aeruginosa* to β-lactams. The antibacterial activity of β-lactams against a resistant clinical isolate, *P. aeruginosa* 100609 (MIC of 1120 μg/ml for ticarcillin) is increased significantly in the presence of a non-inhibitory concentration of PEI (MIC of 20 μg/ml for ticarcilline with 200 μg/ml of PEI), although PEI itself has a very low bactericidal property. MICs of ticarcillin, carbenicillin, ampicillin and piperacillin were efficiently lowered by 5-50 times with 200 μg/ml of PEI. In a kinetic bacterial killing-regrowth study, a combination of ticarcillin (1/8 × MIC) and PEI (200 μg/ml) reduced the viable bacterial count up to 3 log when compared to either ticarcillin or PEI alone. The enhancement of β-lactam bactericidal activity by PEI is synergistic and concentration dependent. It is believed that the synergism observed between β-lactams and PEI is due to an increase in bacterial membrane permeability. The dramatic susceptibility increase of the resistant *P. aeruginosa* by PEI offers an interesting approach for both fundamental research and therapeutic applications. These data provide a rationale for investigating polyethylenimine as a potential permeability-increasing agent against *Pseudomonas aeruginosa.*

## Introduction

*Pseudomonas aeruginosa*, an opportunistic pathogen, plays an important role in chronic pulmonary infections and is considered to be the most important factor determining the prognosis of cystic fibrosis patients (5,15,20). It is also the most frequent bacteria isolated from immuno-compromised hosts and from burn wounds (24). These bacteria are known for their very low nonspecific membrane permeability and/or the presence of membrane-associated energy driven-efflux systems. These mechanisms confers *P. aeruginosa* a resistance to the majority of antibiotics and are thus not easily eradicated even by intensive chemotherapy (18,12,6). Consequently, efficient therapy against *P. aeruginosa* remains difficult and methods to improve bactericidal activity of different antibiotics are currently appealing for both fundamental and clinical research (1,2,21).

It is well known that certain cationic substances are able, in vitro, to enhance the susceptibility of *P. aeruginosa* to antibiotics (22). Polymyxin, polymyxin derivatives, protamine, histones and poly-lysines have been reported to be able to permeabilize *P. aeruginosa* membrane and thus increase the bactericidal action of antibiotic agents. Polyethylenimine, a synthetic polycationic polymer, has also recently been reported to be able to permeabilize Gram-negative bacterial outer membrane. Helander and his co-workers (1997) observed an increase in susceptibility of *E. coli*, *P. aeruginosa* and *S. typhimurium* to erythromycin, novobiocin, rifampin, clindamycin and fucidin in an agar diffusion assay (10). Some studies have indicated that in some cases agar diffusion seems to lead to

false results of bacterial sensitivity with certain frequency (16). To the best of our knowledge there is no report on a systematic investigation of the effect of PEI on the sensitization of bacteria to antibiotics in quantitative microbiological methods such as MIC's and kinetics of bacterial killing. In addition, no studies have been conducted to find whether the bactericidal activity of β-lactam against *P. aeruginosa* can be enhanced by PEI. In the present study, all the lines of evidence suggest that the synthetic polyethylenimine does exhibit a strong effect on the susceptibility of *P. aeruginosa* to β-lactam antibiotics.

## MATERIALS AND METHODS

**Bacterial strains.** A clinical isolate of *Pseudomonas aeruginosa* (PA 100609), originally isolated as a multiple antibiotic resistance strain in Foothills hospital (Calgary, Alberta, Canada), is a gift from Dr. D.E. Woods. Bacteria were stored at –70°C in Brain Heart Infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% glycerol.

**Antibiotics, chemicals and Media.** Piperacillin, ticarcillin, ampicillin, carbenicillin were purchased from Sigma (Oakville, Ontario, Canada), Polyethylenimine (mean molecular weight 10 kDa) was obtained from Polyscience Inc (Warrington, PA). A stock solution of PEI, pH adjusted to 7.4, was prepared in Hepes Buffer Saline (HBS) and filter sterilized through a 0.22 μm filter.

The effect of PEI (200 μg/ml) on four β-lactam antibiotics (1/4 × MIC) was evaluated using only optical density measurement method. The tubes were incubated in a temperature controlled shaker (200 rpm, 37°C). Bacterial growth was monitored by recording the increase in optical density ($OD_{550nm}$). Measurements were made at 1, 2, 4, 6, and 24 h post-inoculation. The bactericidal activity of β-lactams with or without PEI was measured by their logarithmic reduction of the number of bacterial count at different incubation time. In all experiments control made of PEI alone, antibiotics alone or only bacteria were included.

## RESULTS

**Bactericidal effect of PEI on *P. aeruginosa.*** The bactericidal activity of PEI (M.W. 10 kDa) was quantitatively determined by its minimal inhibitory concentration. Previous study has described the experimental use of PEI to increase bacterial membrane permeability (10). They have documented that PEI with a molecular weight of 50 kDa resulted in the precipitation of bacterial cells. We observed the similar phenomena with 50 kDa PEI. It is interesting to note that significant precipitation of bacteria was induced by PEI with a high molecular weight (50 or 750 kDa), but not with smaller ones (M.W. 10 kDa). In order to avoid misinterpretation of bactericidal activity and the susceptibility enhancing property of PEI on bacteria, we used the 10 kDa PEI in the following studies. MIC study indicated that PEI has no anti-*Pseudomonas* activity, at least in the concentration range tested. There was no observed bacterial inhibition when up to

**Minimal inhibition concentration of antibiotics.** The minimal inhibitory concentration of PEI, β-lactams antibiotics and combinations of both was measured by broth microdilution assay in accordance with the procedures recommended by the National Committee for Clinical Laboratory Standards (17). Briefly, 100 µl of an overnight culture grown in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, Mich.) were added to 20 ml of fresh TSB broth. When the inoculum reached an $OD_{550nm}$ of 0.02, corresponding to $5\times10^5$ colony forming units (cfu)/ml, 50 µl was added to 50 µl of serial two-fold dilutions of testing samples (appropriate amount of PEI 200 µg/ml, β-lactam antibiotics or combination of both) into the wells of microtiter plates (Sarstedt, Québec, Canada). The MICs were determined as the lowest antibiotic concentration at which no visible growth was observed after incubation at 37°C overnight. No precipitation was observed at the concentration tested.

**Bacterial killing curve.** *In-vitro* bacterial killing curves were performed as previously described (8). Briefly, fresh TSB was inoculated with a pre-culture of *P. aeruginosa* 100609 giving a suspension containing a final inoculum of $1 \times 10^6$ cfu/ml, as evaluated by $OD_{550nm}$. PEI was added to a final concentration of 200 µg/ml. In order to ascertain the reliability of killing curves obtained by optical density measurement compared to cfu counts, PEI at a final concentration of 200 µg/ml and ticarcillin (1/8 × MIC) were used respectively.

500 μg/ml of PEI were present. Our results are in agreement with previous study obtained with the agar diffusion method (10).

**Effect of PEI on β-lactam bactericidal activity.** The ability of PEI to sensitize *P. aeruginosa* to hydrophobic β-lactams was determined by their corresponding MIC with and without addition of 200 μg/ml PEI. As shown in Table 1, the susceptibility of *P. aeruginosa* toward four β–lactams was increased 56, 37, 22 and 5 folds for ticarcillin, carbenicillin, ampecillin, and pipericillin, respectively. The enhancing effect on antibacterial activity of β–lactams was much more dramatic for ticarcillin, ampicillin and carbenicillin which have the highest MICs against *P. aeruginosa*, while the latter effect was less effective for piperacillin. The reason for such difference could be related to the bacterial mechanism of resistance. It is understandable that the sensitization effect of PEI would have a larger impact on antibiotics against which bacteria are more resistant than ones with a relatively lower resistance.

**Bacterial killing-regrowth study.** The killing and regrowth kinetics of *P. aeruginosa* exposed to sub-MICs of ticarcillin and sub-concentration of PEI are presented in Figure 2. Both optical density measurement and accurate viable bacterial count (cfu/ml) give similar conclusion on the effect of PEI on the susceptibility of *P. aeruginosa* to antibiotic. There was no inhibition effect on bacterial growth with either 140 μg/ml ticarcillin (1/8 × MIC) or 200 μg/ml PEI alone, but this bacterial growth was greatly retarded when a combination of

ticarcillin (1/8 × MIC) and 200 μg/ml PEI was used. Regardless of the monitoring methods used, a significant bactericidal enhancing effect was demonstrated by including PEI in the treatment solutions.

Such efficient bacterial killing of antibiotics in combination with 200 μg/ml of PEI was clearly demonstrated for all four β-lactams tested by killing curve tests as shown in Figure 3. The resultant bacterial killing was not additive, but clearly reflected a synergistic effect. Alone, PEI and each antibiotic tested at sub-MIC concentrations (1/4 × MIC) did not have any bactericidal effect, but excellent bactericidal activity were produced when antibiotics were combined with PEI.

**Correlation between susceptibility and concentration of PEI.** The bactericidal activity was also dependent on the concentration of PEI as shown in Figure 4. Using a constant sub-MIC concentration of ticarcillin (1/4 × MIC), lower PEI concentrations (10 – 50 μg/ml) did not influence the bacterial killing activity of ticarcillin. As PEI concentration was increased, the net killing was proportionally increased. A rough percentage of bacteria killing can be calculated with optical density measurements by equation 1.

$$\text{Bacterial killing (\%)} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100 \qquad [1]$$

where $OD_{control}$ and $OD_{sample}$ are viable bacteria counts measured by optical density for control and antibiotic treated samples, respectively. A correlation

between bacterial killing by sub-MIC of ticarcillin at 24 hours and the amount of PEI is represented in Figure 5.

## DISCUSSION

Although a large number of cationic peptides have been investigated as potential bactericidal agents and some of them also show synergistic effect with hydrophobic antibiotics (22,8,9), very little is known regarding the ability of non-peptide polymers to enhance the bactericidal activity of conventional antibiotics. By using a synthetic polycationic polymer, polyethylenimine, Helander and his co-workers observed the enhanced susceptibility of *E. coli*, *P. aeruginosa* and *S. typhimurium* to erythromycin, novobiocin, rifampin, clindamycin and fucidin in the agar diffusion assay (10). Their results demonstrated that PEI increases the permeability of the outer membrane of Gram-negative bacteria and may be a potent permeabilizer to increase antibiotic activity. Despite such encouraging information, there is still a lack of both quantitative results produced by conventional microbiological methods in a liquid medium and information on synergistic action with β-lactam antibiotics to the clinically isolated resistant *P. aeruginosa*. The current study explored the potential of PEI in increasing the sensibility of a resistant *P. aeruginosa* 100609 strain to conventional antibiotics. We determined the MICs of four β-lactams toward this isolate under different concentrations of PEI and measured bacterial killing-regrowth kinetics. *In-vitro* studies confirmed the potential of polyethylenimine to enhance the susceptibility of the resistant *P. aeruginosa* isolate to β-lactam antibiotics. With 200 μg/ml of

PEI, the bactericidal activities of ticarcillin, carbenicillin, ampicillin, and piperacillin were all greatly increased in a range of 5-50 times.

β-Lactams are the most frequently used class of antibiotics, but resistance is also associated with them. Accumulated evidences suggest that three mechanisms may be involved in the β–lactam resistance: deactivation of β-lactams by β–lactamase, decreased outer membrane (OM) permeability, and efflux pump (4,13,14,19). The use of β–lactamase inhibitors in the combination therapy tackles the β–lactam hydrolysis enzyme, avoiding inactivation of β–lactams and allowing the antibiotics to proceed with their normal bactericidal activity. This approach has little or no effect on the drug resistance caused by low permeability and efflux pump. Using polycationic molecules to permeabilize the outer membrane of Gram-negative bacteria has previously been demonstrated to be a rational strategy for enhancing penetration of hydrophobic antibiotics. This strategy is more efficient to overcome either low permeability and efflux pump. More or less, it has also shown some degree of reduction on the enzyme deactivation reactions. As a highly functionalized polycationic polymer, PEI resembles the action mechanisms of other polycationic compounds such as the polycationic peptides discovered by Vaara and coworkers (23). PEIs are good OM permeabilizers, but lack the direct bactericidal activity as reported by Helander *et al.* (10,11). It is believed that binding between amino groups of polyethylenimine and anionic lipopolysaccharides (LPS) of *P. aeruginosa* will disrupt the outer membrane structure, resulting in increased permeability, allowing

hydrophobic β-lactams to enter the cell and inhibit specific bacterial metabolic processes. Figures 1 and 2 indicate that PEI is a potent permeabilizer for disturbing the OM of *P. aeruginosa* to allow hydrophobic β-lactams to penetrate through the bacterial LPS protection layer. At sub-MIC concentrations, the bactericidal activity of ticarcillin was proportional to the amount of PEI. This is in agreement with the results of enhanced hydrophobic antimicrobial activities assessed with the agar diffusion assay reported by Helander *et al.* (10).

PEIs are a class of polymer which are commercially produced through the acid-catalyzed ring-opening polymerization of monomeric ethylenimine. Contrary to other cationic bacterial sensitization agents, PEIs hold the following advantages: 1). most commercial PEI is branched and consists of primary, secondary and tertiary amines in the approximate proportion of 1:2:1, while most cationic peptides such as polylysine are linear, with only one type of amino group; 2). PEI has high pH-buffering capacity and the highest cationic density (one positive charge per 34 Da molecular weight); 3). It is neither a peptide nor protein. Therefore, it does not have a potential immunogenic side effect; and 4). PEI is cheap and easy to synthesize in a variety of molecular size. It is interesting to note that PEI has already been a common ingredient in a variety of cosmetic formulations (7) and has been used as carriers for DNA and oligonucleotide delivery (3). As demonstrated in this study and other reports (10,11), PEI may be also a potential agent as an additive in different antibiotic formulations to overcome or reduce drug resistance. Taken together, subjects regarding it's

formulation and bactericidal enhancing properties with different classes of antibiotics toward several clinically important bacterial strains should be systematically investigated.

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Figure 1. A partial chemical structure of polyethylenimine under either extremely basic or acidic conditions. The branched PEI has primary, secondary and tertiary amines in the approximate proportion of 1:2:1. PEI has no pKa. The percentage of cationic charges of PEI is proportional to pH. Around 20% of amino groups of PEI are protonized at pH 7.4.

Figure 2. In-vitro growth and killing curves of *Pseudomonas aeruginosa* 100609 measured by (A) optical density at 550 nm and (B) viable bacterial counts (cfu/ml). (○) Control growth curve of the strain in absence of antibiotics and PEI, (■) with PEI (200 μg/ml), (△) with ticarcillin (1/8 × MIC), ( ● ) with ticarcillin (1/8 × MIC) and PEI (200 μg/ml). Data are means ± SD of an experiment performed in triplicate.

Figure 3. In-vitro growth and killing curves of *Pseudomonas aeruginosa* 100609 exposed to 4 β-lactams synergistic with PEI. (●) Control growth curve of the strain in absence of antibiotics and PEI, (□) with PEI (200 μg/ml), (▲) with antibiotic (1/4 × MIC), ( ◇ ) with antibiotic (1/4 × MIC) and PEI (200 μg/ml). Data are means ± SD of an experiment performed in triplicate.

Figure 4. Effect of PEI concentration on the bacterial killing and regrowth. The samples were incubated at 37°C and bacterial growth was quantified by

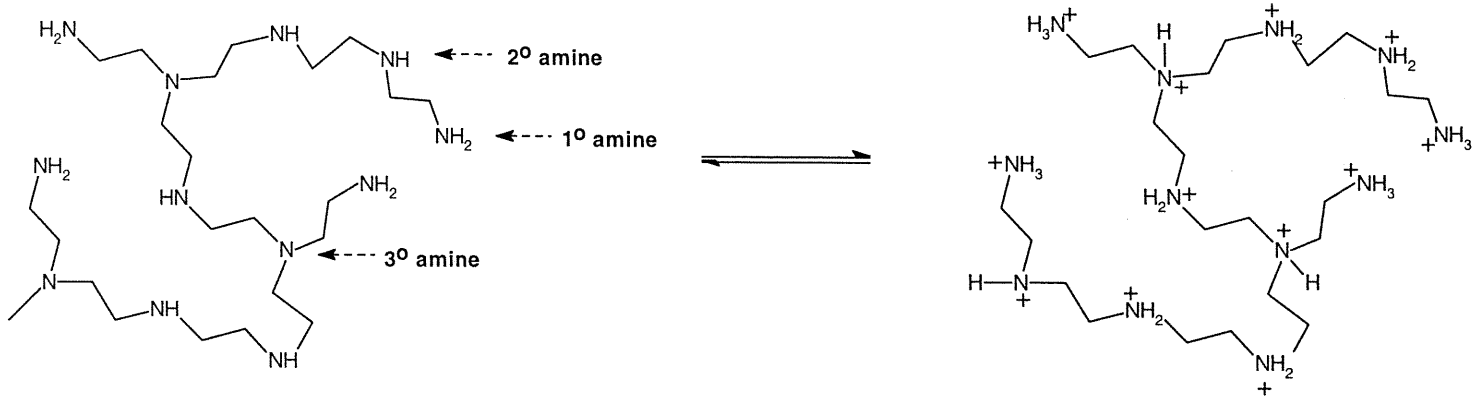
measuring the optical density at the wavelength of 550 nm. Each experiment except of bacteria alone was treated with ticarcillin (Atb) (1/8 × MIC) and the different concentrations of PEI as indicated in figure. Control growth curve were obtained without any antibiotic or PEI. Growth curve in presence of PEI overlapped control growth curve (data not shown).

Figure 5. Efficiency of bactericidal enhancement of ticarcillin by PEI. The efficiency was calculated by equation 1 as described in the text. The viable bacterial count was determined by measure of the optical density at 550 nm after incubation of samples at 37°C for 24 h. Each experiment was treated with ticarcillin (1/8 × MIC) and the different concentrations of PEI as indicated in figure.

TABLE 1. MICs of β–lactam antibiotics alone and in combination with 200μg/ml of PEI against a clinically isolated resistant *P. aeruginosa*.

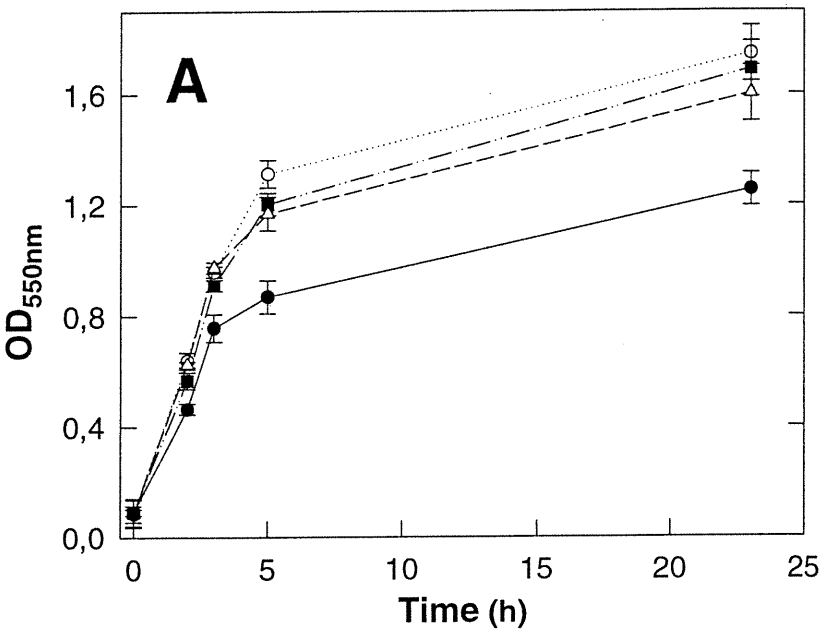
| Antibiotic | MIC (μg/ml) | |
|---|---|---|
| | Without PEI | With PEI* (μg/ml) |
| Carbenicillin | 2240 | 60 |
| Ticarcillin | 1120 | 20 |
| Ampicillin | 2240 | 100 |
| Piperacillin | 40 | 7.5 |

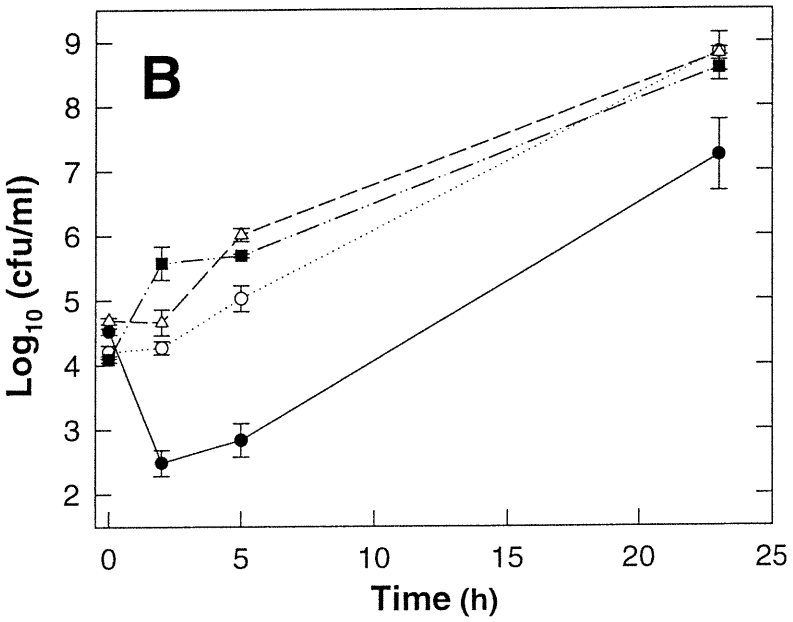
*PEI with a molecular weight of 10 kDa was used for these experiments. The MIC were measured as described in the text.

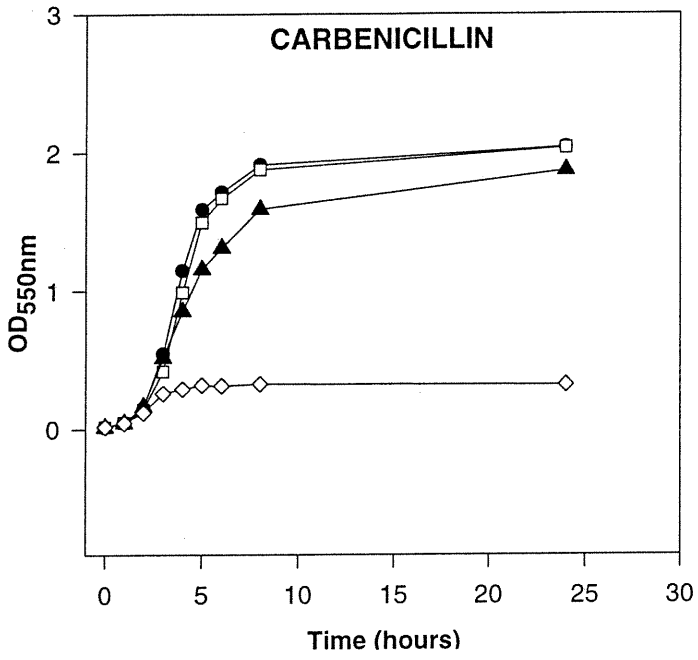


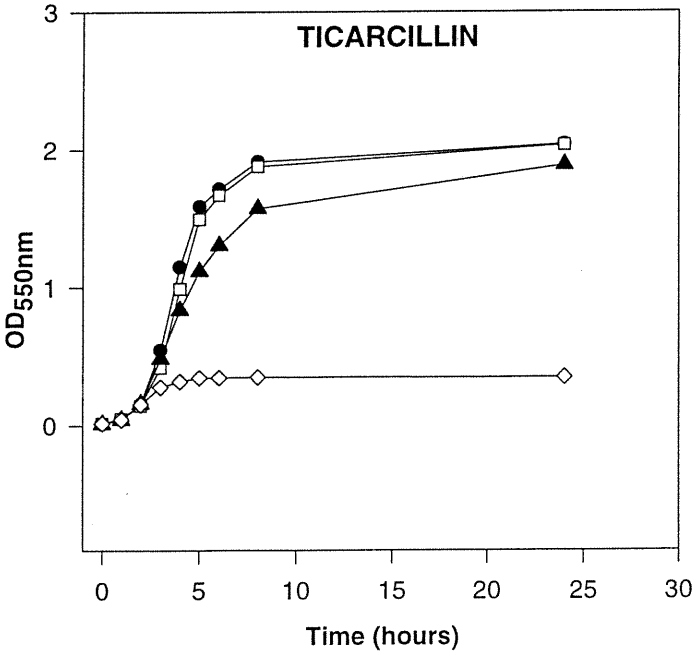

**Basic condition (pH > 14)**

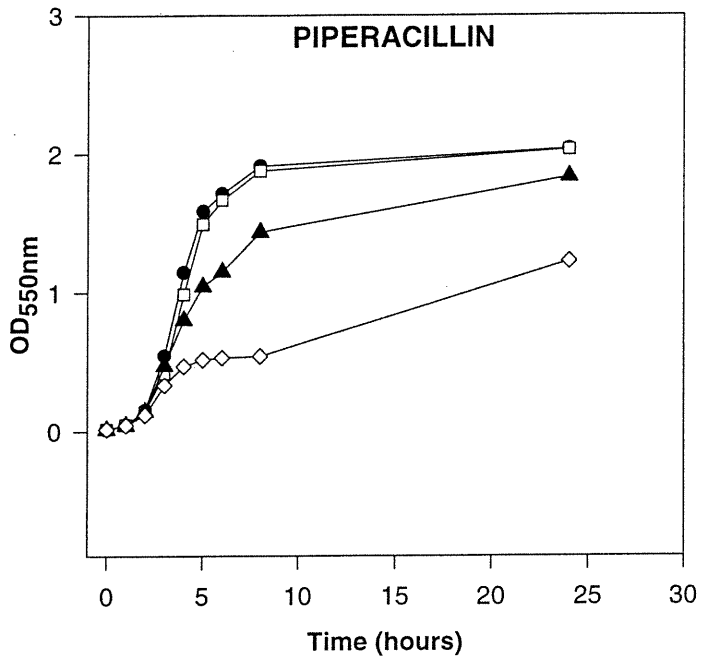
**Acidic conditions**

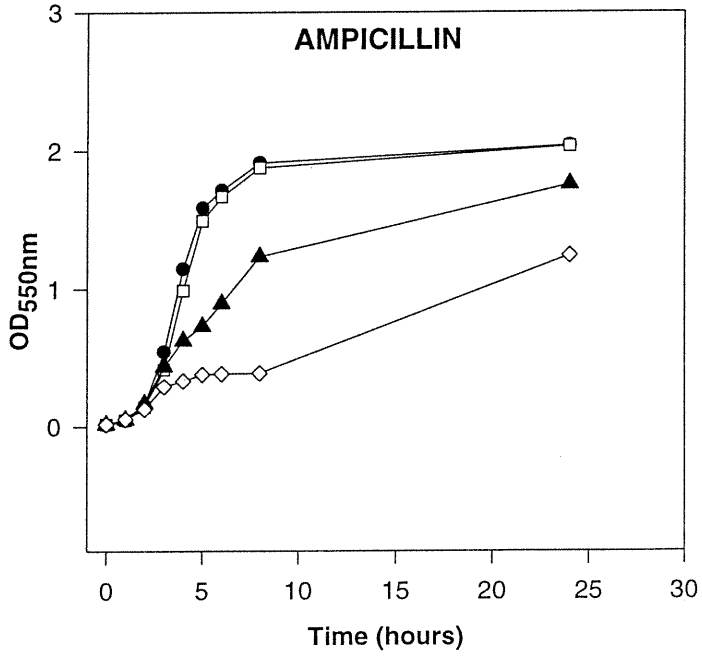

A
1,6
1,2
0,8
0,4
0,0
OD550nm
0
5
10
15
20
25
Time (h)


B
9
8
7
6
5
4
3
2
Log10 (cfu/ml)
0
5
10
15
20
25
Time (h)


CARBENICILLIN
3
2
1
0
OD550nm
0
5
10
15
20
25
30
Time (hours)


TICARCILLIN
3
2
1
0
OD550nm
0
5
10
15
20
25
30
Time (hours)


PIPERACILLIN
3
2
1
0
OD550nm
0
5
10
15
20
25
30
Time (hours)


AMPICILLIN
3
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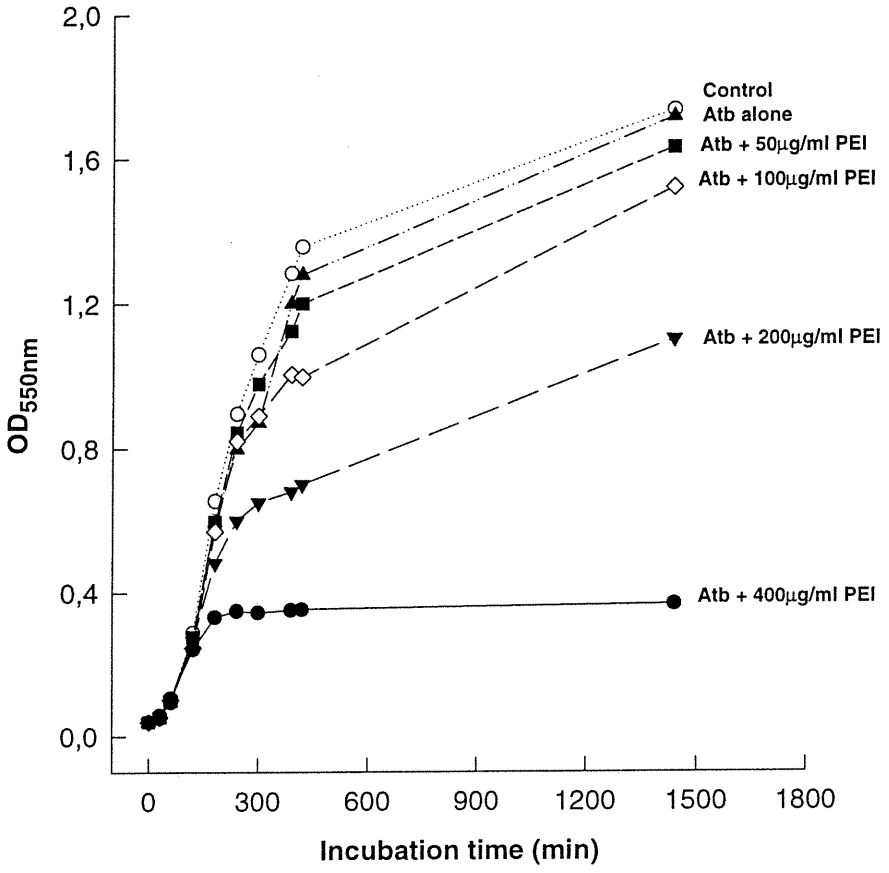
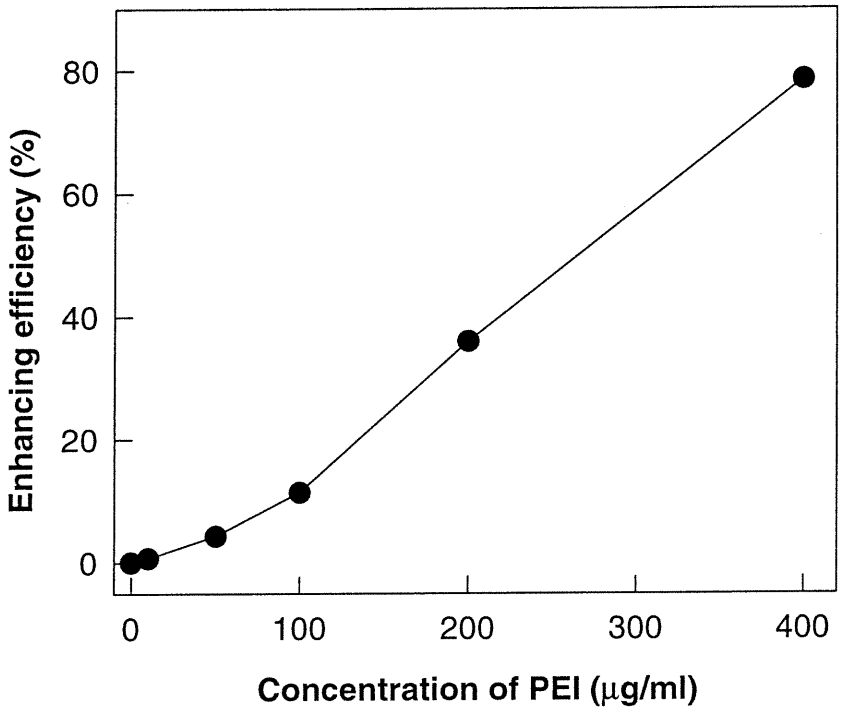
1
0
OD550nm
0
5
10
15
20
25
30
Time (hours)


2,0
1,6
1,2
0,8
0,4
0,0
OD550nm
0
300
600
900
1200
1500
1800
Incubation time (min)
Control
Atb alone
Atb + 50µg/ml PEI
Atb + 100µg/ml PEI
Atb + 200µg/ml PEI
Atb + 400µg/ml PEI


Enhancing efficiency (%)
80
60
40
20
0
0
100
200
300
400
Concentration of PEI (μg/ml)

# CHAPITRE 6- DISCUSSION

Au cours des deux dernières décennies, il a été mis en évidence que *P. aeruginosa* est un pathogène opportuniste majeur chez les patients atteints de fibrose kystique. Malgré des antibiothérapies extrêmement agressives, *P. aeruginosa* est très rarement éradiqué, principalement à cause de sa résistance intrinsèque très élevée aux antibiotiques. Il semble de plus en plus évident que ce phénomène de résistance est attribuable à la membrane externe de ce microorganisme. La membrane externe de *P. aeruginosa* constitue une véritable barrière à l'entrée des antibiotiques hydrophyliques et hydrophobiques.

Pour contrer le haut niveau d'imperméabilité de *P. aeruginosa* aux antibiotiques notre laboratoire a travaillé aux cours des dernières années à développer une formulation liposomale fluide, véritable cheval de Troie contenant de la tobramycine. Cette formulation a démontré *in-vitro* et *in-vivo* une efficacité supérieure à celle de l'antibiotique libre contre *P. aeruginosa* et d'autres bactéries d'importance médicale. Il a ensuite été mis en évidence qu'un mécanisme de fusion entre les liposomes et les bactéries permettait d'atteindre ces résultats.

Le principe de fusion membranaire entre deux liposomes ou entre des liposomes et des membrane biologiques a été le sujet de nombreuses études (Sanchez-Migallon et al., 1995; Bailey and Cullis, 1997; Wilschut, 1990), mais très peu de ces études sont consacrées aux cellules procaryotes.

Conjointement avec un autre membre du laboratoire (Dr. Sébastien Sachetelli), nous avons procédé à des études de microscopie électronique, de cytométrie et de fluorescence afin de mettre en évidence ce phénomène de fusion entre les liposomes fluides et les bactéries. On a ainsi réussi à confirmer les résultats obtenus en microscopie électronique et en cytométrie à l'aide d'une technique de transfert d'énergie de fluorescence par résonnance appliquée à une méthode de mesure de l'échange lipidique entre deux membranes. Nous avons ainsi démontré que le niveau de fusion des liposomes avec les bactéries était proportionnel à leur niveau de résistance membranaire aux antibiotiques. Un taux d'environ 55% de fusion était observé en présence d'une souche sauvage de *P. aeruginosa* (ATCC 25619) comparativement à 29% pour une souche résistante par imperméabilité (Chapitre 4).

Afin de comprendre et de pouvoir optimiser l'interaction entre les liposomes fluides et les bactéries, nous avons étudié l'implication de différents paramètres physico-chimiques dans ce phénomène. Plusieurs facteurs clés ont alors été mis en évidence. C'est ainsi que la stabilisation des liposomes fluides par l'addition de 10% de cholestérol, diminue drastiquement de 44% à 5% le niveau de fusion avec *P. aeruginosa* (Figure 1. Chapitre 2). Une observation similaire a été obtenue en variant la température. A des températures inférieures à la température de transition de phase (37-40°C), on observe une diminution de la capacité de fusion (Figure 5, Chapitre 2). De plus la démonstration que les liposomes ne fusionnent

pas entre eux (Figure 1B, Chapitre 2) indique que la membrane bactérienne est un facteur indispensable pour induire la fusion des liposomes fluides. Dans cette optique, nous avons mis en évidence qu'il semble y avoir une corrélation entre le niveau de fusion et le pourcentage de phosphatidylethanolamine dans la membrane bactérienne. Le phophatidylethanolamine est un phospholipide très étudié dans les liposomes articifiels pour son caractère fusogénique (Siegel and Epand, 1997)

Outre les caractéristiques des liposomes et de la membrane bactérienne, d'autres facteurs environnementaux peuvent affecter une fusion membranaire. Il a notamment été démontré que le calcium peut induire une fusion membranaire dans le cas des vésicules liposomales modèles (Ortiz et al., 1999). Nous avons démontré dans notre étude que le potentiel de fusion des liposomes fluides avec les membranes bactériennes était directement proportionnel à la concentration en calcium du milieu. Un des effets du calcium consiste à neutraliser les charges négatives à la surface des membranes ce qui entraîne une déshydratation de celles-ci. Cette déshydratation contribue à réduire les forces répulsives d'hydratation.
Lors de fusion membranaire, il a été démontré que les phospholipides doivent passer par un état intermédiaire appellé « phase hexagonale II » (Siegel and Epand, 1997), or le calcium est capable d'induire cet état chez certain phospholipides notamment le phosphatidylethanolamine. Ceci suggérerait que la fusion entre les liposomes et les bactéries gram négatives est dépendante de la présence de phospholipides dans le feuillet externe de la membrane externe ce qui

va à l'encontre des études démontrant l'absence de phospholipides dans ce feuillet (Lugtenberg and Van Alphen, 1983). D'un autre côté, il a été mis en évidence que le calcium en se liant aux LPS entraîne une perte des protéines de la membrane externe, provoquant une redistribution des phospholipides au niveau de la membrane externe (Stan-Lotter and Sanderson, 1981)

L'implication de différents cations divalents a également été analysé. Il a été démontré que le niveau de fusion entre les liposomes et les bactéries était amplifié dans la séquence $Fe^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$. Cette différence d'efficacité entre les cations est probablement due à leurs différentes capacités à causer une agrégation puis une fusion. Chacun de ces cations possède une affinité électronégative pour la phosphatidylcholine diminuant selon la séquence $Mg^{2+} > Ca^{2+} > Ba^{2+}$ tel que démontré par McLaughlin et collaborateurs. De plus, nous avons établi que le degré de fusion était inversement proportionnel au rayon atomique des cations (Figure 3, Chapitre 2). Plus le cations est petit, plus les liposomes seraient proches de la membrane bactérienne, et donc fusionneraient plus facilement. A notre connaissance, il s'agit de la première étude démontrant l'importance des cations dans un processus de fusion entre les liposomes et les bactéries.

Le pH de la solution est un autre paramètre qui affecte la fusion liposome-bactérie. Un pH acide ou basique induit respectivement 19% et 10% plus de fusion qu'un pH neutre (Figure 4, Chapitre 2). Une des hypothèses expliquant ce phénomène est apportée par le groupe de Coughlin et collaborateurs (1985). Ils ont démontré

que la structure lamellaire des LPS est stabilisée à des pH neutres par des interactions ioniques à l'intérieur du LPS et par des ponts de cations divalents entre les LPS. A des pH basiques, les liaisons hydrogènes seraient perdues entraînant une hydratation et une augmentation de la charge des LPS. Ceci causerait alors la formation de structures instables dans la membrane externe. A des pH acides, il y aurait une diminution de la répulsion par les charges et une déshydratation due à la perte des liaisons hydrogènes. Ceci conduirait également à la formation de complexes insolubles et donc à une membrane externe instable. Ces membranes altérées seraient donc plus susceptibles de fusionner facilement avec des liposomes. Cette étude démontre clairement que plusieurs paramètres influencent positivement le caractère fusogénique des liposomes fluides se traduisant par une meilleure pénétration intracellulaire de la drogue et une éradication des bactéries.

L'autre volet de ce travail concernait l'étude des effets synergiques du PEI grâce à son caractère perméabilisant. Notre laboratoire s'est interessé au PEI à la suite des travaux de Helander et collaborateurs (1997, 1998). Ce groupe de recherche avait démontré que le PEI d'un poids moléculaire de 50 Kda permet d'augmenter la susceptibilité de *E. coli, P. aeruginosa et S. typhimurium* à l'érythromycine, la rifampine, la clyndamycine et la fucine dans des tests de diffusion en agar.

Nous avons alors voulu savoir si le PEI pouvait être utilisé en combinaison avec des β-lactams contre *P. aeruginosa*. C'est ainsi que par des courbes de croissance

*in-vitro* et des mesures de CMI nous avons démontré que le PEI (25 Kda) augmente l'effet bactéricide de la ticarcilline, carbenicilline, ampicilline et piperacilline de 5 à 50 fois. Aux concentrations testées, le PEI ne possède aucun effet bactéricide par lui-même, son action est due uniquement a son caractère perméabilisant. L'effet synergique observé est dépendant de la concentration en PEI utilisée (Figure 5. Chapitre 5). Le mode d'action du PEI serait vraisemblablement relié aux charges négatives présentes sur les LPS. Le PEI competitionnerait avec les cations divalents situés entre les LPS. En conséquence, il y aurait formation d'agrégats de LPS grâce aux nombreuses branches polycationiques du PEI conduisant à l'apparition de nombreuses brèches au niveau de la membrane externe. Cette perturbation de l'agencement des LPS par le PEI semble être responsable de l'augmentation de la perméabilité membranaire aux β-lactams.

Afin de mettre en évidence un tel phénomène, nous avons testé une vaste gamme d'antibiotiques en combinaison avec le PEI (Figure 1. Chapitre 3). Nous avons alors constaté que le PEI est plutôt un antagoniste de toute molécule chargée positivement, dont le mode de pénétration est dépendant des charges négatives des LPS. Ceci est en accord avec l'hypothèse que le PEI se lie électrostatiquement aux LPS et entraîne la formation de complexes.

Ce travail a mis en évidence que le PEI possède un effet synergique avec les molécules chargées négativement ou neutres, que ces dernières soient

hydrophobiques ou hydrophiliques. Le PEI augmente notamment de 1,5 à 50 fois l'effet bactéricide de la novobiocine, ceftazidime, ampicilline, ticarcilline, carbenicilline, piperacilline, cefotaxime, chloramphenicol, rifampicine et norfloxacine. Dans des études basées sur la courbe de mortalité, la combinaison du PEI avec la novobiocine, ceftazidime, chloramphenicol et rifampicine diminue de 5 à 8 $\log_{10}$ le nombre de CFU/ml après 9 heures lorsque seulement 25% de la CMI de chaque antibiotique est utilisée (Figure 2. Chapitre 3).

Les infections à *P. aeruginosa* sont difficiles à traiter dû à leur haut niveau de résistance intrinsèque aux antibiotiques. La principale cause d'une telle résistance a été attribuée à une imperméabilité membranaire anormalement élevée. Nous avons dans ce présent travail étudié deux nouvelles approches dans l'optique de vaincre cette imperméabilité. Nous avons démontré que les liposomes fluides fusionnent avec les membranes des bactéries déversant leur contenu tel un cheval de Troie. Nous avons également étudié les différents paramètres physico-chimiques impliqués dans ce processus de fusion. Des études futures seront nécessaires afin d'évaluer l'importance de ces paramètres sur l'efficacité *in-vivo et in-vitro* des liposomes fluides. Dans cette optique, il sera important d'évaluer l'impact, des paramètres physico-chimiques mis en évidence, sur les liposomes contenant de la tobramycine. Toutes ces études ayant été effectué sur des liposomes vides, il serait intéressant de savoir si le contenu des liposomes influence également le degrés de fusion. De plus, lorsque de la tobramycine est encapsulé dans des liposomes, il reste toujours des molécules en surface, malgré

les lavages. Quel est l'importance des charges positives de la tobramycine sur le processus de fusion ? Ces molécules cationiques pourraient-elles avoir le même effet que des cations divalents ? Afin d'évaluer l'impact des antibiotiques à la surface des liposomes, des études de fusion en présence ou non de tobramycine serait envisageable.

Toutes nos études démontrent clairement que la fluidité des liposomes est un des paramètres les plus important lors de la fusion avec les membranes bactériennes. Des études ultérieures seront nécessaires afin d'évaluer l'impact de la tobramycine sur la fluidité membranaire des liposomes. Finalement, la combinaison de toutes les conditions idéales de fusion (fluidité, pH, cations, température) permettra peut-être d'augmenter la capacité bactéricide des liposomes fluides.

D'un autre côté, nous avons mis en évidence que l'effet bactéricide de nombreux antibiotiques pouvait être augmenté lorsque combiné au PEI. Cet agent polycationique, non bactéricide, et non immunogénique possède donc la capacité de vaincre la barrière membranaire si importante dans la résistance de *P. aeruginosa*. D'autres études seront nécessaires afin d'évaluer la possibilité d'utiliser le PEI dans des infections *in-vivo*. Une des première étude envisageable serait l'administration de PEI en combinaison ou non avec un antibiotique dans les voies pulmonaires de rats infectés par *Pseudomonas aeruginosa*. De nombreux obstacles sont envisageables, notamment le degrés de toxicité du PEI. Cette molécules étant disponible sous une multitude de poids moléculaires ( 0,8 à 200

kDa) et de formes ( linéaires ou enbranchés), un compromis entre l'efficacité synergétique et la toxicité pourrait surement être trouver.

Finalement, l'efficacité du PEI pour vaincre la perméabilité membranaire pourrait servir à délivrer des molécules chargés négativement dans les bactéries. Cette aspect n'a pas été couvert au court de ce travail, mais de nombreuses possibilités existent. Une des voies qui semble la plus prometteuse concerne l'utilisation du PEI comme agent de transformation bactérienne par des plasmides. En effet de nombreuses études on démontré la transfection de cellules de mammifères par des plasmides envellopés de PEI. Une telle approche au niveau bactérien n'a jamais été rapporté. Tout laisse croire que des plasmides recouvert de PEI, et qui forment des particules d'environ 20 nanomètres chargés positivement, pourraient facilement traverser la barrière de LPS des bactéries gram-négatives. La transformation de bactéries Gram négatives par des plasmides recouvert de PEI permettrait peut-être d'obtenir de meilleur résultats que les techniques actuelles. Ces dernières sont extrêmement limités par la barrière naturelle de LPS des bactéries Gram négatives.

L'utilisation de nouvelles techniques comme les liposomes fluides et le polyethylenimine permettra de lutter plus efficacement contre certaines infections bactériennes. En effet, ces méthodes permettent de surmonter un problème de taille qui est de pouvoir passer à travers la barrière naturelle des bactéries . En augmentant de façon significative la pénétration des antibiotiques à l'intérieur des

pathogènes visés, il sera possible d'optimiser leur utilisation. Ceci permettra sûrement de réduire la dose d'antibiotique administrée, ce qui implique une diminution de la toxicité pour le patient et par le fait même un réduction du coût de la thérapie. Dans la lutte constante de la médecine face à des pathogènes de plus en plus résistants, ces nouvelles approches constituent certainement un grand avantage pour des applications futures.

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