

# Enhanced bactericidal potency of nanoliposomes by modification of the fusion activity between liposomes and bacterium

Yufan Ma<sup>1</sup>  
Zhao Wang<sup>1,2</sup>  
Wen Zhao<sup>1</sup>  
Tingli Lu<sup>1</sup>  
Rutao Wang<sup>1,2</sup>  
Qibing Mei<sup>1</sup>  
Tao Chen<sup>1-3</sup>

<sup>1</sup>Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, People's Republic of China; <sup>2</sup>Shaanxi Liposome Research Center, Xi'an, Shaanxi, People's Republic of China; <sup>3</sup>Xi'an Libang Pharmaceuticals Co, Ltd, Xi'an, People's Republic of China

**Background:** *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. The acute and growing problem of antibiotic resistance of *Pseudomonas* to conventional antibiotics made it imperative to develop new liposome formulations to overcome these mechanisms, and investigate the fusion between liposome and bacterium.

**Methods:** The rigidity, stability and charge properties of phospholipid vesicles were modified by varying the cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and negatively charged lipids 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol sodium salt (DMPG), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine sodium salt (DMPS), 1,2-dimyristoyl-sn-glycero-3-phosphate monosodium salt (DMPA), nature phosphatidylserine sodium salt from brain and nature phosphatidylinositol sodium salt from soybean concentrations in liposomes. Liposomal fusion with intact bacteria was monitored using a lipid-mixing assay.

**Results:** It was discovered that the fluid liposomes-bacterium fusion is not dependent on liposomal size and lamellarity. A similar degree of fusion was observed for liposomes with a particle size from 100 to 800 nm. The fluidity of liposomes is an essential pre-request for liposomes fusion with bacteria. Fusion was almost completely inhibited by incorporation of cholesterol into fluid liposomes. The increase in the amount of negative charges in fluid liposomes reduces fluid liposomes-bacteria fusion when tested without calcium cations due to electric repulsion, but addition of calcium cations brings the fusion level of fluid liposomes to similar or higher levels. Among the negative phospholipids examined, DMPA gave the highest degree of fusion, DMPS and DMPG had intermediate fusion levels, and PI resulted in the lowest degree of fusion. Furthermore, the fluid liposomal encapsulated tobramycin was prepared, and the bactericidal effect occurred more quickly when bacteria were cultured with liposomal encapsulated tobramycin.

**Conclusion:** The bactericidal potency of fluid liposomes is dramatically enhanced with respect to fusion ability when the fusogenic lipid, DOPE, is included. Regardless of changes in liposome composition, fluid liposomes-bacterium fusion is universally enhanced by calcium ions. The information obtained in this study will increase our understanding of fluid liposomal action mechanisms, and help in optimizing the new generation of fluid liposomal formulations for the treatment of pulmonary bacterial infections.

**Keywords:** liposomes, fusion, bacteria, *Pseudomonas aeruginosa*, lipid composition

Correspondence: Tao Chen  
School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, People's Republic of China  
Tel +86 29 8833 2932  
Fax +86 29 8831 4307  
Email taochen@libang.com.cn

## Introduction

The potential of fluid liposomes to effectively deliver antibiotics into bacteria and dramatically increase their killing has been previously explored for a broad range of

bacterial species including antibiotic resistant strains.<sup>1-6</sup> It was found that such enhanced bactericidal activity of fluid liposomal formulations is mainly due to their ability to fuse with bacterial cells and the fluid liposomes-bacterium fusion process has been confirmed by several different techniques.<sup>7-9</sup>

It has recently been shown that several factors, such as bacterial membrane property, divalent cations, bacterial surface pH, and temperature can play important roles in fusion of fluid liposomes with bacteria.<sup>10</sup> There is a clear relationship between the degree of fluid in the liposomes-bacterium fusion and the nature of the bacterial membrane, especially bacterial membrane lipid composition. For example, the high phosphatidylethanolamine content in Gram-negative bacteria seems to enhance fusion with fluid liposomes. However, the effect of the individual properties of the liposomes themselves on the degree of fluid liposomes-bacterium fusion is less clear. In addition, the properties of liposomes also have a great impact on their antibacterial performance. Therefore, understanding the importance of different liposomal parameters on the extent of fusion with bacteria is imperative in order to have an optimized vesicle that will efficiently fuse with the intact bacteria, deliver its contents into the cytoplasm, and kill the bacteria.

In this work, we systematically changed the liposome composition to evaluate the effects of different modifications on the degree of fusion with intact bacterial cells. Among all the parameters that could be manipulated we focused on the effects of liposomal size, lipid composition, and surface charge. Furthermore, the fluid liposomal encapsulated tobramycin was later prepared and the in vitro bactericidal efficacy of it on *Pseudomonas aeruginosa* was also investigated.

## Material and methods

Natural and synthetic phospholipids are commercially available, the following of which were used in this study: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol sodium salt (DMPG), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine sodium salt (DMPS), 1,2-dimyristoyl-sn-glycero-3-phosphate monosodium salt (DMPA), 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), natural phosphatidylserine sodium salt from brain (L-PS), and

natural phosphatidylinositol sodium salt from soybean (L-PI) were all obtained from Northern Lipids (Vancouver, BC, Canada). Tobramycin, T-Octylphenoxypolyethoxyethanol (Triton X-100), cholesterol, Sepharose CL-6B were obtained from Sigma Chemicals (St Louis, MO, USA). Finally, we also used Mueller Hinton broth (MH; Difco Laboratories, Detroit, MI, USA), HEPES buffered saline and brain heart infusion broth (Difco Laboratories).

## Bacterial culture

A bacterial reference strain, *P. aeruginosa* (ATCC 25619) was obtained from American Type Culture Collection (Manassas, VA, USA), and used in this study. The microorganisms were stored at  $-70^{\circ}\text{C}$  in brain heart infusion broth supplemented with 10% glycerol. The growth medium in all liquid cultures was MH broth. For the experiments, the bacteria were cultured in MH broth overnight and the next day 30  $\mu\text{L}$  of this culture was used to inoculate 20 mL of fresh MH broth during agitation at  $37^{\circ}\text{C}$ . Experiments were always carried out when the culture reached a mid-logarithmic phase as measured by an optical density (OD) at 660 nm of 0.6.

## Liposomes preparation

Liposomes were prepared using hydration-extrusion as described by Hope et al.<sup>11</sup> Appropriate amounts of lipid mixtures were dissolved with a solution of methanol/chloroform (1:2). The fluorescence-labeled lipid marker solutions (NBD-PE and Rh-PE, at a 0.5% molar ratio [mol] each) were added to the lipid mixture tubes and the solvents were evaporated under nitrogen stream in a warm water bath until a homogeneous lipid film was produced. The resultant film was dried under vacuum overnight. Each specific lipid composition was hydrated with HBS. The hydration is performed by vigorous mixing, followed by five times freeze-and-thaw (frozen with dry-ice/acetone and thawed in  $65^{\circ}\text{C}$  water). The samples are then extruded through two stacked polycarbonate filters (Nucleopore Costar, Cambridge, MA, USA) ten times at  $60^{\circ}\text{C}$  with pore sizes of 1, 0.6, and 0.4  $\mu\text{m}$  to obtain a specific size.

## Determination of liposome size, zeta-potential, and polydispersity index determination

Liposome size, zeta-potential, and polydispersity index determination was determined using Zetasizer Nano ZS system (Malvern Instruments Ltd, Worcestershire, UK). The emulsion samples were diluted 1:10 with purified water before measurement.

## Fusion assay

Liposomal fusion with intact bacteria was monitored using a lipid-mixing assay based on the extent of resonance energy transfer between the lipid headgroup-labeled probes, Rh-PE and NBD-PE, as described by Struck et al.<sup>12-14</sup> All fluorescence measurements were carried out with a F-7000 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan).

The Rh-fluorescence decrease due to fusion was measured and used for calculation of degree of fusion. Briefly, the NBD/Rh-labeled liposomes were prepared as described above containing both NBD-PE and Rh-PE at 0.5% mol each. The NBD/Rh labeled vesicles (10 µg/mL final concentration) were quickly mixed with 1.9 mL of bacteria (OD<sub>660</sub> = 0.6) in a cuvette under controlled temperature at 37°C under continuous stirring. The final incubation volume in all measurements was 2 mL. Continuous monitoring of rhodamine fluorescence (590 nm) was done at 1-minute intervals under steady-state excitation at 475 nm (NBD maximal excitation). The final fluorescence intensity (F<sub>max</sub>) which represents maximal fluorescent lipid probe dilution in each sample was determined following the solubilization of vesicles with Triton X100 detergent (0.2% volume). The percentage of fusion (or lipid dilution) was calculated using equation 1:

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

where F<sub>t</sub> is the fluorescence intensity at each time point and F<sub>o</sub> is the initial fluorescence intensity. Each experiment was repeated three times.

## Preparation of fluid liposomal encapsulated tobramycin

The fluid liposomal encapsulated tobramycin was prepared using a dehydration-rehydration vesicle method as previously described.<sup>12-14</sup> The compositions of the liposome encapsulated tobramycin were DPPC, DMPG, and/or DOPE the molar ratios of which are shown in Table 1. The content of NBD-PE and Rh-PE were each constantly 0.5% molar ratio. The capsules had an overall low gel-liquid crystalline transition temperature (*phase transition temperature (TC)* < 37°C). The quantification of tobramycin in the liposome was performed by high-performance liquid chromatography analysis as previously described.<sup>12-14</sup>

## Antibacterial experiment in vitro

In vitro antimicrobial effect of liposome encapsulated tobramycin were performed in the presence of subinhibitory concentrations (sub-MIC) concentrations of each liposomes in Table 1. *P. aeruginosa* were inoculated in 250 mL of MH broth for 17 hours to obtain a log-phase cell density of approximately 10<sup>5</sup> colony forming units/mL by OD at 660 nm. Aliquots of 18 mL of the cultures described above were transferred to 50 mL conical tubes. Bacteria were incubated with 200 µL of each liposomes to obtain a final concentration of 4 × 10<sup>-6</sup> M. Phosphate-buffered saline was then added to complete the volume to 20 mL. *P. aeruginosa* received 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.031 mg/L of liposome encapsulated tobramycin as determined by high-performance liquid chromatography analysis. These values were lower than the MIC for each

**Table 1** Fluidity, MIC and degree of fusion to liposome encapsulated tobramycin

Strain	Composition (%)*				Fusion of fluid liposomes (%)	MIC to liposome encapsulated tobramycin (µg/mL)	
	Ca <sup>2+</sup> (mM)	DSPC	DPPC	DOPE			DMPG
		Transformation temperature (°C)					
		55	41	-16	23		
<i>Pseudomonas aeruginosa</i> ATCC 25619	0	89	0	0	10	10 ± 3	64
	0	0	79	10	10	20 ± 4	32
	0	0	59	30	10	30 ± 2	16
	3	0	79	10	10	56 ± 5	0.20
	3	0	59	30	10	72 ± 3	0.10
	5	0	94	0	5	55 ± 3	0.20
	5	0	89	0	10	64 ± 6	0.125
	5	0	79	0	20	66 ± 4	0.125
	5	0	69	0	30	73 ± 4	0.10

**Notes:** Data are shown as mean ± standard deviation (n = 3). \*The composition of liposomes was without cholesterol.

**Abbreviations:** DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol sodium salt; MIC, minimal inhibitory concentration.

bacterial strain. The cultures were then incubated on a shaker at 37°C for 16 hours. Samples of 2.0 mL were collected after 6 hours of incubation. One milliliter was read at 660 nm and 0.5 mL was used immediately for serial dilutions in cold sterile phosphate-buffered saline. Appropriate dilutions were plated and cultured in triplicate on MH broth. After overnight incubation at 37°C in 5% CO<sub>2</sub>, the number of colony forming units was calculated.

## Data analysis

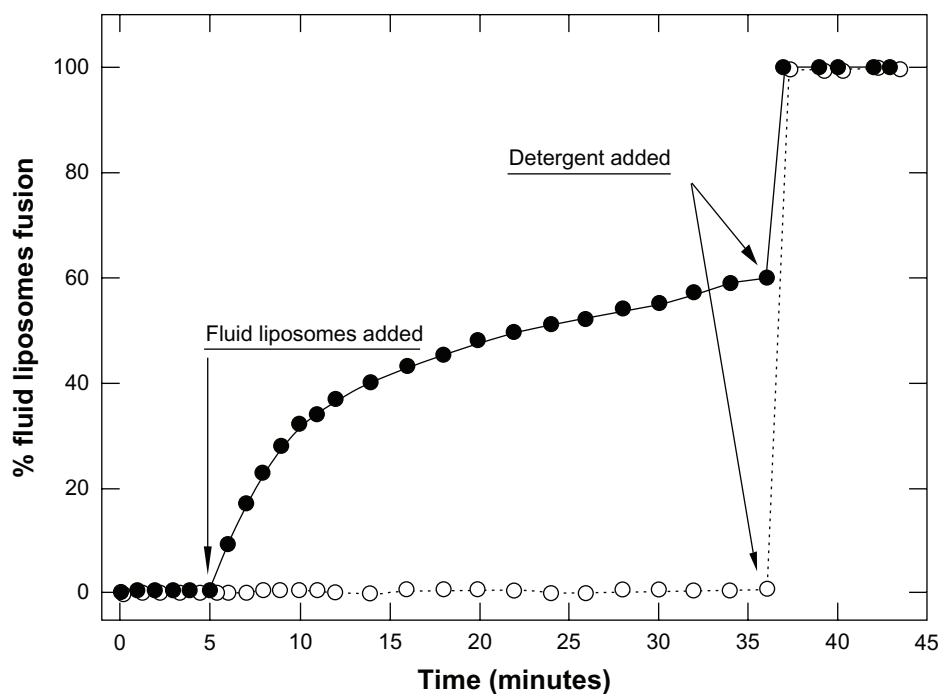
Bacterial counts were expressed as six standard errors of the means obtained from at least three plates per dilution. All computer analysis was performed with a linear regression system using statistical package for the social sciences 11.5 for windows (SPSS; IBM Corporation, Armonk, NY, USA).

## Results

### Monitoring the fusion between liposomes and bacteria

As described previously, the fusion between fluid liposomes and bacteria was quantitatively measured using a lipid-mixing assay with Rh-PE and NBD-PE as fluorescent probes.<sup>7-10</sup> Upon incubation of a mixture of labeled

liposomes and bacteria at 37°C, fluorescence intensity of Rh (590 nm) decreased and the NBD signal (520 nm) increased as time increased.<sup>4</sup> This indicates that a significant fusion occurred when fluid liposomes were mixed and incubated with bacteria at 37°C. We chose to monitor fluid liposomes-bacterium fusion with Rh emission peak at 590 nm because *P. aeruginosa* have a relatively strong background fluorescence that interferes with NBD measurement, and Rh has a higher sensitivity in this study. Figure 1 represents the typical profile of fluid liposomes-bacterium fusion as calculated by equation 1 with Rh-fluorescence intensity (with constant excitation at 475 nm). In control experiments with a mixture of NBD/Rh labeled fluid liposomes and non-labeled fluid liposomes, no fluorescence signal change was detectable under the same experimental conditions as the fluid liposomes-bacterium fusion. The liposomes-bacterium fusion has been previously confirmed with other techniques, such as negative staining, immunoelectron microscopy, and fluorescence-activated cell sorting.<sup>7-9</sup> The observed fusion has recently been independently verified by successfully transferring encapsulated DNA into intact bacteria.<sup>15</sup> Delivery of encapsulated non-permeable fluorescent antisense oligonucleotides into bacteria was significantly



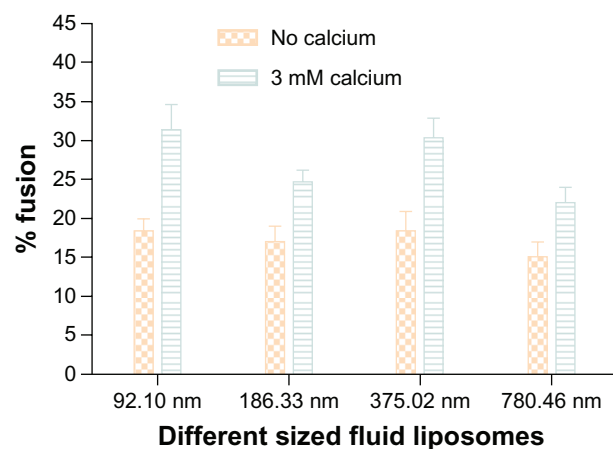
**Figure 1** A typical fusion profile between fluid liposomes and the intact Gram-negative bacteria, *Pseudomonas aeruginosa* 25619 under 37°C and 5 mM Ca<sup>2+</sup> with continuous stirring. **Notes:** Fluid liposomes with 200 nm in size were prepared by hydration-extrusion method. 45 μmol of fluid liposomes were added into bacterial solution (OD<sub>660 nm</sub> 0.6) after 5 minutes and the mixture was then continuously monitored for another 30 minutes. (••), fusion between fluid liposomes and bacteria; (○•), labeled fluid liposomes and unlabeled fluid liposomes.

**Abbreviation:** OD, optical density.

enhanced compared to free antisense.<sup>15</sup> When encapsulated, anti-LacZ antisense molecules were incubated with bacteria, bacterial LacZ expression was specifically inhibited. Such antisense delivery and inhibition of gene expression by encapsulation in fluid liposomes must be a result of liposome-bacterium fusion since oligonucleotides molecules are non-permeable and their uptake by bacteria is insufficient for inhibition.

### Effect of liposomal size on liposomal fusion with bacteria

When liposomes are made using the extrusion method, 800 nm sized liposomes are considered multilamellar vesicles, 400 nm sized liposomes exhibit an oligolamellar nature, and 200 and 100 nm preparations consist of largely single bilayer vesicles.<sup>16</sup> As the vesicle size increases, the degree of lamellarity of liposomes is also increased. It is not clear whether different sized fluid liposomes (or different lamellarity) influence fluid liposomes-bacterium fusion. Previous studies have indicated that in some cases, liposome size is a key characteristic in liposome-liposome fusion.<sup>17,18</sup> This is due to the packing constraints experienced by phospholipids in vesicles with different sizes (due to the acute radius of curvatures), which can induce instability therefore resulting in different fusion potential. In order to gain firsthand data, we measured fusion of bacteria with fluid liposomes ranging from 100 to 800 nm in size. The size of the liposomes used in this test was 92.10, 186.33, 375.02, and 780.46 nm, respectively. The polydispersity index of each liposome was 0.12, 0.11, 0.23, and 0.34, respectively. The zeta potential of each liposome was  $-15.23$ ,  $-17.10$ ,  $-13.56$ , and  $-12.14$  mv, respectively. In a typical experiment, the NBD/Rh-labeled liposomes were prepared as described previously. The NBD/Rh labeled vesicles were mixed with bacteria (OD 660 nm = 0.6) giving a final volume of 2 mL (10 g/mL final vesicle concentration) and incubated at 37°C under continuous low speed stirring. The fluorescence scans of solutions were measured at a wavelength range of 500 to 610 nm under steady-state excitation at 475 nm (maximal excitation peak for NBD). The data shown in Figure 2 demonstrate that the degree of fusion between fluid liposomes composed of DPPC/DMPG (90:10, mol/mol) and bacteria is not dependent on either vesicular size or lamellarity. Similar degrees of fusion were observed for liposomes with size ranging from 100 to 800 nm. Addition of calcium ions increased the degree of fusion of the whole size range of vesicles tested to similar values.

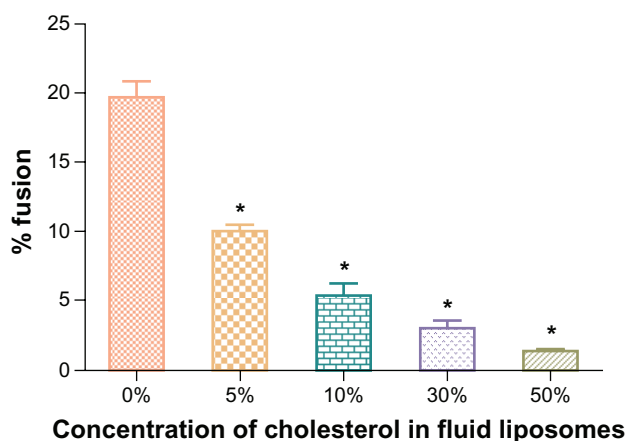


**Figure 2** Effect of liposome size on the degree of fusion between fluid liposomes and bacteria at 37°C for 30 minutes.

**Notes:** Data are shown as mean  $\pm$  standard deviation ( $n = 3$ ). The vesicles were extruded through specific sized filters ten times. *Pseudomonas aeruginosa* was used in this study.

### Effect of liposomal rigidity on liposomal fusion with bacteria

Previous studies have shown that fluidity of liposomes is very important for fluid liposomes-bacteria fusion. In order to fully characterize the rigidity response, fusion of liposomes with bacteria after 30 minutes of incubation at 37°C was measured for vesicles containing different amounts of cholesterol as shown in Figure 3. There is an extremely sharp transition in fusion properties of liposomes made with and without cholesterol as a liposomal component. A significant reduction in the fusion potential of liposomes was observed when only 5% mol of cholesterol was added into multicomponent mixture of liposomes. Fusion was reduced as much as 70% when



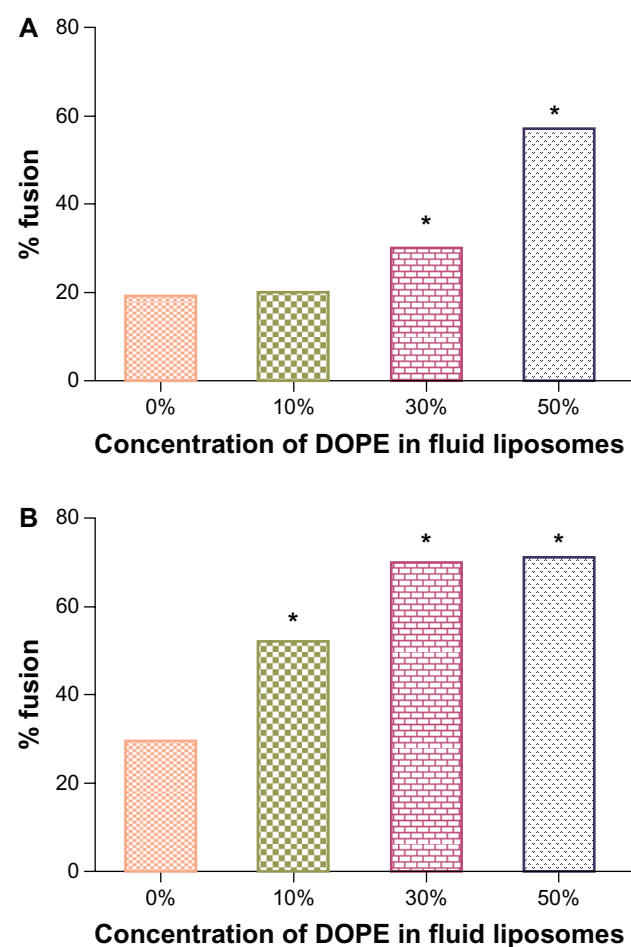
**Figure 3** Effect of liposome rigidity on the degree of fluid liposomes-bacterium at 37°C for 30 minutes.

**Notes:** Data are shown as mean  $\pm$  standard deviation ( $n = 3$ ). \* $P < 0.05$ . The vesicles were extruded through specific sized filters ten times. *Pseudomonas aeruginosa* 25619 was used in this study.

only 10% of cholesterol was added. Further increase in the cholesterol content did not result in substantial fusion decrease. Results from liposomal fluidity measurement obtained by fluorescent anisotropy technique indicated that fluid liposomes have a relative fluidity of 20 compared to 10 for rigid liposomes with 5% cholesterol. It is clear that fluidity is one of the most important factors in liposome-bacterium fusion.

## Fusion potential of DOPE-containing liposomes

It has been well documented that fusogenic liposomes can be constructed with DOPE and a number of pH-sensitive liposomal drug carriers have been intensively investigated.<sup>19–26</sup>



**Figure 4** Effect of DOPE in liposomes on the degree of fluid liposomes-bacterium fusion at 37°C for 30 minutes.

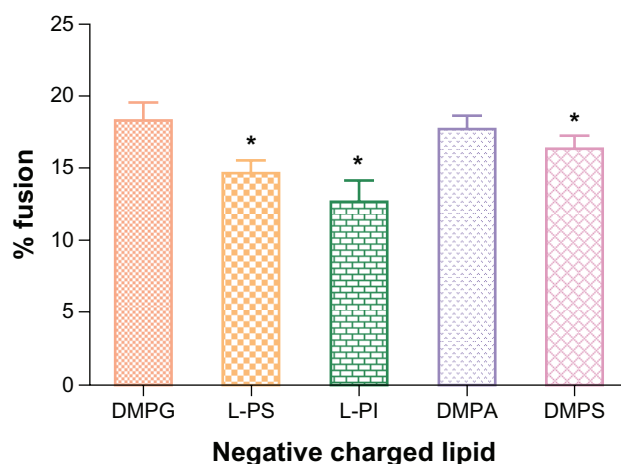
**Notes:** (A) Without the addition of calcium. (B) With the addition of calcium. Data are shown as means  $\pm$  standard deviation ( $n = 3$ ).  $*P < 0.05$ . Fluid liposomes with lipid composition of DPPC/DOPE/DMPG/NBD-PE/Rh-PE (the percentage of DMPG at 10% molar ratio, NBD-PE and Rh-PE each at 0.5% were kept constant) were prepared using the hydration-extrusion method. The vesicles were extruded through specific sized filters ten times. *Pseudomonas aeruginosa* 25619 was used in this study.

**Abbreviations:** DMPG, 1,2-dimyristoyl-sn-Glycero-3-phosphatidylcholine sodium salt; DOPE, 1,2-dioleoyl-sn-Glycero-3-phosphatidylethanolamine; DPPC, 1,2-dipalmitoyl-sn-Glycero-3-phosphatidylcholine; NBD-PE, 1,2-dioleoyl-sn-Glycero-3-phosphatidylethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); Rh-PE, 1,2-dioleoyl-sn-Glycero-3-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonate).

DOPE does not form a bilayer when dispersed in aqueous solution, but it can be stabilized into liposomes with the addition of other lipids, which form a bilayer. We anticipated that fluid liposomal fusion capacity may be further enhanced by introducing the fusogenic DOPE. As indicated in Figure 4A, fluid liposomes containing various amounts of DOPE can be constructed and DOPE-containing fluid liposomes are stable at 37°C without bacteria. This means that the DOPE-containing fluid liposomes do not fuse with each other. When incubated with *P. aeruginosa*, they fuse with the bacterial membrane at a faster and higher degree compared to the original fluid liposomes (DPPC/DMPG). The effect of DOPE on fluid liposomal fusion was dramatic and the potential of fluid liposomal fusion was increased 20% to 50% as the percentage of DOPE increased. The negatively charged DMPG was used as a stabilizer for constructing stable fluid liposomes. The addition of calcium cations bind to the phosphorous group and reduce the stabilizer ability of DMPG and, therefore, the extent of fusion reached was as high as 72% (Figure 4B).

## Effect of different negative phospholipids on the liposome-bacterium fusion

We also formulated different fluid liposomes by adding the negative charged lipids (DMPG, DMPS, DMPA, L-PS, and L-PI) to liposomes. The sizes of the liposomes containing DMPG, DMPA, DMPS, L-PS, or L-PI were 154.20,



**Figure 5** Effect of different negatively charged phospholipids on the degree of fluid liposomes-bacterium fusion at 37°C for 30 minutes without the addition of calcium.

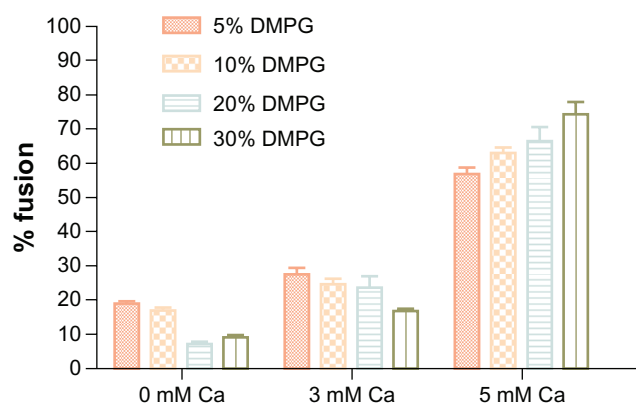
**Notes:** Data are shown as means  $\pm$  standard deviation ( $n = 3$ ).  $*P < 0.05$ . The vesicles were extruded through specific sized filters ten times. *Pseudomonas aeruginosa* 25619 was used in this study.

**Abbreviations:** DMPA, 1,2-dimyristoyl-sn-Glycero-3-phosphatidylcholine monosodium salt; DMPG, 1,2-dimyristoyl-sn-Glycero-3-phosphatidylcholine sodium salt; DMPS, 1,2-dimyristoyl-sn-Glycero-3-phosphatidylcholine-L-serine sodium salt; L-PI, natural phosphatidylinositol sodium salt from soybean; L-PS, natural phosphatidylinositol sodium salt from brain.

128.31, 133.62, 136.23, and 126.75 nm, respectively. The polydispersity index of each liposome was 0.17, 0.15, 0.23, 0.19, and 0.14, respectively. The zeta potential of each liposome was  $-13.46$ ,  $-15.22$ ,  $-14.16$ ,  $-15.62$ , and  $-15.23$  mv, respectively. The fusion potential of fluid liposomes-bacteria with liposomes containing different negative charged lipids was compared and is illustrated in Figure 5. There was a statistically significant difference between DMPG, L-PS, L-PI, and DMPS ( $P < 0.01$ ). There was no significant difference between DMPG and DMPA ( $P > 0.05$ ). Therefore, among the negative phospholipids examined, PG gave the highest fusion, PA and PS had intermediate fusion levels, and PI resulted in the lowest fusion.

### Effect of DMPG content on liposome-bacterium fusion

The drug encapsulation of tobramycin has been facilitated and the gel-liquid transition temperature of fluid liposomes has been regulated when including the negatively charged DMPG.<sup>27,28</sup> At the same time, the amount of negatively charged head groups on the surface of fluid liposomes would certainly affect the degree of fluid liposomes-bacterium fusion. Figure 6 shows the observed degree of fusion versus amount of DMPG in the fluid liposomes under different concentrations of additional calcium cations. The zeta-potential of liposomes before and after treated with calcium cations were  $-23$  and  $-6$  mv, respectively. As anticipated, higher negative charges increased the electrostatic repulsion between negatively charged liposomes and negatively charged bacteria. Such repulsion force is so strong that it



**Figure 6** Effect of DMPG content in fluid liposomes on the degree of fluid liposomes-bacterium fusion at 37°C for 30 minutes.

**Notes:** Data are shown as mean  $\pm$  standard deviation ( $n = 3$ ). Vesicles were extruded through specific size filters ten times. *Pseudomonas aeruginosa* 25619 was used in this study.

**Abbreviation:** DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol sodium salt.

overlays the benefit of the lowered liposomal transition temperature resulting from the increased amount of DMPG when the fusion was measured without additional calcium cations.

A previous study from our laboratory has shown that  $\text{Ca}^{2+}$  enhances fluid liposomes-bacterium fusion.<sup>7</sup> It was thus interesting to measure the effect of  $\text{Ca}^{2+}$  on the fusion properties of fluid liposomes with different amounts of DMPG. As shown in Figure 6, the degree of fusion for all four fluid liposomes with different amounts of DMPG increased with 3 mM calcium cations. In contrast to the behavior of fusion without calcium cations, much higher fusion enhancement is observed for fluid liposomes with higher content of DMPG when sufficient calcium cations, such as 5 mM, was present in the incubation solutions. A possible explanation is that the calcium cations, which carry two positive charges, reduces the electrostatic repulsion and help to bridge the liposomes and bacteria together. The degree of fusion increased and was proportional to the amount of calcium cations. A similar high level of fusion of fluid liposomes-bacterium has been achieved with the addition of calcium cations to all four fluid liposomes containing various percentages of DMPG.

### In vitro bactericidal efficacy of liposome encapsulated tobramycin

The MICs of tobramycin for *P. aeruginosa* are shown in Table 1. Sterilizing effects of bacteria exposed to sub-MIC of encapsulated tobramycin were evaluated by counting colony forming units on agar plates. As indicated in Table 1, the fusion potential of DOPE-containing liposomes increased to 20% under experimental conditions as the amount of DOPE content increased to 30%. But, with 3 mM calcium cations, the fusion potential of DOPE-containing liposomes increased to 72%, and the MIC of liposome encapsulated tobramycin decreased to 0.031  $\mu\text{g}/\text{mL}$ . This means that calcium ions improved the fusion of the liposomes with bacteria.

Without DOPE, the degree of fusion for all four fluid liposomes increased when the amount of DMPG increased. Also, much higher fusion enhancement was observed for fluid liposomes with a higher content of DMPG when 5 mM calcium cations were present in the incubation solutions. Therefore, the MIC of liposome encapsulated tobramycin decreased and this was proportional to the degree of fusion. Therefore, the fluidity or rigidity of liposomes is the most important factor controlling fluid liposomes-bacterium fusion and the antibacterial effect of liposome encapsulated tobramycin.

## Discussion

In this study, we showed that fluidity or rigidity of liposomes is the most important factor controlling fluid liposomes-bacterium fusion. A significant reduction in the fusion potential of liposomes was observed when only 5% mol of cholesterol was added to multicomponent mixtures of liposomes (data not shown), while the fusion of liposome-bacteria was almost completely inhibited by 10% mol or more of cholesterol. It is known that cholesterol-containing vesicles reduce vesicular content leaking and fusion in artificial liposomal systems.<sup>29–32</sup> The results reported here are the first systematic investigation of the effects of cholesterol on liposome-bacterium fusion. Cholesterol is the predominant sterol in the mammal cell membranes. Its 3-OH, 5-double bond, central planar ring, and branched aliphatic chain at C-17 provide the perfect alignment with the phospholipid acyl chains and results in tidy membrane packing. Cholesterol increases both the rigidity and the degree of order of the lipid layer in the fluid phase of lipids.<sup>33–35</sup> A reduced liposome-bacterium fusion with cholesterol-containing liposomes was anticipated, but such a dramatic reduction resulting from only 10% mol cholesterol was still somewhat surprising since in general a stable liposome requires much higher cholesterol content (from 30% to 45% mol).

The addition of DOPE is another prominent determinant of liposome-bacterium fusion observed in this study. DOPE is a non-bilayer forming lipid that can efficiently modulate the fusion potential of liposomes. The fusion potential of DOPE-containing liposomes increases from the original 20% to 56% under experimental conditions as the amount of DOPE content increases up to 50% mol (Figure 4A). When combining DOPE (at concentration of 10% to 50% mol) with DPPC and DMPG stable bilayer structured liposomes were formed. However, upon mixing without calcium cations bridges, the fusion between bacteria with membranes was reduced. This reduces the ability of the stabilizing lipids, and thus DOPE-containing liposomes go from a bilayer to an  $H_{II}$  structure triggering fusion. In addition, a further fusion increase was observed in the presence of extra calcium cations (Figure 4B). In our previous study, the influence of divalent cations, such as calcium cations, on the fluid liposomes-bacterium fusion was demonstrated. Calcium cations-enhanced fusion between bacteria and DOPE-containing liposomes would be expected through bridging of negatively charged liposomes and bacteria, lateral phase separation, and bilayer to hexagonal phase transition as reported by Cullis and his coworkers.<sup>36–40</sup>

Two parameters regarding the effect of acidic phospholipids on fluid liposomes-bacterium fusion were investigated in the present report: the type of negatively charged lipids and amount of negatively charged lipids in liposomes. Among different negative phospholipids examined, DMPA gave the highest level of fusion, DMPS and DMPG had intermediate fusion levels, and DMPI resulted in the lowest fusion (Figure 5). As illustrated in Figure 6, without the addition of extra calcium cations, the degree of fluid liposomes-bacterium fusion decreases as the net DMPG increases. The first step for any fusion process is contact between targeting and receptor membranes. The increased amount of negatively charged DMPG results in an enhanced repulsion force between fluid liposomes and bacteria. Subsequently, the fusion potential was reduced. Calcium cations will neutralize the net negative charges on both bacteria and fluid liposomes, reduce the electric repulsion, and at the same time bridge together fluid liposomes and bacteria. These result in the increase in fluid liposomes-bacterium fusion and the final levels of fusion for formulations with different amounts of DMPG reaching almost the same value.

In addition, the results obtained following the use of sub-MIC quantities of encapsulated tobramycin against *P. aeruginosa* are striking since this group is recognized for its very low nonspecific permeability and/or the presence of an efflux pump in its external membrane. The fusion potential of DOPE-containing liposomes increased 10% under experimental conditions as the amount of DOPE content increases up to 30% mol. But, with 3 mM calcium cations, the fusion potential of DOPE-containing liposomes increased to 72%, and the MIC of liposome encapsulated tobramycin decreased to 0.031  $\mu\text{g}/\text{mL}$ . The MIC of liposome encapsulated tobramycin decreased to 0.031  $\mu\text{g}/\text{mL}$  when liposomes incubated with higher content of DMPG and 5 mM calcium cations.

## Conclusion

In this study, we discovered that the fluidity of liposomes is an essential pre-request for liposome fusion with bacteria. An increase in the amount of negative charges at the surface of fluid liposomes reduces fluid liposomes-bacterium fusion, but the extent of fusion is restored in presence of extra calcium cations. Fluid liposomes-bacterium fusion is not negative charge lipid specific. Substitution of DMPG with other negative phospholipids such as DMPA, DMPS, or PI resulted in a similar extent of fusion. Regardless of changes in liposome composition, fluid liposomes-bacteria fusion is universally enhanced by calcium ions. Fusion ability of



fluid liposomes is dramatically enhanced when fusogenic lipid, DOPE, is included as a lipid component. Finally, fluid liposomes-bacterium fusion is not dependent on either liposomal size or lamellarity, but liposomes with multi-lamellar might be better to delivery antibiotics into the cytoplasm by penetrating the double bacterial membranes of Gram-negative bacteria.

Moreover, the bactericidal effect occurred more quickly when bacteria were cultured with liposomal encapsulated tobramycin. This cannot be explained only as a result of prolonged residence time of liposome-encapsulated tobramycin and the resulting release of entrapped antibiotic at the bacterial site; rather, direct interaction of liposomes and bacteria, probably by a fusion process, may explain the bactericidal effect of the sub-MIC antibiotic doses used.

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## Disclosure

The authors report no conflicts of interest in this work.

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