Cationic Liposomes as Antimicrobial Agents

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This document contains a mini-review of research work on bilayer-forming cationic lipids which self-assemble in water medium as closed bilayer membranes or liposomes and behave as very potent antimicrobial agents. In contrast to conventional liposomes, the large but differential cytotoxicity of cationic liposomes has been advantageously used against pathological microrganisms. This action has been described both in vitro against Gram-negative or positive bacterial strains and in vivo, against fungus. In certain instances, synergistic drug action against yeasts for fungicidal drugs carried by the antimicrobial bilayers has been described. Given the inexpensive character of certain synthetic cationic lipids and the multiple utility of liposomal models in drug delivery, many pharmaceutical applications are to be anticipated for this convenient joint venture.

Keywords cationic lipid; microbicidal action; drug delivery; antimicrobial agents

1. General remarks

Spontaneous non-covalent interactions between molecules leading to supramolecular assemblies is ubiquitous in living organisms because intermolecular interactions are central to the formation of a wide variety of complex biological structures [1 -6]. Association of molecules under equilibrium conditions leading to stable, structurally well-defined, non-covalently bound aggregates allows highly specific recognition, reaction, transport and regulation processes, such as substrate binding to enzymes, transcription and translation of genetic code, receptor-ligand and antigen-antibody recognition [1 -7]. Biologically produced organic-inorganic composites, such as bone, teeth and sea shells, could be formed through template-assisted self-assembly where self-assembled lipids, or proteins, or both offered the scaffolding for the deposition of inorganic material [4]. Lately, the importance of self-assembly went far beyond the biological limits. New and non-biological advanced materials have been obtained, which would be inaccessible solely via chemical synthesis [4 -7].

The liposomes first produced in Cambridge UK in 1965 by Alec Bangham looked like myelin figures forming coherent and closed concentric spheroidal bilayers [8]. From lipid self-assembly in water solution [9, 10], organized lamellar, myelin - like structures might result, which reminds the one of cells. From those early days up to the present, the development and diversification of the liposome "membrane" model was astonishing. Much of our present knowledge of membrane properties has been obtained with models prepared with phospholipids [11]. In addition to these model systems, other synthetic amphiphiles and lipids were introduced in the late 1970`s to mimic membrane properties. A variety of bilayer structures made up of synthetic amphiphiles such as some dialkyldimethylammonium halides [12] provided unique opportunities to investigate structure-function relationships [13 -17].

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2. Antimicrobial quaternary ammonium compounds and cationic liposomes

Among the classical cationic surfactants, quaternary ammonium compounds (QACs) are the most useful antiseptics and disinfectants [18,19]. QACs are membrane active agents [20,21] and cause lysis of spheroplasts and protoplasts suspended in sucrose [22,23-26]. The cationic agents hypothetically react with phospholipid components in the cytoplasmic membrane, thereby producing membrane distortion and protoplast lysis under osmotic stress [27,28]. On the other hand, the positive charge on microbial cells has often been correlated to the biocidal action [29-51]. The deposition of organic monolayers onto solid surfaces containing quaternary ammonium groups has been shown to prevent deposition and growth of bacterial biofilms [29]. Various cationic and antimicrobial architectures have been tested such as polyelectrolyte layers [39,42-44, 51] or hyperbranched dendrimers[45-46].

Dioctadecyldimethylammonium bromide (DODAB) is a cationic, bilayer-forming synthetic lipid with a high chemical stability [52]. **Fig 1** shows DODAB chemical structure and self-assembly in dispersion forming closed vesicles or bilayer fragments. Many papers characterized DODAB self-assembly in water solution [53 -61, 62 -64, 65]. Depending on the DODAB dispersion method, large or small vesicles or bilayer fragments are obtained. Using ultrasonic disruption with tip, which introduced a high energy input in the DODAB powder/water system, DODAB bilayer vesicles were not only formed but also disrupted; bilayer fragments conveniently offered a very large area of hydrophobic nanosurfaces suitable to solubilize hydrophobic substances or drugs [66, 67]. Due to DODAB chemical structure with saturated hydrocarbon chains and a stable quaternary ammonium polar head, it is not prone neither to acid/base catalyzed hydrolysis [68-71] nor to lipoperoxidation [72,73]. However, in contrast to phospholipids, which form colloidally stable dispersions in 150 mM monovalent salt, pH 7.4, colloid stability of DODAB vesicles is low and their stability in the biological milieu, is poorly investigated. In contrast to innocuous phospholipid dispersions, DODAB dispersions present remarkable microbicidal activity [34-38,47-50].

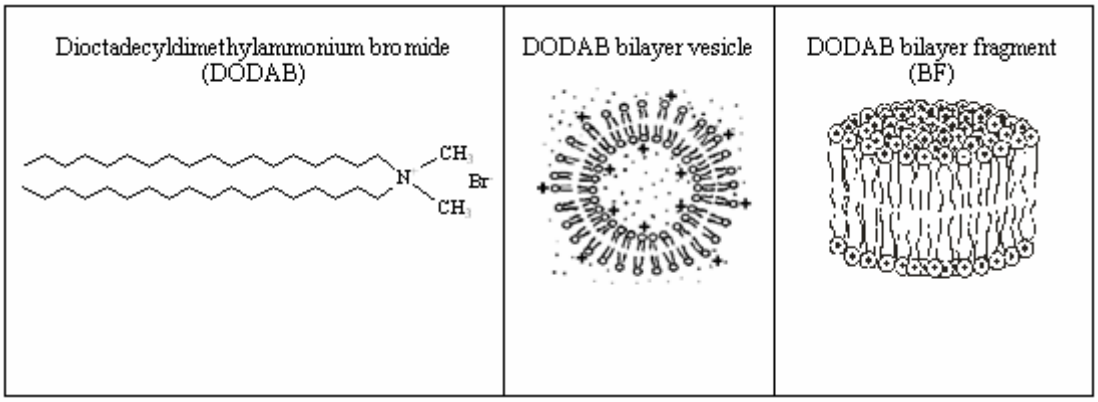

Fig. 1 DODAB chemical structure and DODAB bilayer assemblies in aqueous dispersions.

Adsorption of DODAB cationic bilayers onto bacterial cells changes the sign of the cell surface potential from negative to positive with a clear relationship between positive charge on bacterial cells and cell death.[47]. The adsorption isotherms pointed to an interaction of high affinity with limiting adsorption values compatible with DODAB vesicle adhesion to bacteria without vesicle rupture [34, 48] The flocculant and bactericidal effects of DODAB bilayers on E. coli were not related to each other; cell death taking place at much smaller DODAB concentrations than those required for bacterial flocculation [35]. Regarding the mechanism of DODAB action, neither bacterial cell lysis nor DODAB vesicle disruption takes place [48]. The fact that these cationic vesicles in the rigid gel state did not disintegrate upon adsorption to the bacterial cell surface was significant for deliverance of drugs inside the vesicle aqueous compartment by endocytosis. One should notice that absence of vesicle leakage was done with

large, closed DODAB bilayer vesicles, ca. 400 -500 nm mean diameter, obtained by injection of a DODAB chloroformic solution in water at a temperature above the solvent boiling point [48]. From the bacterial cell point of view, cell lysis was also absent as determined by absence of leakage of phosphorilated compounds from the cells. This is nicely correlated with the extremely low critical micelle concentration for DODAB, and its existence in dispersion as bilayers with absence of micelles or individual monomers in solution. The absence of bacterial cell lysis in the presence of DODAB bilayers contrasts with the effect of other micelle-forming amphiphiles where lysis occurs [48]. For *E. coli* and *S. aureus,* simultaneous determination of cell viability from plating and electrophoretic mobility as a function of DODAB concentration yielded a very good correlation between cell surface charge and cell viability. Negatively charged cells were 100 % viable whereas positively charged cells did not survive [47]. Thus, an interesting correlation between sign of the cell surface charge and cell viability was found; life or death controlled by the sign of surface charge on the bacterial cell [47]. Would there be a DODAB induced, lethal conformational change in protein structure of a vital, essential protein at the cell surface?

The bactericidal effect of DODAB bilayers was evaluated against *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in order to establish susceptibilities of different bacteria species towards DODAB at a fixed concentration of viable bacterial cells ($2.5X10^7$ viable bacteria/mL) [47]. For the four species, susceptibility towards DODAB increased from *E. coli* to *S. aureus* in the order above. Typically, cell viability decreased 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 or bilayer fragments was shown to be controlled be cell diffusion [47]. **Fig 2** illustrates the in vitro efficacy of cationic DODAB bilayers against four bacteria species clinically important.

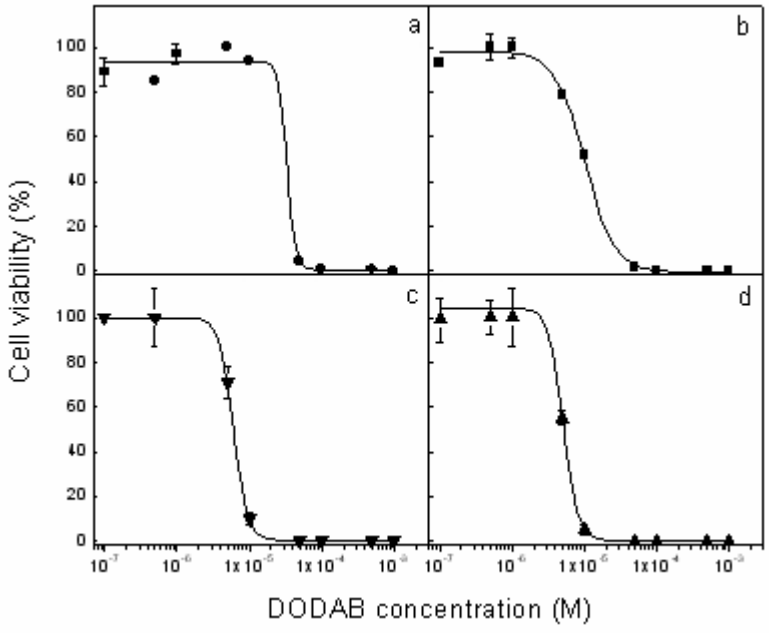

Fig. 2 DODAB bilayers as bactericides against *E. coli* (a); *S. thyphimurium* (b); *S. aureus* (c) and *P. aeruginosa* (d) as adapted from [47].

DODAB also affected viability of *C. albicans* [36]. Simultaneous determination of cell viability and electrophoretic mobility as a function of DODAB concentration yielded a very good correlation between yeast surface charge and yeast viability. Upon increasing DODAB concentration, the cell surface charge decreased and changed its sign to yield positively charged cells. However, in contrast to the DODAB bactericidal property, DODAB effect on fungus over 1 h of interaction time was only fungistatic at 1

mM DODAB and ca. 10^6 cells/mL. The compared effect of DODAB lipid and the micelle-forming surfactant hexadecyltrimethylammonium bromide (CTAB) on *Candida albicans* or erythrocytes cells was recently evaluated from adsorption isotherms, cell viability, electrophoretic mobility (EM) and fungus cell lysis as compared to haemolysis clearly showing that the critical phenomenon determining antifungal effect of cationic surfactants and lipids is not cell lysis but rather the reversal of cell surface charge from negative to positive [43].

3. DODAB differential cytotoxicity and DODAB -based formulations for drug delivery

The inexpensive DODAB synthetic lipid is a potent bactericide and exhibits cytotoxicity against mammalian cells in culture that is much lower than against prokaryotic or yeast cells. Bacteria are very susceptible to DODAB, with micromolar DODAB concentrations effectively killing four bacteria species of clinical importance. Table 1 shows DODAB concentrations for 0% survival of *C. albicans* ATCC 90028 at 0.8 mM DODAB. Therefore, DODAB concentrations for killing yeast cells are much higher than those required to kill bacteria. Regarding kidney epithelial cells, the DODAB concentration required for 50% survival of cultured cells was 5.4 mM, i.e. these cells were much less susceptible to DODAB than bacteria or yeast cells. **Table 1** shows that toxicity against mammalian cells in culture [75] was much lower than the one exhibited against bacteria or fungae.

Table. 1 Differential cytotoxicity of cationic DODAB liposomes. Interaction time between small DODAB vesicles and cells was fixed at 1 h. The data were taken from the references quoted in the last column.

Cell type	Viable cells concentration (cells/mL)	DODAB concentration for 50% survival (mM)	References
Kidney epithelial cells	10^5	5.400	38
Normal Balb-c 3T3 (clone A31) mouse fibroblasts	10^4	1.000	75
SV40-transformed SVT2 mouse fibroblasts	10^4	1.000	75
C. albicans	$2X10^6$	0.010	36
E. coli	$2X10^7$	0.028	47 and 48
S. typhimurium	$2X10^7$	0.010	47
P. aeruginosa	$3X10^7$	0.005	47
S. aureus	$3X10^7$	0.006	47

The fungistatic DODAB effect in-vitro was first reported against *C. albicans* in pure water [36]. In order to achieve complete fungicidal action, miconazole and amphotericin B were temptatively associated with DODAB and the composition tested against *C. albicans* in-vitro so that 0 % fungus viability could finally be achieved. This antifungal activity for DODAB bilayers by themselves could be associated with their positive charge which drove the bilayer to adsorption onto the oppositely charged fungus cells. In vivo, however, physiological ionic strength (150 mM NaCl) was much higher than that in pure water and the positive charge on the DODAB bilayer was practically zero. Therefore, in vivo DODAB cytotoxicity was expected to be null. In vivo effects were thus ascribed only to the carried drug [36]. DODAB as a carrier in vivo, was very successful, e.g, for presenting antigen as an immunoadjuvant [76,77]. In summary, the ionic strength is an important parameter that controls DODAB activity as a bactericide [34,35,47,48,52] or a fungistatic agent [36]. Ionic strength has to be low and over the 0 –1 mM range of monovalent salt concentrations.

Solubilization of amphotericin B (AB) by synthetic bilayer fragments from dioctadecyldimethylammonium bromide (DODAB) or sodium dihexadecylphosphate (DHP) dispersions in water was followed from dynamic light scattering and optical spectroscopy [66]. Since solubility of AB in water is very poor (10-7 M), size distribution for AB aggregates in water were determined by

dynamic light-scattering and compared to those in the presence of DODAB or DHP nanosized bilayer fragments [66]. There was disappearance of the large drug aggregates upon their incubation with the nanosized bilayer fragments. Light absorption spectra for AB in a poor solvent (water), in a good organic solvent (dimethylsulfoxide; methanol 1:1) and in different lipid dispersions also showed that solubilization strictly depended on the presence of bilayer fragments. AB was poorly soluble in dispersions formed of closed DODAB, sodium dihexadecylphosphate (DHP), phosphatidylcholine (PC) or asolecithin vesicles [66]. Solubilization could be understood from AB chemical structure and an increased hydrophobicity at the borders of bilayer fragments. These hydrophobic borders interacted with the polyenic moiety of the antibiotic leaving the hydroxylated moiety free to face the surrounding water. AB solubilization in DODAB bilayer fragments is illustrated in Fig. 3.

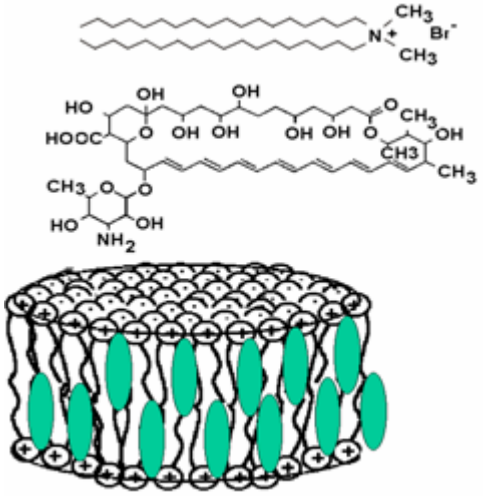

Fig. 3 DODAB bilayer fragments and solubilization of amphotericin B (in green). Chemical structure of amphotericin B is also shown. The drug hydrophobically attached to the rim of DODAB bilayer fragments. Adapted from reference [66].

Similarly to AB, an increased hydrophobicity at the borders of bilayer fragments explained miconazole (MCZ) solubilization [67]. Aqueous MCZ aggregates were solubilized and/or colloidally stabilized by assemblies of bilayer-forming synthetic amphiphiles, such as DODAB or DHP dispersions. Particle sizing, light absorption and scattering from drug particles, zeta-potentials determination and drug aggregation kinetics from turbidity changes in presence or absence of lipid dispersions were obtained over a range of drug and synthetic lipid concentrations [67]. The very low solubility of MCZ in water allowed determination of size distribution for drug particles in water and comparison of the one in the presence of DODAB or DHP nanosized bilayer fragments or entire and closed bilayer vesicles. There was disappearance of large MCZ aggregates upon its incubation with nanosized bilayer fragments produced by ultrasonic dispersion with tip. Light absorption spectra for MCZ in a poor solvent (water), in a good organic solvent (methanol) and in different lipid dispersions showed that solubilization depended on the presence of bilayer fragments. MCZ was poorly soluble in dispersions formed of closed bilayers (vesicles) of DODAB or DHP in the gel state and in PC vesicles in the liquid-crystalline state. At [MCZ] >0.4 mM, kinetics of drug aggregation, zeta-potential measurements and size minimization were obtained upon adding minute amounts of oppositely charged bilayer fragments ([DHP] = 0.05 mM) allowing determination of a remarkable stabilizing effect of drug particles by coverage with anionic bilayer fragments. High drug colloid stability in the presence of charged bilayer fragments was achieved by two different means: 1) at large amounts of bilayer fragments, drug solubilization in its monomeric form at the borders of bilayer fragments; 2) at large drug concentrations and small concentrations of bilayer fragments, coverage of large drug particles with bilayer fragments [67]. Fungicides formulations at low and high molar proportions drug: lipid (P) are shown in **Fig 4.** At high P, there is another example

in the literature of drug aggregates being surrounded by a lipid bilayer; this was described for cisplatin which is a anticancer drug very difficult to formulate as a water dispersion [78].

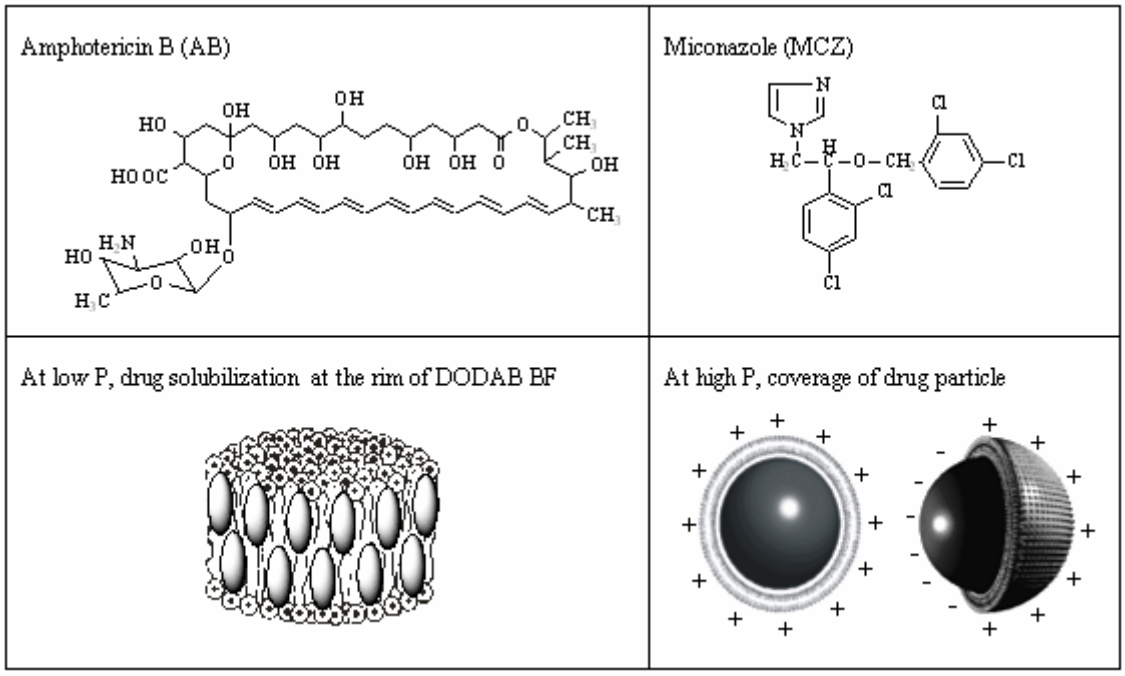

Fig. 4 Chemical structure of amphotericin B and miconazole, the two fungicides formulated with DODAB bilayer fragments at low and high drug to lipid molar proportions (P). Adapted from [66], [67], [81].

In vivo activity of the DODAB/AB formulation against systemic candidiasis in a mouse model was evaluated from survival and tissue burden experiments in comparison to the classical drug formulation named Fungizon [37]. At ≤0.1 mg/mL AB and 10 mg/mL DODAB (high P), the fungicidal drug was solubilized at the rim of the cationic bilayer fragments and used for treating the mice. Effective AB dose in the novel DOD/AB formulation (0.4 mg/kg/day given i.p. for 10 days) was lower than AB dose in Fungizon but gave the same therapeutic result: 100 % survival [37]. From tissue burden experiments, DOD/AB efficacy was also equivalent to the one exhibited by Fungizone regarding elimination of *Candida* colonization in spleen and kidneys. In summary, from mice survival experiments, DOD/AB, was as effective as Fungizon for treating systemic candidiasis in a mouse model [37]. Main DOD/AB drawback was related to its limited capacity to carry the monomeric form of the drug: 10 mg/mL DODAB dispersion efficiently solubilized AB over a drug concentration range that had to be smaller than 0.1 mg/mL [37]. Therefore, if the DOD/AB eventually comes to be successful also for use in humans, the treatment will possibly have to be prolonged to administer AB small doses daily distributed over a larger number of days.

The mechanism by which AB was delivered to the *Candida* cell in vivo possibly involved the following steps. The complex DOD/AB-albumin [79] travelled in blood until finding the ergosterol or colesterol rich cell membrane of the fungus or of the host, respectively. AB readily available from the bilayer disk would complex with ergosterol in the fungus membrane upon disk adsorption to the cell. Contrary to other lipidic carriers, such as fusogenic liposomes or cochleates, the DOD/AB bilayer, in the rigid gel state, would not fuse with the cell membrane.

The question of nephrotoxicity for the novel DOD/AB was addressed both in vitro against cultured kidney epithelial cells in culture and in vivo in a mice model [38]. For in vivo experiments, Swiss Webster female mice were injected intraperitoneally for 10 consecutive days with 0.4 mg/kg/day AB in the form of traditional bile salt desoxycholate (DOC)/AB or DOD/AB [38]. Body and spleen weight,

and biochemical and histopathological data were obtained at days 11 and 180 after injection keeping the experimental conditions where 100 % survival is obtained [37]. At day 11, DODAB and DOD/AB cause loss of body weight and increase in spleen weight, which are not observed for DOC/AB, although the changes are reversible and weights return to control values at day 180. Ten days after injection, biochemical parameters for hepatic and renal function remained unaltered [37]. The nephrotoxicity of the novel formulation is lower than that of Fungizone (DOC/AB), which is the traditional AB formulation using deoxycholate [38]. Dose-dependent cytotoxicity of DOD/AB is lower than that exhibited by DOC/AB [38]. At day 180, renal cortex histopathology reveals leukocytic infiltration and moderate hydropic degeneration of the renal tubules in the DODAB and DOD/AB groups, in contrast to more severe lesions observed for the DOC/AB group such as tubular cystic degeneration and glomerular injury, which are absent for the former groups. The assembly DOD/AB was therefore excellent in vivo [37] presenting low nephrotoxicity in a mouse model [38] but still requiring high DODAB:AB molar proportions in order to carry sufficient amount of drug. In vivo evaluation of hepatotoxicity, spleen damage and blood changes in comparison to DOC/AB (Fungizone) was recently performed ultimately showing toxic effects associated to DODAB carrier alone at the low drug to lipid molar ratios employed [80]. Given the valuable anti-infective properties of cationic lipids [50], there is a need of further toxicity studies employing DODAB in vivo. In conclusion, the DOD/AB formulation exhibits differential cytotoxicity and low nephrotoxicity, though there are still important aspects of general toxicity requiring evaluation with full-scale toxicity protocols. **Fig 5** shows the activity in vivo of the DOD/AB formulation at low P.

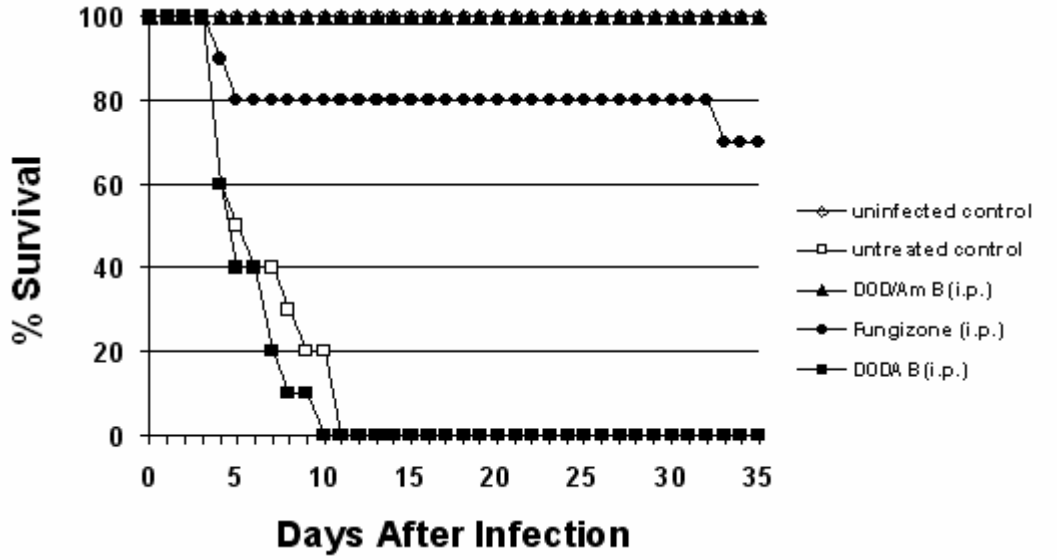

Fig. 5 Activity in mice of the DOD/AB formulation against systemic candidiasis. Adapted from [37].

Synthetic and charged bilayer fragments are opening new perspectives for delivery of water insoluble drugs. In the specific case of the synthetic cationic lipid DODAB, DODAB bilayer fragments (BF) present antimicrobial activity, solubilize fungicides, e.g., amphotericin B and miconazole (MCZ), stabilize hydrophobic drug particles, are therapeutically effective in vivo, and sometimes exhibit synergism with the drug to be carried [74, 81]. Minimal fungicidal concentrations against *C. albicans* are synergically reduced by 10 times for the combination miconazole/DOBAB bilayer fragments [74, 81]. The main drawback of the formulations at low drug to lipid molar ratio is the limited loading capacity of the bilayer fragments with drug. DODAB BF could be loaded with miconazole up to 1 MCZ molecule per 10 DODAB molecules [74]. Amphotericin B was carried by the bilayer fragments up to a molar

proportion of 1:20 drug to DODAB molar ratio [81]. Fig. 6 shows the light-scattering experiment where loading capacity of DODAB BF with AB was determined.

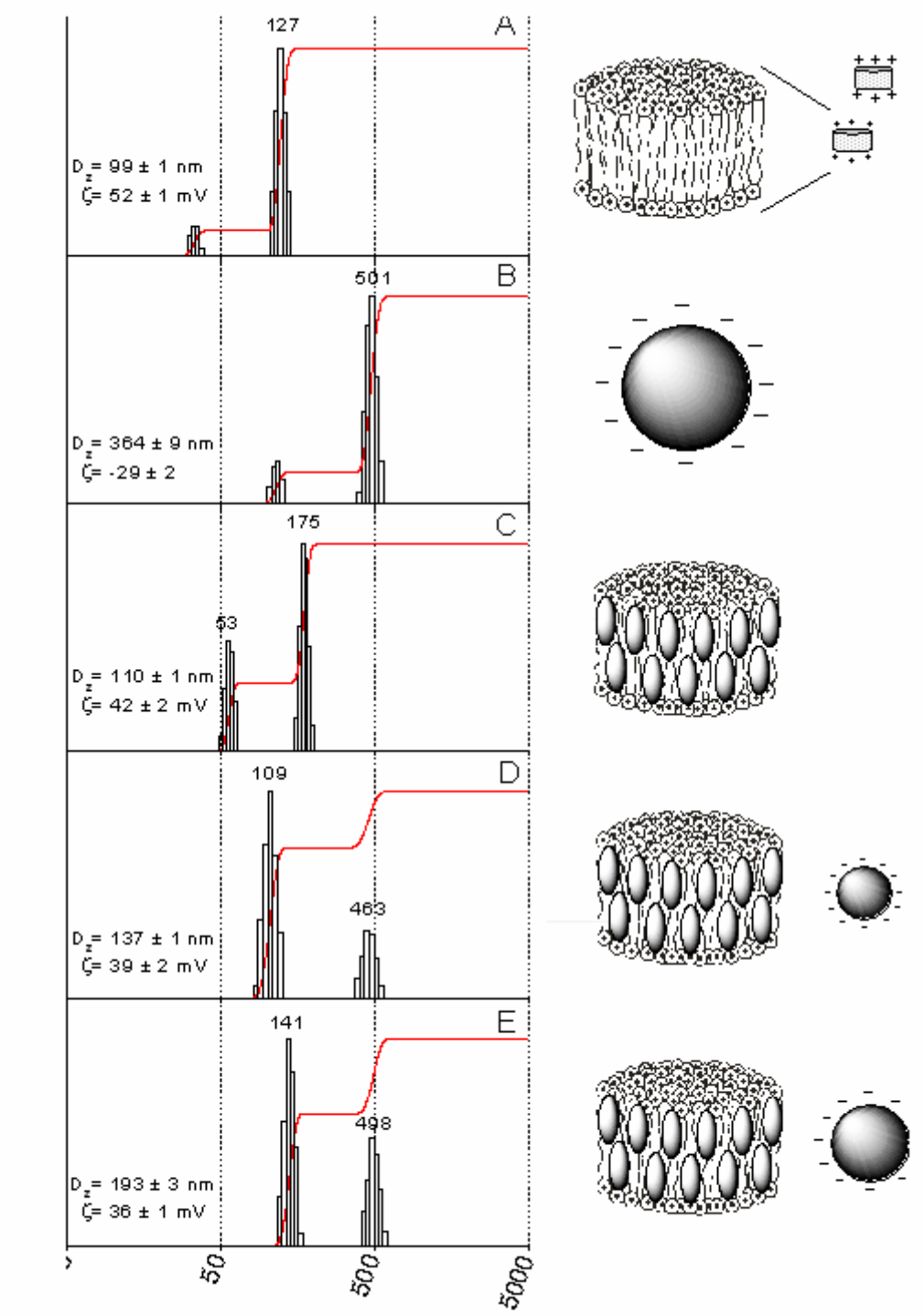

Fig 6 Loading capacity of DODAB BF with AB from size distributions in [81]. In (A), DODAB BF dispersion at 2 mM DODAB. In (B), 0.01 mM AB in water. From (C) to (E), at 2 mM DODAB, [AB] increased yielding P equal to 1:40 (C); 1:20 (D) and 1:13 (E). Mean zeta-potentials in mV are quoted in each subfigure.

4. Cationic bilayers in perspective

At present, there is an unprecedented level of interest in the properties and structures of complexes consisting of DNA mixed with oppositely charged cationic liposomes (CLs). The interest arises because the complexes mimic natural viruses as chemical carriers of DNA into cells in worldwide human gene therapy clinical trials. However, since our understanding of the mechanisms of action of CL-DNA complexes interacting with cells remains poor, significant additional insights and discoveries will be required before the development of efficient chemical carriers suitable for long-term therapeutic applications [82]. Previously the development and applications of an important new platform system for gene delivery known as liposome-mu-DNA (LMD) was described, prepared from cationic liposomes (L), plasmid DNA (D) and the mu(M) peptide derived from the adenovirus core [83]. Nonviral gene delivery relies mainly on the complexes formed from cationic liposomes (or cationic polymers) and DNA, i.e., lipoplexes (or polyplexes). Many lipoplex formulations have been studied, but in vivo activity is generally low compared to that of viral systems [84]. After administration, nonviral vectors encounter many hurdles that result in diminished gene transfer in target cells. Cationic vectors sometimes attract serum proteins and blood cells when entering into blood circulation, which results in dynamic changes in their physicochemical properties. To reach target cells, nonviral vectors should pass through the capillaries, avoid recognition by mononuclear phagocytes, emerge from the blood vessels to the interstitium, and bind to the surface of the target cells. They then need to be internalized, escape from endosomes, and then find a way to the nucleus, avoiding cytoplasmic degradation. Successful clinical applications of nonviral vectors will rely on a better understanding of barriers in gene transfer and development of vectors that can overcome these barriers [85]. Furthermore, the toxicity of, and the induction of an inflammatory response in association with the administration of lipoplexes were described [84].

Other very useful applications of liposomes are in delivery of peptides [83] or proteins or antimicrobial agents. After i.v. administration colloidal carriers are preferentially taken up by the reticuloendothelial system (RES). For this reason nanoparticles and liposomes may be used as antimicrobial drug carriers in the treatment of infections involving the RES [86] For example, the tetrapeptide tuftsin (Thr-Lys-Pro-Arg) specifically binds to monocytes, macrophages, and polymorphonuclear leukocytes and potentiates their natural killer activity against tumors and pathogens [87]. The tuftsin-bearing liposomes not only enhance the host's resistance against a variety of infections but also serve as useful vehicles for the site-specific delivery of drugs in a variety of macrophage-based infections, such as tuberculosis and leishmaniasis [87]. Promising alternative therapies in cutaneous and visceral leishmaniasis, specially in cases resistant to antimony treatment, have employed liposomal amphotericin [88].

Encapsulation of certain antibiotics in liposomes can enhance their effect against microorganisms invading cultured cells and in animal models. For example, the incorporation of amikacin, streptomycin, ciprofloxacin, sparfloxacin, and clarithromycin in a variety of liposomes was described [89]. The liposomes can be used for carrying oil-soluble bactericides (e.g., Triclosan) or water-soluble antibiotics (e.g., vancomycin or benzylpenicillin) and targeted to immobilized bacterial biofilms of oral or skin-associated bacteria. Biofilms have been modeled due to the importance of studying the delivery of oil-soluble or water-soluble bactericidal compounds to a wide range of adsorbed bacteria responsible for infections in implanted devices such as catheters, heart valves, and artificial joints [90]. Biofilms can be also associated with chronic infections such as those occurring in the respiratory tract [91]. A number of elements in the process of biofilm formation were studied as targets for novel drug delivery technologies. These include surface modification of devices to reduce bacterial attachment and biofilm development as well as incorporation of antimicrobials -again to prevent colonization. Liposomal systems were widely studied, either to target antibiotics to the surface of bacterial biofilms, or by virtue of their property of being taken up by cells of the reticuloendothelial system, to target antibiotics towards intracellular bacteria [91]. The delivery of antiseptic and topical antimicrobials has been also employing liposomes for hand scrub with antiseptic liquid soap (e.g. chlorhexidine, PVP-iodine, triclosan) and alcohol-based hand rub [92]. Industrial applications for liposomes include encapsulation of pharmaceuticals and

therapeutics, cosmetics, and anticancer and gene therapy drugs. In the food industry, liposomes have been used to deliver food flavors and nutrients [93-95]. More recently, liposomes have been investigated for their ability to incorporate food antimicrobials, such as the polypeptide antimicrobial nisin, that could aid in the protection of food products against growth of spoilage and pathogenic microorganisms [96-99].

All these applications for conventional liposomes are in perspective for the cationic systems discussed in this review. The special advantages of these DODAB systems would be the low cost and extra antimicrobial activity, which is not provided by conventional liposomes.

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