Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action

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Abstract **The bactericidal effect of dioctadecyldimethylammonium bromide (DODAB), a liposome forming synthetic amphiphile, is further evaluated for *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in order to establish susceptibilities of different bacteria species towards DODAB at a fixed viable bacteria concentration (2.5×10^7 viable bacteria/mL). For the four species, susceptibility towards DODAB increases from *E. coli* to *S. aureus* in the order above. Typically, cell viability decreases to 5% over 1 h of interaction time at DODAB concentrations equal to 50 and 5 μM for *E. coli* and *S. aureus*, respectively. At charge neutralization of the bacterial cell, bacteria flocculation by DODAB vesicles is shown to be a diffusion-controlled process. Bacteria flocculation does not yield underestimated counts of colony forming units possibly because dilution procedures done before plating cause deflocculation. The effect of vesicle size on cell viability demonstrates that large vesicles, due to their higher affinity constant for the bacteria ($45.20\ M^{-1}$) relative to the small vesicles ($0.14\ M^{-1}$), kill *E. coli* at smaller DODAB concentrations. For *E. coli* and *S. aureus*, simultaneous determination of cell viability and electrophoretic mobility as a function of DODAB concentration yields a very good correlation between cell surface charge and cell viability. Negatively charged cells are 100% viable whereas positively charged cells do not survive. The results show a clear correlation between simple adsorption of entire vesicles generating a positive charge on the cell surfaces and cell death.**—Campanhã, M. T. N., E. M. Mamizuka, and A. M. Carmona-Ribeiro. **Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action.** ***J. Lipid Res.*** **1999.** 40: **1495–1500.**

Supplementary key words dioctadecyldimethylammonium bromide vesicles • Gram-positive and Gram-negative bacteria • electrophoretic mobility for bacteria • correlation cell charge / cell viability • bacteria aggregation

Liposomes are relatively well established as carriers for antimicrobial and anticancer agents (1, 2). They reduce toxicity of drugs in the target organ by modifying drug distribution and improve the therapeutic index observed with several antimony salts (3, 4), immunomodulators (5, 6), antifungal agents (7, 8), and antibiotics (9, 10). Liposome encapsulation results in sustained local concentrations of antimicrobial agents (11, 12). After in vivo administration via the intravenous route, conventional liposomes are taken up by the reticuloendothelial system (RES) and are potentially useful as antibiotic carriers for treatment of infections involving the RES (13). Alternatively, vesicle size and phospholipid composition may be controlled to change liposome biodistribution and circulation time (14). A general property of conventional liposomes is that by themselves they are generally innocuous.

This work further elucidates the mechanism responsible for the antimicrobial properties of some synthetic cationic liposomes (15) which, by themselves, are not innocuous (15–19). Dioctadecyldimethylammonium bromide (DODAB) or chloride (DODAC) are quaternary ammonium compounds that form stable and closed bilayers (vesicles) in aqueous solutions (15). The bactericidal, flocculant, and cytotoxic effects of these cationic vesicles on bacteria and cultured mammalian cells were partially described, a differential toxicity being ascribed to DODAB towards bacteria or mammalian cells (16–19). In this work, we determine: *1*) dose and time effects on four different bacteria species of clinical importance at a fixed bacteria concentration; *2*) effect of vesicle size on *E. coli* viability; and *3*) simultaneous effect of DODAB vesicles on bacteria electrophoretic mobility and viability. An interesting correlation between sign of the cell surface charge and cell viability is found: negatively charged cells are 100% viable whereas positively charged cells do not survive (ca. 0% viability).

MATERIALS AND METHODS

Organisms and culture conditions

Escherichia coli O111H$^-$ isolated from diarrheic human feces were obtained from the culture collection at the Universidade

Abbreviations: DODAB, dioctadecyldimethylammonium bromide; RES, reticuloendothelial system; CFU, colony forming units; SV, small unilamellar vesicles; LV, large unilamellar vesicles; EM, electrophoretic mobility; DODAC, dioctadecyldimethylammonium chloride.

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Federal de São Paulo (UNIFESP), São Paulo, S.P., Brazil. *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, or *Pseudomonas aeruginosa* ATCC 27853 were reactivated for 2–5 h at 37°C in 3 mL of Tryptic Soy Broth, TSB (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK). Thereafter, bacteria were spread on plates of MacConkey agar (Difco Laboratories, Detroit, MI) for the Gram-negative bacteria or on plates of blood agar for *S. aureus* and incubated (37°C/24 h). Two colonies of each species were taken from the plates and incubated in 3 mL of liquid TSB (150 rpm, 37°C, 2 h). Thereafter, 3 mL of each inoculum was added to 27 mL of liquid TSB for further incubation (150 rpm, 37°C, 3 h). Finally, 25 mL of each culture was pelleted and separated from its nutritive medium by centrifugation (8000 rpm/15 min). The supernatant was replaced by sterile water and the bacteria pellet was resuspended. The centrifugation/resuspension procedure was repeated twice before using the bacteria for evaluating DODAB bactericidal effects. Bacteria number densities were determined from agar plating and colony forming units (CFU) counting, turbidity against a MacFarland scale, and a correlation established between bacteria number density (obtained by CFU counting) and turbidity at 400 nm. For *E. coli*, a growth control was done which established the culture obtained as above as a suspension in the stationary phase.

Chemicals

Dioctadecyldimethylammonium bromide 99.9% pure (DODAB) was obtained from Fluka Chemie AG (Switzerland) and used as such without further purification. All other reagents were analytical grade and were used without further purification. Water was Milli-Q quality.

Vesicles preparation

Small unilamellar DODAB vesicles (SV) with 86 ± 1 nm mean diameter (20) were prepared by ultrasonic dispersion with tip in water or in an isotonic 0.264 m d-glucose solution (21). As no effects on viability were obtained for suspensions in water or in 0.264 m d-glucose, most assays for bacteria were performed in water or water solutions. SV were centrifuged at 10,000 rpm for 1 h at 15°C to precipitate multilamellar liposomes and titanium particles ejected from the titanium probe during sonication. The supernatant containing the unilamellar vesicles was used within 1 h of the preparation. Large unilamellar DODAB vesicles (LV) with 250 ± 12 nm mean diameter were prepared by vaporization of a chloroformic DODAB solution as previously described (21). DODAB concentrations were determined by microtitration (22). One should notice that DODAB vesicles contain 100% DODAB.

Viability assays

Colony forming units (CFU) counting was obtained as a function of DODAB concentration at 1 h of interaction time between bacteria and vesicles or as a function of time at 5 μm DODAB. Both time and dose effects were obtained at a fixed bacteria concentration of 2.5×10^7 viable bacteria/mL. After interaction between vesicles and cells, mixtures were diluted (1:20000) and 0.1 mL of the diluted mixtures was spread on agar plates. After spreading, plates were incubated for 24 h at 37°C. CFU counts were made using a colony counter.

Microelectrophoresis of *E. coli* and *S. aureus* in the presence of the cationic liposomes

DODAB vesicles over a range of DODAB concentrations (or interaction times) and bacteria were mixed to yield a final bacteria concentration of 2.5×10^7 bacteria/mL, allowed to interact for 1 h at room temperature, and placed in a flat cell to determine bacteria electrophoretic mobility (EM). One should notice that the measurements were done precisely at the same experimental conditions used to obtain the viability curves for *E. coli* or *S. aureus* as a function of DODAB concentration in the mixtures. Mobilities were determined using a Rank Brothers microelectrophoresis apparatus (Cambridge, England) with a flat cell at 25°C. The sample to be measured was placed into the electrophoresis cell, electrodes were connected, and a voltage of 60 V was applied across the cell. Velocities of individual bacteria over a given tracking distance were recorded, as was direction of bacteria movement. Average velocities were calculated from data on at least 20 individual bacteria. EM was calculated according to the equation EM = cm(u/V)(1/t), where u is the distance over which the particle is tracked (micrometers), cm is the interelectrodes distance (7.27 cm), V is the voltage applied (±60 V), and t is the average time in seconds required to track one particle a given distance u.

Determination of viability for flocculated bacteria

DODAB—induced bacteria flocculation is described elsewhere (18). Assuming neutralization of the cell surface charge by DODAB and an interaction time between bacteria and vesicles larger than the half-time for flocculation, bacteria flocculation should be diffusion-controlled (18, 19). From the DODAB concentration necessary to neutralize *E. coli* cells at EM = 0 (18) and flocculation kinetics followed over times larger than the half-time for flocculation, the flocculation effect on bacteria viability determined by plating was evaluated. Bacteria flocculation was determined by measuring turbidity at 400 nm as a function of time after vesicle addition using a Hitachi U-2000 spectrophotometer in the double beam mode. In the cuvette used as a reference, a bacteria suspension at the same bacteria concentration as that in the sample cuvette was added of pure water instead of adding the DODAB vesicles dispersion. Thus, turbidity measured after vesicle addition is basically due to bacteria aggregation induced by the vesicles. The time lag between mixing and recording was usually smaller than 10 s. Before dilution and plating of the flocculated samples, the occurrence of flocculation was double checked by direct microscopic visualization using an optical microscope.

RESULTS AND DISCUSSION

Susceptibilities of different bacteria species towards DODAB cationic vesicles

The effect of DODAB concentration (C) on cell viability at a fixed bacteria concentration (ca. 2.5×10^7 bacteria/mL) and interaction time (1 h) for 4 different model microorganisms is shown in **Fig. 1**. As cell concentration is the same for the 4 species, all other experimental conditions also being the same, susceptibilities of the 4 bacteria towards DODAB can be compared for 50% and 5% viability (**Table 1**). The more resistant bacteria is *E. coli*, which requires ca. 50 μm DODAB to remain 5% viable (Table 1). *E. coli* is followed by *S. typhimurium*, *P. aeruginosa*, and the Gram-positive bacterium *S. aureus* (Table 1). Table 1 also illustrates the consistency between the present data and data previously published by our group. For an interaction time of 2 h, at 8 μm DODAB, 0% viability for *S. aureus* was previously obtained (17) consistent with the 5% viability at 12 μm DODAB for a smaller interaction time, 1 h (Table 1). One should notice the very large cell number density in the interaction mixtures with

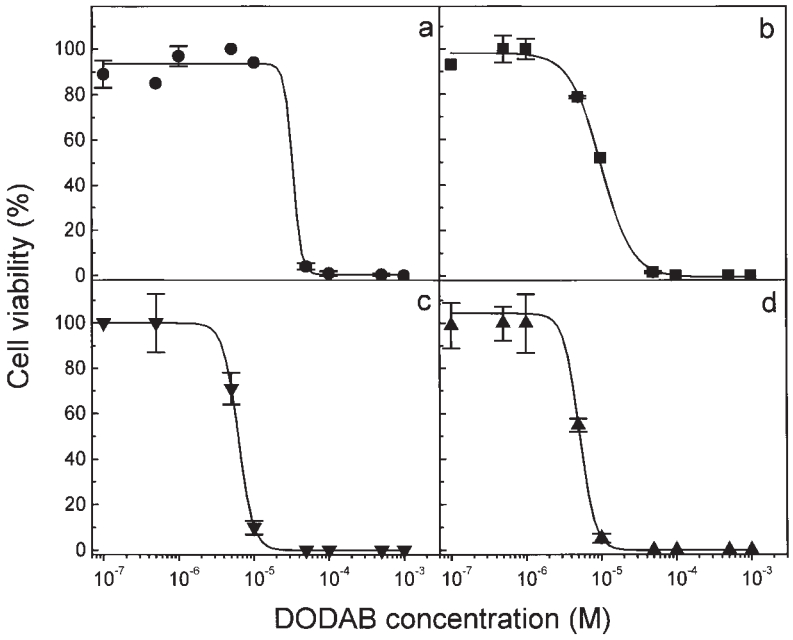

Fig. 1. Cell viability (%) as a function of DODAB concentration at 1 h of interaction time between DODAB SV and bacteria. About 100% of viability was obtained for each control sample containing bacteria only (in absence of DODAB). Before plating 0.1 mL in agar, interaction mixtures were diluted 1:20000. Viable bacteria number densities in each interacting mixture were fixed at ca. 2.5×10^7 CFU/mL for the four bacteria species. For *E. coli* 2.2×10^7 CFU/mL (a), *S. typhimurium* 2.0×10^7 CFU/mL (b), *S. aureus* 2.5×10^7 CFU/mL (c), and *P. aeruginosa* 3.0×10^7 CFU/mL (d).

DODAB (Fig. 1). Because DODAB-induced bacteria flocculation was previously described at these high number densities, plating of aggregated viable cells could yield underestimated CFU counts, as an aggregate of several viable cells would result in a single count on the plate. In order to investigate this possibility, viability for flocculated against non-flocculated bacteria samples was determined. Two different experimental conditions at charge neutralization on the cell surface were tested: one yielding extensive flocculation at 4×10^8 bacteria/mL and the other yielding basically no flocculation at all, at 4×10^7 bacteria/mL (**Table 2**). Because the expected viability of 50% was indeed obtained for both samples, viability underestimation due to DODAB-induced clustering of several viable cells yielding a single count is not a possibility. Possibly, the dilution procedure done before plating caused bacteria defflocculation.

Susceptibilities of the 4 different bacteria species towards DODAB are possibly higher than shown in Table 1. Because our cultures are in the stationary phase and DODAB interacts equally well with viable or nonviable cells in the final bacteria suspension (19), the DODAB dose required for killing a certain number of bacteria in exponential growth, a growth phase for which all cells are viable, is certainly smaller than the dose required to kill all alive cells in the same total number of dead and alive cells at the stationary phase. This reasoning is further confirmed from the comparison between viabilities for *E. coli* in exponential growth (6.4×10^7 bacteria/mL) (17) and the present results for *E. coli* in the stationary phase (2.5×10^7 CFU/mL) (Fig. 1), both suspensions in the presence of 5 μm DODAB. For the former, a viability of 0% (17) contrasts with 100% of viability obtained for the latter suspension (Fig. 1).

TABLE 1. DODAB concentration required for 5% and 50% cell viability for four different bacteria species of clinical importance

Microorganism	Cell Concentration	Interaction Time	DODAB Concentration	Cell Viability
	(CFU/ml) $\times 10^7$	*h*	*μM*	*%*
E. coli	2	1	28	50
			48	5
S. typhimurium	2	1	10	50
			41	5
P. aeruginosa	3	1	5	50
			10	5
S. aureus	3	1	6	50
			12	5
S. aureus[a]	5	2	8	0

In the bacterial/vesicles mixture, bacteria concentration was fixed at ca. 2.5×10^7 CFU/mL for 1 h of interaction time.

[a] Data taken from reference 17.

The effect of time on cell viability was obtained at a fixed DODAB (5 μm) and bacteria concentration (**Fig. 2**). One should notice that a comparison between different species cannot be performed if cell number densities are not the same or, at least, very similar. For this reason we fixed the bacteria concentration in order to establish an order for bacteria susceptibilities towards the bactericidal vesicles. The most resistant among the 4 species tested is *E. coli* (Fig. 2, Table 1).

Effect of bacteria surface charge and vesicle size on viability

In **Fig. 3**, the simultaneous effect of DODAB concentration on cell viability and electrophoretic mobility for *E. coli* and *S. aureus* suspensions at ca. 2.5×10^7 cells/mL is presented. Negatively charged cells have survival percentages close to 100% whereas positively charged cells do not survive. This is better seen by plotting electrophoretic mobility against cell viability for both bacteria species in **Fig. 4**.

TABLE 2. Cell viability for flocculated against nonflocculated bacteria

E. coli Concentration	DODAB Concentration	$t_{1/2}$	Interaction time t	Turbidity T at 400 nm		Cell Viability
				T at t_0	T at t	
CFU/mL	*M*	*min*	*min*			*(%)* $\pm \sigma$
4×10^7	1×10^{-5}	83.33	90	0.036	0.055	56 ± 6
4×10^8	6.5×10^{-5}	8.33	30	0.015	0.483	51 ± 8

Bacteria flocculation was induced by DODAB vesicles that were added at a DODAB concentration required to yield electrophoretic mobility of the bacterial suspension equal to zero (see plot for logarithm of DODAB concentration required for zero of electrophoretic mobility as a funtion of the logarithm of bacteria concentration in reference 18). Flocculation kinetics were followed turbidimetrically over interaction times t which were larger than the half-time required for flocculation ($t_{1/2}$); t_0, starting time (about 10 sec after mixing vesicles and bacteria).

DODAB concentration required for neutralization of the cell surface charge at an *E. coli* concentration of ca. 2×10^7 bacteria/mL is ca. 7 μm DODAB (18). Because positively charged cells die (Figs. 3 and 4), at charge neutralization (EM = 0), when 50% of the cells are negatively charged whereas the other half is positively charged, viability has to be close to 50%. In fact, results in **Table 3** confirm this prediction. At 5 μm DODAB, one is slightly below the DODAB amount required to cause charge neutralization, which is 7 μm, and viability is ca. 35 ± 5%, i.e., slightly below the 50% predicted (Table 3).

From adsorption isotherms for small DODAB vesicles or large DODAC vesicles onto *E. coli* (19) and assuming the Langmuir adsorption model, it is possible to calculate affinity constants for both vesicle types. Affinity of small DODAB vesicles is much lower than affinity exhibited by the large ones. Therefore, the bactericidal effect of large vesicles should be more pronounced than the bactericidal effect exhibited by the small vesicles. The experiment shown on **Fig. 5** confirms this reasoning. Large vesicles are indeed more effective for inducing the bactericidal effect than small vesicles, as expected from the higher affinity constant of the large vesicles towards the cell surface.

In summary, DODAB seems to act differently when compared with other cationics that disrupt the cell membrane. The mechanism of DODAB bactericidal action possibly involves damage to protein function at the bacterial external wall level, where vesicles do adhere without vesicle rupture or cell lysis (17).

The aim of this study was threefold: *1*) to reconfirm the bactericidal effect of cationic vesicles previously described for *E. coli* (18, 19) establishing an order of bacterial susceptibilities towards DODAB dispersions; *2*) to observe whether vesicle size would be important in determining the bactericidal action; and *3*) to investigate the mechanism of death as related to the cell surface charge. We have shown that bacterial death caused by DODAB vesicles is associated with a positive charge on the cell surface (Figs. 3 and 4).

From the point of view of antibiotics incorporation in DODAB or DODAC liposomes, conventional cationic liposomes usually encapsulate antibiotics such as amikacin,

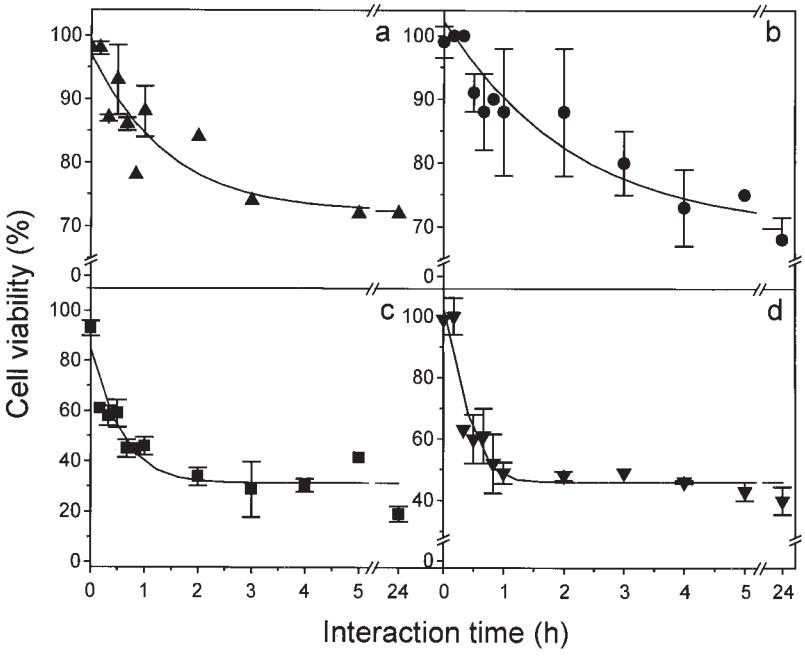

Fig. 2. Cell viability (%) as a function of interaction time between bacteria and DODAB SV at 5 μm. Viable bacteria number densities in each interacting mixture were fixed at ca. 2.1×10^7 CFU/mL for *E. coli* (a), 2.8×10^7 CFU/mL for *S. typhimurium* (b), 2.7×10^7 CFU/mL for *S. aureus* (c), and 2.8×10^7 CFU/mL for *P. aeruginosa* (d). Plating on agar (0.1 mL) was done after a 1:20000 dilution of the mixture. Viability determined for control mixtures (in absence of DODAB) was close to 100%.

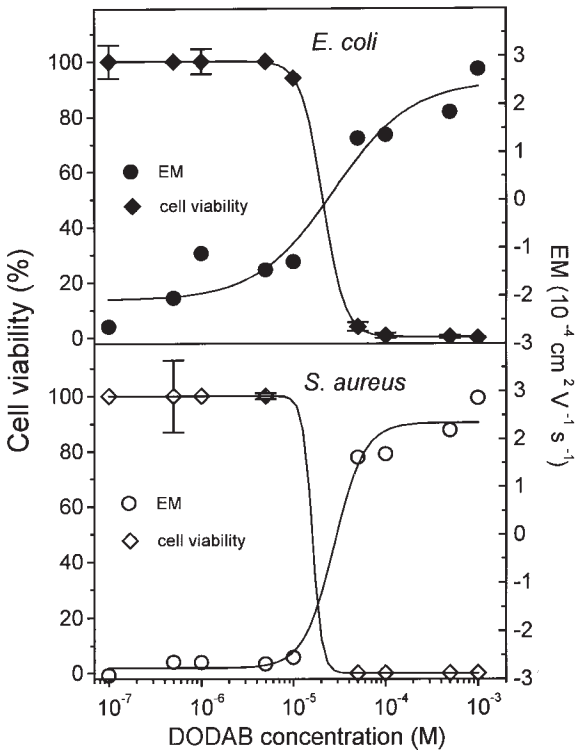

Fig. 3. Electrophoretic mobility (EM) and cell viability for *E. coli* (2.2×10^7 CFU/mL) and *S. aureus* (2.5×10^7 CFU/mL) as a function of DODAB concentration. Bacteria and vesicles in the mixtures interacted for 1 h before dilution and plating or EM measurements.

netilmicin and tobramycin (23) more efficiently, a fact that reinforces the importance of prospective applications of our bactericidal liposomes as antibiotics encapsulators. A possible synergistic action between antimicrobial liposomes such as DODAB SV or LV and antibiotics may become useful in clinics. Also, bacterial cell lysis was demonstrated to be absent both for a Gram-negative (*E. coli*) and a Gram-positive (*S. aureus*) bacterium, in clear contrast to observations by other authors that detected lytic effects induced by micelle-forming quaternary ammonium surfactants (24, 25). Thus, bilayer-forming surfactants such as DODAB and DODAC clearly behave differently and must cause death via a mechanism that does not include cell lysis. Consistently, interaction between bacteria and large DODAC vesicles did not induce leakage of intravesicle contents (17). Vesicle fusion with the cell membrane would necessarily lead to leakage. Therefore, lipids from the cell membrane and DODAB molecules from the vesicle do not seem to mix, as expected for lipids that are in different physical states (lipids from the cell membrane are in the liquid–crystalline, more fluid state whereas DODAB or DODAC are in the gel, rigid state). In fact, DODAC or DODAB are not micellizing agents capable of disrupting cell membranes. Furthermore, we have previ-

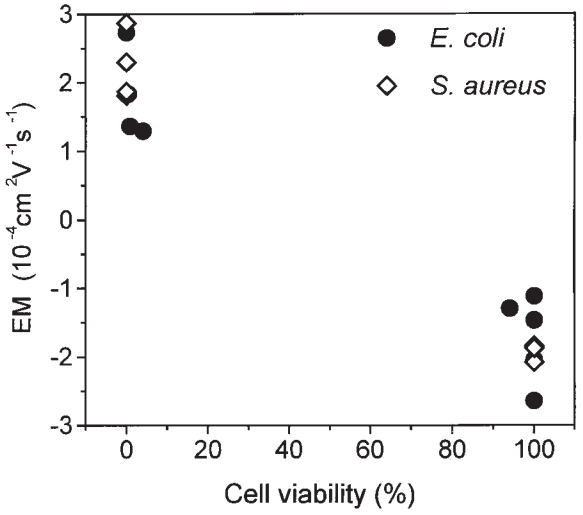

Fig. 4. Correlation between sign of the cell surface charge and life or death. For both *E. coli* and *S. aureus*, positively charged cells do not survive. The correlation was found from data in Fig. 3.

TABLE 3. Effect of *E. coli* concentration on viability at 5 μm DODAB and 1 h of interaction time

E. coli	Bacterial Viability	DODAB Concentration Required for EM = 0[a]
CFU/mL	%	μm
4×10^8	95 ± 8	65
1×10^8	87 ± 5	35
1×10^7	35 ± 5	7

One should notice that 5 μm is a DODAB concentration very close to 7 μm which is the DODAB concentration required for neutralization of the cell surface charge (EM = 0) at 1×10^7 bacteria/mL. Under these conditions, a viability of 50% is to be expected. In fact, in the third line of this table, at 5 μm DODAB, we are slightly below the amount required for charge neutralization so that viability should be close to 50% as indeed obtained (35 ± 5).

[a] Data taken from reference 18.

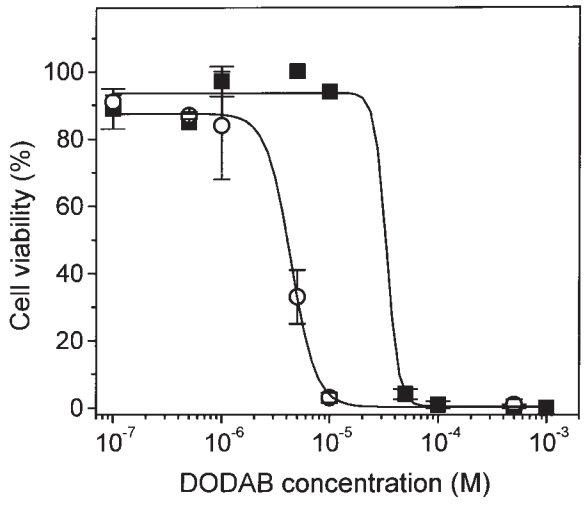

Fig. 5. *E. coli* viability as a function of DODAB concentration for small (■) or large (○) DODAB vesicles prepared using sonication with tip or vaporization of a chloroformic solution, respectively. In comparison to the small vesicles, large vesicles have a larger affinity constant (19) for the bacterium surface consistent with their higher efficiency as bactericides.

ously quantified the adsorbed DODAB amount on the surface of the bacterial cells (19). At limiting adsorption, a palisade of vesicles, i.e., a single layer of adjacent vesicles, was demonstrated to surround the cell surface (19). The palisade model adequately explained the amphiphile amount adsorbed on the bacterial surface. Thus, mere adhesion of entire vesicles to the cell seems to have devastating effects that lead to cell death. A strong possibility is functional damage of membrane proteins responsible for transport through the bacterial cell wall in the case of the Gram-negative bacteria. Would cell death involve obstruction of porins by entire, non-disrupted vesicles? This is a possibility that requires further investigation.

The results presented in this work establish DODAB or DODAC liposomes by themselves as effective bactericides, pointing out the differential killing effect of a positive charge on the cell surface, in contrast to the usually innocuous effect of conventional liposomes used as drug carriers. Regarding the potential clinical utility of DODAB as a bactericide, the present work conducted in water at practically zero ionic strength may be at odds with any clinical use as under physiological conditions ionic strength is much higher. The low colloidal stability of DODAB vesicles in the presence of salt could be circumvented by using DODAB in a mixture of lipids capable of yielding vesicles of high colloidal stability under physiological conditions. This possibility is being investigated in our laboratory. Furthermore, a bactericidal polyelectrolyte, which as a cationic vesicle is active at low concentrations and ionic strength, may have potential use in preventing enteropathogenic bacterial contamination of water. More importantly, DODAB concentrations required for the bactericidal effect are of the order of 0.01–0.1 mm (Fig. 1) whereas 40–50% of death for cultured mammalian cells was measured by flow cytometry in the presence of 1 mm DODAB (16). Hence, the bactericidal cationic liposomes do present a potential therapeutic use hitherto unexplored.

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