Fungicidal Nanoparticles of Low Toxicity from Cationic Lipid and Polyelectrolytes

L. D. Melo* and A. M. Carmona-Ribeiro*

*Biocolloids Lab, Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, CEP 05513-970, São Paulo, SP, Brazil, lemelodias@usp.br, amcr@usp.br

ABSTRACT

In this work, the assembly of the cationic lipid dioctadecyldimethylammonium bromide (DODAB) and polyelectrolytes such as carboxymethylcellulose (CMC) and polydiallyldimethylammonium chloride (PDDA) produced small (SP) and large stable particles (LP) that presented activity against *Candida albicans* in complete absence of antibiotics. These nanoparticles have also been evaluated regarding toxicity against mammalian red blood cells. SP and LP exhibit 108 and 473 nm mean diameter, respectively, and 30 and 52 mV of zeta-potential, respectively. The results showed that the active layer in the DODAB/CMC/PDDA assembly was the outermost PDDA layer, and that PDDA effective dose was smaller for SP than for LP. Each element in the assembly and the assembly itself displayed very low hemolytic activity at concentrations well above the minimal fungicidal concentration. The microbicidal and nontoxic particles may find interesting applications in antimicrobial chemotherapy.

Keywords: dioctadecyldimethylammonium bromide, polydiallyldimethylammonium chloride, bilayer fragments, low hemolytic activity, fungicidal effect.

1 INTRODUCTION

Major strategies for formulating particles as carriers for bactericidal or fungicidal agents or as antimicrobial agents themselves have been based on a variety of materials [1]. Major approaches used metals, metal oxides or composite materials [2-8], oil-in-water emulsions or solid-lipid nanoparticles [9-15] or polymeric particles [16-20]. In a few cases, the polymeric particle itself was designed to be the active antimicrobial agent [21,22]. Quaternary ammonium compounds such as mono- and dialkyl dimethylammonium salts and polymeric quaternary ammonium ionenes display antimicrobial, antifungal and tumoricidal properties with the polymers being more active than the corresponding monomers due to favored polymer adsorption onto the microbial cell surface and the subsequent disruption of cytoplasmic membrane integrity [23,24]. Besides the quaternary polyionenes, other synthetic and interesting antimicrobial polymers with dark and light activated biocidal activity have been reported [25]. In particular, the polyelectrolyte poly (diallyldimethylammonium) chloride (PDDA) with permanently charged quaternary ammonium groups in its cyclic unities has been considered safe for human health and is widely used in paper manufacturing, water treatment, mining industries and food processing. PDDA antimicrobial activity has been incidentally reported [26-28].

Among the lipids, dioctadecyldimethylammonium bromide (DODAB) is a cationic, bilayer-forming synthetic lipid with a high chemical stability and well-described anti-infective properties [29]. Depending on DODAB dispersion method, large vesicles or bilayer fragments (BF) are obtained [30]. DODAB BF display antimicrobial activity both *in vitro* and *in vivo*, solubilize water insoluble drugs, stabilize hydrophobic drug particles, are the basis of an effective amphotericin B formulation against systemic candidiasis in mice and exhibit synergism while carrying miconazole against *C. albicans* [31-33]. However, DODAB activity against fungus is only fungistatic [34]. Recently, the layer-by-layer (LbL) procedure [35] was employed to produce hybrid antimicrobial and cationic particles from DODAB BF supporting consecutive layers of anionic carboxymethylcellulose (CMC) and cationic PDDA [22]. The poor activity of DODAB against fungus inspired this evaluation of DODAB BF/CMC/PDDA assemblies against *Candida albicans*. The assemblies revealed a potent fungicidal activity and a low toxicity against red blood cells.

2 METHODS

2.1 Preparation and characterization of hybrid particles from cationic lipid and polyelectrolytes

DODAB was dispersed in 0.264 M D-glucose solution using a titanium macrotip probe powered by ultrasound. This procedure dispersed the lipid powder in aqueous solution using a high-energy input, which not only produced bilayer vesicles but also disrupted these vesicles, thereby generating open bilayer fragments (BF) [29,30]. The dispersion was centrifuged in order to eliminate residual titanium ejected from the macrotip. Stock solutions of CMC and PDDA were prepared in isotonic D-glucose 0.264 M aqueous solution. CMC stock solution was added to aliquots of the DODAB BF dispersion over a range of CMC concentrations and allowed to interact for 20 min. before adding PDDA solution. After 20 min. of interaction, small (SP) and large stable particles (LP) from DODAB BF/ CMC/ PDDA were obtained and characterized

regarding their zeta-average diameters (Dz), zeta-potentials (ζ) and antimicrobial activity. Final concentrations for preparing SP were 0.063 mg/mL DODAB, 0.100 mg/mL CMC and 0.100 mg/mL PDDA. For LP they were 5 times larger, i.e., 0.315 mg/mL DODAB, 0.500 mg/mL CMC and 0.500 mg/mL PDDA. Sizes and zeta-potentials were determined by means of a ZetaPlus Zeta-Potential Analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) equipped with a 570 nm laser and dynamic light scattering at 90° for particle sizing [36].

2.2 Determination of antifungal activity of SP or LP

Some isolated colonies of *Candida albicans* ATCC 90028 were transferred to 10 mL of Sabourad broth and incubated under stirring for 24 h/37°C. Thereafter, the cell suspension was centrifuged and the pellet resuspended in 0.264 M D-glucose. This last procedure was repeated twice. The cell suspension was then adjusted to 0.5 of the McFarland scale and further diluted 10 times to yield a final cell concentration of 2 x 10^6 UFC/mL. Interaction between fungus and dispersions of SP or LP proceeded for 1 h at room temperature after mixing 0.5 mL of the cell suspension and 0.5 mL of each dispersion over a range of particles dilution. Thereafter, 0.1 mL of each particles/cells mixture dilutions were plated on Sabouraud ágar so that each plate received about 100 cells. After incubation (37 °C/ 48 h) CFU counting was performed. Cell viability (%) was presented as a mean value ± mean standard deviation.

2.3 Determination of hemolysis induced by SP or LP

Toxicity of the assemblies was evaluated from hemolysis of red blood cells induced by the fungicidal particles. An aliquot of 0.5 mL of a 1% cell suspension in 0,264 M D--glucose was added to 0.5 mL of particles dispersion and the mixture interacted for 1 h. Thereafter, samples were centrifuged and the absorbance (A) of the supernatant at 411 nm was determined. This wavelength was chosen due to occurrence of the maximal absorbance for the red cells suspension after complete hemolysis achieved in the presence of 1% Triton X-100. This positive control yielded 100 % hemolysis. The negative control taken as 0% hemolysis was given by the absorbance at 411 nm of the supernatant of red blood cells suspension centrifuged in isotonic D-Glucose solution (A_{411sup}). The hemolysis percentile (%H) was calculated from equation (1):

$$\%H=100(A_{411sup} - A_{411negative\ control}) / (A_{411\ positive\ control}) \quad (1)$$

Hemolysis was evaluated both as a function of concentration of components of the particles and as a function of time at a given particle concentration.

3 RESULTS AND DISCUSSION

3.1 Physical properties of small particles and large particles from DODAB BF/ CMC/ PDDA assemblies

Our previous work showed the effect of CMC concentration on sizes (Dz) and zeta-potentials of DODAB BF/CMC assemblies and the effect of PDDA concentration on Dz and zeta-potentials of DODAB BF/ CMC/ PDDA assemblies [22]. From the region of high colloid stability for the negatively charged DODAB BF/CMC assemblies, two CMC concentrations were selected: 0.1 and 0.5 mg/mL CMC. These anionic assemblies further interacted with the cationic polyelectrolyte PDDA at 0.1 and 0.5 mg/mL PDDA which were the concentrations for size minimization and colloidal stabilization [22]. Figure 1 shows the size distributions, mean Dz and zeta-potentials for the layered small (SP) and large (LP) nanoparticles. Small and large DODAB BF/CMC/PDDA cationic assemblies to be tested against *Candida albicans* were selected at 0.1 (Fig. 1 A) and 0.5 mg/mL PDDA (Fig. 1 B).

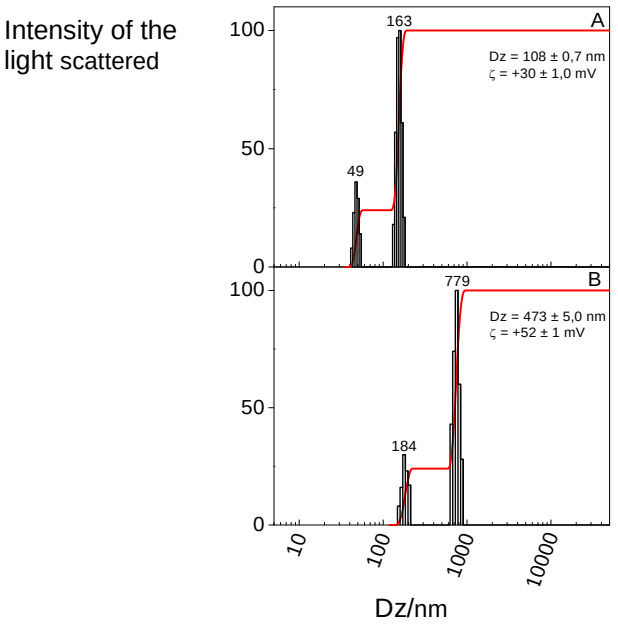

Figure 1: Size distributions, mean z-average diameter (Dz) and zeta-potential (ζ) of DODAB BF/CMC/PDDA assemblies prepared to yield small particles, SP (A) or large particles LP (B).

3.2 Fungicidal activity of DODAB BF/ CMC/ PDDA SP or LP

Cell viability as a function of [PDDA] only, [DODAB] only or [PDDA] and [DODAB] combined as small or large particles revealed the potent effects of PDDA by itself and the fungicidal activity of SP and LP against the fungus *Candida albicans* (Figure 2).

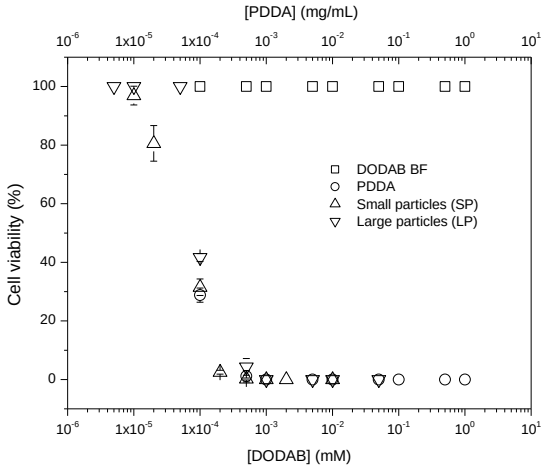

Figure 2: *C. albicans* viability (%) as a function of PDDA and DODAB concentration in small (Δ) or large particles (▽). Controls are DODAB BF only (□) or PDDA only (○). Interaction time is 1 h at 1 – 2x10^6 CFU/mL.

Doses of DODAB, PDDA or DODAB/PDDA in particles required for killing 99% and 50% of fungal cells after 1 h interaction time are summarized in Table 2 and compared with previous data for bacteria. DODAB BF and PDDA doses required to kill 99% of C. albicans cells (Table 2) can be considered as minimal fungicidal concentrations (MFC) determined, however, there are certain special conditions for the cationic fungicides to be effective. These conditions are the absence of the cell culture medium and a low ionic strength. The low ionic strength would avoid the screening of the electric double layer of the cationic assemblies. The electrostatic attraction certainly is a major driving force directing the cationic assembly to the bacterial cells.

The fungicidal effect of PDDA by itself or as the outer layer on particles occurs at small doses. For killing *C. albicans*, these doses are even smaller than those required for killing bacteria (Tab. 2). The fungus is much more susceptible to the cationic polyelectrolyte than the bacteria are. The PDDA dose needed to kill the fungus increases from PDDA only to SP to LP (Tab. 2), suggesting that not all PDDA in SP or LP is available to adsorb and kill the cells. The PDDA interaction with CMC or the PDDA bridging two or three small particles to yield a large particle reduce the net cationic charge at the outer PDDA layer that is so important for the interaction with the cells.

The cationic compounds and assemblies can be ordered after their fungicidal efficiency against fungus: PDDA > DODAB BF/CMC/ PDDA >>>DODAB BF (Table 2). Among other factors, the lower efficiency of DODAB BF when compared to PDDA alone may be related to the different counterions: bromide and chloride, respectively. The chloride binds with lower affinity to the quaternary ammonium moiety than the bromide does [38].

Liposomal or particulate delivery systems have been successfully targeting antibiotics to bacteria *in vivo* since the seventies due to co-localization of infecting microorganisms and liposomes or particles in the macrophages after phagocytosis [39, 40]. SP and LP nanoparticles are also expected to co-localize with the infecting bacteria inside the macrophages.

3.3 Hemolytic activity of small and large particles of DODAB BF/ CMC/ PDDA

The toxicity of SP or LP against red blood cells was determined from hemolysis over a range of particle concentrations at 1 h of interaction time between cells and particles. PDDA, SP and LP barely caused hemolysis whereas DODAB BF yielded 0.5-2.5 % hemolysis (Fig. 3).

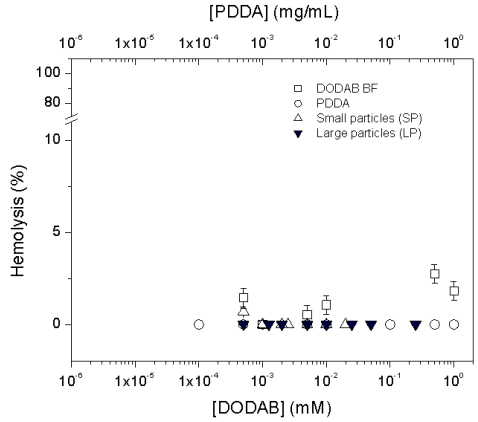

Figure 3 : Hemolysis (%) as a function of final [PDDA] and [DODAB] assembled as DODAB BF/CMC/PDDA SP (△) or LP (▽). Interaction time was 1 h.

The determination of hemolysis as a function of time was also performed at particles concentrations above the minimal fungicidal concentration (MFC) and for DODAB BF or PDDA alone as controls (not shown).

Dispersion	**$[PE]_{99/50}$ $[CL]_{99/50}$ (μg/mL)** ***P. aeruginosa***		**$[PE]_{99/50}$ $[CL]_{99/50}$ (μg/mL)** ***S. aureus***		**$[PE]_{99/50}$ $[CL]_{99/50}$ (μg/mL)** ***C. albicans***	
DODAB BF		6.3[a]/3.1[a]		7.5[a]/4.4[a]		
DODAB BF		3.0[b]/1.2[b]		-/1.8[b]		>>>630
PDDA	1.0[b]/0.5[b]		10.0[b]/0.5[b]		0.4/0.06	
SP	2.0[b]/0.9[b]	1.0[b]/0.5[b]	10.0[b]/0.5[b]	6.0[b]/0.3[b]	0.5/0.08	0.32/0.05
LP	2.0[b]/0.7[b]	1.0[b]/0.4[b]	10.0[b]/0.4[b]	6.0[b]/0.3[b]	1.0/0.10	0.63/0.06

Table 2: PDDA [PE] and DODAB concentrations [CL] that kill 99 and 50 % of microrganisms.
[a]data from [37]; [b]data from [22]

SP and LP induce 0-5 % hemolysis at concentrations above MFC whereas DODAB BF yield 15 % of hemolysis after 24 h interaction with red blood cells. Thus PDDA was less hemolytic than DODAB BF.

PDDA at the outermost layer of a particle can be considered more interesting than PDDA in solution since the macrophages are specifically designed by nature to the uptake of foreign particles in general. Since this is the first systematic description in the literature of the PDDA fungicidal action, several applications for these and others microbicidal nanoparticles based on PDDA can be foreseen in disinfection, prophylaxis and even clinical treatment of infectious diseases caused by fungi.

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