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Original article

Comparative study of free and liposome-entrapped chloramphenicol against biofilms of potentially pathogenic bacteria isolated from cooling towers

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ABSTRACT

This study aimed to investigate for the first time the *in vitro* antibiofilm effectiveness of two chloramphenicol liposome formulations against biofilms of potentially pathogenic bacteria associated to corrosion isolated from the water of cooling towers from a Brazilian industry. Antibiofilm assays with liposomes were performed in 96-wells microtiter plates, and data was compared to free chloramphenicol treatment. Chloramphenicol-loaded liposomes were successfully produced using the dehydration-rehydration method, with vesicle diameters of 131 nm (100 nm membrane extrusion) and 182 nm (200 nm membrane extrusion) assessed by dynamic light scattering. The liposomes obtained by 100 nm membrane extrusion were more effective than 200 nm membrane extrusion vesicles against the biofilms after overnight exposure, and the free drug had no antibiofilm effect. Our study open doors for more investigations on liposome entrapment of antimicrobial compounds such as biocides of industrial use, for controlling biofilm formation in aquatic environments.

1. Introduction

Cooling towers are important devices used in industries in which exceeding heating of equipments is avoided by water circulation, such as oil refinery plants, thermal and nuclear power stations, and even hospital settings (Ning et al., 2015). Generally, the tower removes heat from water to the atmosphere using a fan system, especially in open recirculation systems. To counterbalance water concentration due to evaporation, a new amount of water should enter the tower, thus maintaining the concentration cycle at a desired state. The water for replacement of the original volume or near it is called makeup water, and its adequate quality in cooling towers can be reached by treatment methods that include filtration, clarification and correction of alkalinity (Babu et al., 2013; Ning et al., 2015).

The recirculation of water at different pH and temperatures in the tower, besides the presence of organic matter, provide the ideal environment for microbial growth. Moreover, microorganisms can gain entry into the cooling systems by the air, water and soil nearby (Hsieh et al., 2010; Babu et al., 2013; He et al., 2015). As a consequence, biofilms may grow inside the tower. Biofilms are complex microcolonies, generally of polymicrobial composition in the environment, which are embedded in a matrix of extracellular polymeric substances (EPS), composed by polysaccharides, lipids, proteins, and nucleic acids (Hall-Stoodley et al., 2004). The levels of each of these biomolecules in the EPS are influenced by factors such as availability of nutrients, temperature, and the species present at the biofilm. Biofilm formation in cooling towers can increase the risk of microbiologically induced corrosion, what impairs the adequate functioning of the tower because of biofouling. Furthermore, pathogenic species might be present in these biofilms (Hall-Stoodley et al., 2004; Flemming and Wingender, 2010). In systems with steam production, they can be dispersed to the environment. Due to exposure to aerosols, legionellosis outbreaks associated to cooling towers are not a recent problem (Cooling Tower Institute, 2008).

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Several alternatives have been explored for controlling biofilm formation in cooling towers. However, biofilms show greater resistance to conventional biocides when compared to their planktonic counterparts, mainly by the barrier effect of the EPS (Hall-Stoodley et al., 2004; Flemming and Wingender, 2010; Hsieh et al., 2010). This explains in parts the low effectiveness of most of the currently available alternatives for microbial growth control in coolling towers (Cooling Tower Institute, 2008; Hsieh et al., 2010). Therefore, novel antibiofilm strategies have been proposed and tested in recent years, including the use of substances entrapped in liposomes.

Liposomes are spherical artificial membranes with internal hydrophilic compartment arranged in spontaneously formed bilayers when their components are hydrated and subjected to agitation (Gregoriadis, 2007). Liposomes were firstly developed for the study of the behavior of biological membranes; however, since the first encapsulation studies, liposomes have been highly employed in pharmaceutical, cosmetics and food industries, in micro and nanometric scales (Gregoriadis, 2007; Rushmi et al., 2017; Ingebrigtsen et al., 2016). The entrapment of substances in liposomes can increase their effectiveness by offering physical protection against factors such as radical species and chelating agents, and by improving parameters such as solubility and Interaction with the target (Rushmi et al., 2017).

In this study, we present the first evidence of the effectiveness of liposome-entrapped chloramphenicol against ten different bacterial species isolated from cooling towers of a Brazilian industry. Such species are associated to several diseases of clinical relevance. We developed 100 and 200 nm liposomes using the dehydration-rehydration method, and tested them against overnight-formed biofilms of each isolate in an *in vitro* model. Our study open doors for exploring other drugs and antimicrobial substances entrapped in liposomes against potentially pathogenic microbial isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 10 isolates from our bacterial collection were used in this study (Table 1). They were obtained from the circulating water from industrial scale cooling towers (called towers 1 and 2) of a Brazilian industry. These isolates are part of the microbiota associated to corrosion of these towers, and were isolated as previously described by our group (Santos et al., 2015). All bacteria were identified by partial sequencing of the 16S rRNA (Dias-Souza et al., submitted to publication). The water from tower 1 consists in a secondary effluent generated in the Industrial Waste Treatment Plant of the oil refinery, treated with treated in a prototype reuse unit. The water of tower 2 is collected from an artificial lagoon, which undergoes a pre-chlorination process, being then clarified and chlorinated, reaching a free residual chlorine content ranging between 0.5 and 1.0 mg/L. The water is directed to sand filters to remove particulate matter and then supplies the tower.

Table 1
Bacterial isolates investigated in this study.

Isolate number	Identification
8	*Elizabethkingia meningoseptica*
20	*Acinetobacter junii*
40	*Kluyvera cryocrescens*
63	*Exiguobacterium mexicanum*
94	*Stenotrophomonas maltophilia*
110	*Bacillus* sp.
116	*Lysinibacillus sphaericus*
125	*Acinetobacter haemolyticus*
152	*Enterobacter hormaechei*
157	*Bacillus* sp. Cereus Group

2.2. Preparation and characterization of liposomes

Liposomes were prepared with Phosal 75 SA (proliposome system, Lipoid GmbH, Germany) using the dehydration-rehydration method as described by Frézard et al. (2011) with slight modifications. Phosal 75SA is composed of Lecithin in alcohol (75%), safflower (*Carthamus tinctorius*) oil, glyceryl stearate, coconut (*Cocos nucifera*) oil and ascorbyl palmitate, and does not demand the use of organic solvents such as chlorophorm for the preparation of liposomes. We mixed Phosal with Mili-Q water at 1:10 ratio at home temperature, and stirred at maximum speed for 1 h, forming empty multilamellar vesicles (MLVs). Following, MLVs were downsized by a pre-calibration step consisting in extrusions across polycarbonate membranes of 450 nm (five times), and 200 nm (five times), using a mini-extruder 1 mL syringe system (Avanti Polar Lipids, Canada). In order to reach 100 nm vesicles, liposomes were extruded across 100 nm polycarbonate membranes. Following, pre-calibrated liposomes were mixed with a sucrose criopreservative solution (100 mM) at 1:1 ratio, frozen at −80 °C and freeze-dried for 48 h. Rehydration of dry liposomes was performed with a 1 mg/mL aqueous solution of chloramphenicol (Sigma, St Louis, USA). The suspension was vortexed at maximum speed until reach homogeneity, and then incubated for 1 h at home temperature.

2.3. Estimation of the entrapment efficiency

Following the steps in previous section, the non-entrapped content was separated from liposome-entrapped content by centrifugation (20,000*g*, 90 min, 4 °C) (Frézard et al., 2011). Free chloramphenicol was dosed in the supernatant by spectrophotometric UV readings (CPS 240-A, Shimadzu) at 278 nm (Rimawi and Kharoaf, 2011). Results were used to calculate the entrapment efficiency using a calibration curve, considering the total amount of drug used for preparation of liposomes (Rushmi et al., 2017).

2.4. Determination of liposomes size

The liposomes obtained after the previous steps were ressuspended in Mili-Q water and pre-extruded five times across a polycarbonate membrane of 450 nm, and then, extruded ten times in 200 or 100 nm polycarbonate membranes (Mini-extruder System, Avanti Polar Lipids, Canada), and were then kept at 4 °C. The size of the liposomes and the polydispersion index (PDI) were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern). A total of 10 readings with five repeats each were obtained for each formulation.

2.5. Biofilm formation assay

Strains were cultured in nutrient broth (Difco, USA) at 37 °C overnight. Following, cultures were centrifuged, washed and adjusted to reach 0.5 Mac Farland scale turbidity in nutrient broth. Aliquots of 200 μL of the cultures were dispensed in untreated sterile 96-well flat bottom polystyrene plates (Sarsted, Germany), using fresh broth as negative control. Plates were then incubated in humidified conditions at 37 °C overnight. Next, the wells were aspirated and washed three times with 100 μL of sterile saline (0.85%). A total of 190 μL of sterile saline was added to the biofilms, and 10 μL of resazurin (0.1 g/L) was pippeted in each well in order to assess biofilm viability. Plates were incubated at 37 °C for 10 min, and resazurin (blue) was metabolized to resofurin (pink). Fluorescence of resofurin was measured (λ_{ex}570 nm and λ_{em}590 - nm) by a microplate reader (Varioskan, ThermoFisher, USA) using

sterile saline and resazurin as a control. Readings are expressed as arbitrary fluorescence units (AFU). This experiment was conducted in triplicate.

2.6. Assessment of the effects of free and liposome-entrapped chloramphenicol against biofilms

Biofilms were prepared and washed as described in the previous section. A stock solution of chloramphenicol in its free from was prepared in warm DMSO (Merck, Germany) at the concentration of 4 mg/mL. Serial dilutions were then performed in PBS, in order to reach concentrations ranging from 2 mg/mL to 15.62 μg/mL. After the biofilms were washed, aliquots of 200 μL of each concentration were added to the wells in triplicate. Plates were then incubated at 37 °C overnight. After, the supernatant was aspired, plates were washed with sterile saline, resazurin was added and plates were read as mentioned in the previous section. Chloramphenicol solution and resazurin were used as a control. This assay was performed in triplicate.

Tests with liposome-entrapped chloramphenicol were conducted in triplicate as follows: a total of 100 μL from the liposomes suspensions were added to the wells in triplicate. Plates were then incubated at 37 °C. Following, the supernatant was aspired, plates were washed with sterile saline, and resazurin was added to the plates. The effect of (100 and 200 nm estimated sizes) empty liposomes and chloramphenicol-loaded liposomes against biofilms was monitored hourly for 6 h, then at 8 h, and then overnight. As controls, we used resazurin added to empty liposomes and to liposome-entrapped chloramphenicol. Fluorimetric readings of the plates (λ_{ex}570 nm and λ_{em}590 nm) with free and liposome-entrapped chloramphenicol against biofilms were taken (Varioskan, ThermoFisher, USA) and compared to untreated biofilms.

2.7. Statistics

Homocedasticity was assessed by Bartlett's test. Normality of data was assessed through Shapiro-Wilk test. Differences between AFU obtained from free and liposome-entrapped chloramphenicol (100 and 200 nm estimated sizes), and untreated biofilms were analyzed by ANOVA, followed by Tukey test. Pearson's correlation was used to assess the strength of association between the performance of the liposomes and their sizes. Significance level was set as $P < 0.05$. All analyses were processed in Minitab 17 statistical package for Windows.

3. Results

3.1. Lack of antibiofilm activity of free chloramphenicol and empty liposomes

In its free from, chloramphenicol was not able to eradicate the biofilms of the tested isolates in none of the tested concentrations, and when compared to the untreated biofilms, viability differences were not significant ($p > 0.05$) (Table 2).

Empty liposomes were used as control. As expected, they presented no activity against any of the tested biofilms (data not shown).

3.2. Characterization and antibiofilm effects of liposome-entrapped chloramphenicol

Liposome entrapment efficiency was of 50.6% for 100 nm vesicles, and of 33.3% for 200 nm vesicles. Both presented low PDI values, and in average, reduction of biofilms viability ranged from

Table 2
Viability of chloramphenicol-treated and untreated microbial biofilms.[a] Data is presented as AFU readings averages ± SD in parentheses.

Isolate Number	Control	Chloramphenicol treatment (non-entrapped in liposomes)											
		2 mg/mL		1 mg/mL		500 μg/mL		250 μg/mL		125 μg/mL		62.5 μg/mL		30.25 μg/mL		15.62 μg/mL	
8	2529.16 (45.2)	1154.20	109.02	2473.70	230.74	2560.50	230.74	2489.40	219.66	2484.90	222.81	2448.10	248.86	2479.30	226.76	2249.20	389.46
20	2433.64 (52.82)	1619.30	13.66	2636.60	115.51	2774.60	17.99	2685.50	80.95	2681.00	84.12	2357.30	313.02	2666.20	94.58	2574.40	159.51
40	2425.88 (56.26)	1595.20	420.84	2602.60	139.57	2681.70	83.66	2625.10	123.64	2567.50	164.37	2323.70	336.78	2671.30	91.02	2567.60	164.35
63	2418.01 (62.63)	1186.10	131.59	2346.60	320.60	2478.50	227.33	2391.30	288.99	2404.70	279.49	2272.90	372.72	2381.90	295.66	2235.50	399.13
94	2289.42 (73.21)	1338.70	239.50	2863.00	44.52	2202.10	422.78	2854.60	38.61	2482.80	224.31	2247.00	391.00	2408.00	277.16	2474.50	230.16
110	2516.89 (22.47)	1310.70	219.68	2325.80	335.30	2535.40	187.07	2502.00	210.75	2493.40	216.80	2198.20	425.55	2402.20	281.30	2238.60	396.94
116	2422.67 (28.62)	1466.00	329.54	2454.30	244.46	2486.80	221.48	2489.90	219.27	2488.50	220.24	2491.90	217.85	2482.00	224.87	2487.70	220.83
125	2422.08 (51.76)	1466.90	330.16	2407.90	277.23	2604.80	138.06	2667.60	93.64	2391.60	288.77	2358.10	312.47	2381.30	296.09	2638.20	114.40
152	2410.69 (63.02)	1556.20	393.26	2739.30	42.94	2646.70	108.43	2426.70	263.95	2163.70	449.93	2557.30	171.60	2502.10	210.65	2488.90	219.95
157	2286.90 (95.36)	1477.10	337.35	2421.80	267.46	2269.90	374.84	2177.80	439.96	2091.20	501.17	2064.50	520.05	2472.60	231.52	2470.60	232.89

[a] No statistical significance was observed in this test. Isolates: 8: *Elizabethkingia meningoseptica*; 20: *Acinetobacter junii*; 40: *Kluyvera cryocrescens*; 63: *Exiguobacterium mexicanum*; 94: *Stenotrophomonas maltophilia*; 110: *Bacillus* sp.; 116: *Lysinibacillus sphaericus*; 125: *Acinetobacter haemolyticus*; 152: *Enterobacter hormaechei*; 157: *Bacillus* sp. Cereus Group.

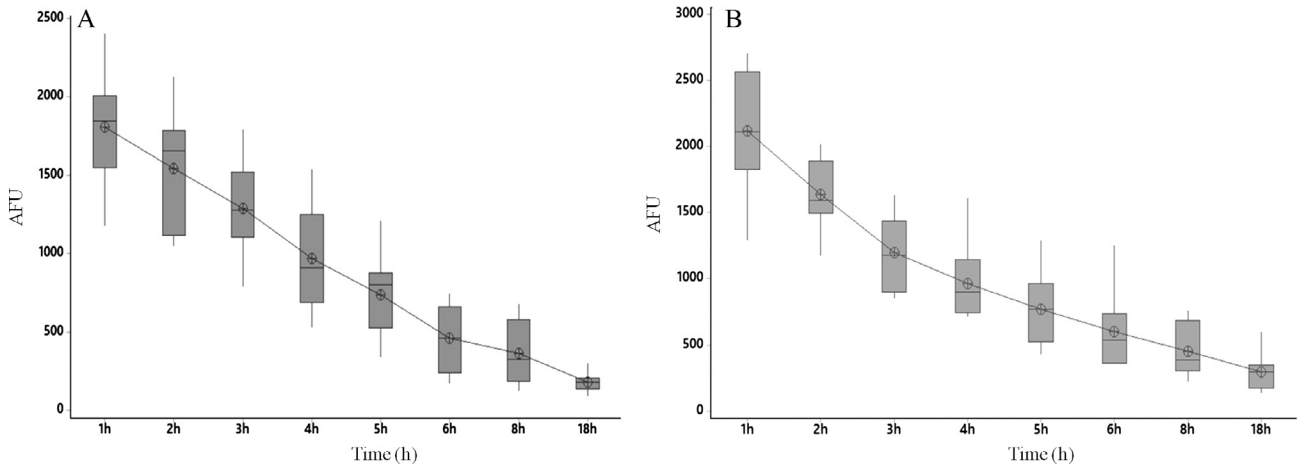

Fig.1. Box-plot analyses of biofilms viability reduction by exposure to 100 nm (A) and 200 nm (B) extruded vesicles. Data is presented as averages of AFU readings of all isolates for each hour of exposure to the liposomes.

Table 3
Liposome entrapped chloramphenicol (100 nm) against biofilms. Fluorescence results are expressed as AFU ± SD. Readings were taken from 1–8 h, and 18 h.

Isolate Number	1 h	2 h	3 h	4 h	5 h	6 h	8 h	18 h
8	2483 (115.6)	2092.6 (142.1)	1305 (107.1)	824.5 (163.5)	550.7 (204)	338.4 (132)	268.3 (106.6)	190 (137.6)
20	2169.9 (194)	1671.4 (102.8)	1350.3 (171.2)	1139.3 (150.4)	1041.3 (132.6)	691.3 (75.2)	521.5 (148.6)	190.2 (127)
40	1699.7 (133.2)	1116.6 (139.5)	875.2 (98.1)	883.8 (151.7)	657.2 (106.5)	479.7 (165.5)	360.4 (141.4)	149.3 (132.1)
63	1891.9 (131.5)	1540.1 !30.4)	1228.4 (140.3)	785.7 (130.4)	934.2 (180.6)	559.2 (112.5)	480.1 (120)	203.4 (59.5)
94	1620.8 (79.2)	1296.8 (146.8)	1567.3 (117.5)	978.2 (109.8)	690.4 (143.4)	494.4 (50.2)	309.6 (127.2)	192.4 (105)
110	1880.8 (169.4)	1887.4 (141.4)	1654 (121.7)	1367.6 (142)	877.1 (120)	345.5 (141)	224.5 (106.8)	177 (115.8)
116	1520.5 (150)	1273.3 (163.1)	1059.6 (139.2)	929.1 (180.3)	701.3 (84.6)	510.4 (71)	279.1 (151)	144.8 (104.7)
125	1203 (135.1)	1147.1 (133.7)	875.3 (118.1)	727.4 (179.3)	550.7 (194)	361 (109)	297.7 (155)	137.4 (140.2)
152	2194.6 (183.6)	1944.5 (124.5)	1322.5 (112.3)	1242.8 (119)	913.6 (102)	647.2 (121.75)	487.6 (140.6)	291.2 (149)
157	1785.3 (149.4)	1433.7 (189.2)	982.9 (187.5)	1305 (107)	944.3 (94.2)	610 (182)	531.6 (210)	247.3 (70.4)

Isolates: 8: *Elizabethkingia meningoseptica;* 20: *Acinetobacter junii;* 40: *Kluyvera cryocrescens;* 63: *Exiguobacterium mexicanum;* 94: *Stenotrophomonas maltophilia;* 110: *Bacillus* sp.; 116: *Lysinibacillus sphaericus;* 125: *Acinetobacter haemolyticus;* 152: *Enterobacter hormaechei;* 157: *Bacillus* sp. Cereus Group.

Table 4
Liposome entrapped chloramphenicol (200 nm) against biofilms. Fluorescence results are expressed as AFU ± SD. Readings were taken from 1–8 h, and 18 h.

Isolate Number	1 h	2 h	3 h	4 h	5 h	6 h	8 h	18 h
8	2415.76 (21.8)	1977.27 (151.69)	1580.35 (159.41)	1252.62 (133.66)	626.33 (264.44)	533.88 (165.75)	417.11 (101.4)	429.5 (118.56)
20	2685.94 (73.9)	1711.865 (154.40)	1386.4 (145.9)	1219.38 (141.84)	1013.7 (275.15)	555.3 (173.06)	336.3 (36.75)	353.37 (105.21)
40	2306.565 (54.8)	1732.19 (181.49)	1475.35 (121.53)	1278.94 (259.51)	1061.6 (175.4)	975.39 (191.04)	659.5 (141.42)	358.25 (55.52)
63	2084.115 (78.54)	1984.91 (143.11)	1241.84 (120.38)	1027.36 (170.26)	847.8 (183)	720.36 (186.23)	588.62 (141.52)	351.7 (88.41)
94	1827.445 (37.4)	1502.08 (188.72)	1398.84 (157.27)	804.79 (101.92)	575.4 (122.8)	484.181 (95.46)	329.5 (22.86)	251.55 (97.61)
110	2196.425 (51.8)	1728.35 (143.6)	870.232 (124.03)	748.38 (233.55)	527.61 (130.25)	388.44 (37.08)	331.51 (20)	296.13 (24.32)
116	1819.615 (90.08)	1430.18 (177.82)	1246.84 (117.88)	1115.58 (144.93)	837.61 (148.35)	556.234 (109.33)	533.494 (112.61)	437.23 (123.87)
125	1677.605 (53.89)	1178.15 (156.31)	958.85 (198.08)	838.8 (170.85)	703.55 (122.86)	558.26 (52.70)	217.82 (99.13)	203.6 (79.02)
152	2315.765 (54.89)	1593.8 (135.87)	1173.15 (176.47)	916.68 (282.84)	739.5 (178.43)	657.13 (143.95)	526.4 (123.02)	292.76 (74.81)
157	1972.83 (35.92)	1626.15 (175.62)	1079.81 (181.61)	804.92 (165.28)	642.64 (149.54)	523.024 (127.42)	294.7385 (95.43)	141.71 (72.62)

SD: Standard deviation. Isolates: 8: *Elizabethkingia meningoseptica;* 20: *Acinetobacter junii;* 40: *Kluyvera cryocrescens;* 63: *Exiguobacterium mexicanum;* 94: *Stenotrophomonas maltophilia;* 110: *Bacillus* sp.; 116: *Lysinibacillus sphaericus;* 125: *Acinetobacter haemolyticus;* 152: *Enterobacter hormaechei;* 157: *Bacillus* sp. Cereus Group.

1804 to 181 AFU (Fig. 1a) for 100 nm extruded vesicles (average size = 131 nm, PDI = 0.158), and from 2139 to 283 AFU (Fig. 1b) for 200 nm vesicles (average size = 182 nm, PDI = 0.195). A significant (although not perfect) negative correlation was observed regarding the association of liposome sizes and reduction of biofilms viability ($r^2 = -0.810$, $p < 0.05$). Furthermore, 100 nm liposomes were more effective than 200 nm liposomes regarding the reduction of biofilms viability after the overnight treatment ($p < 0.01$).

Liposomes were monitored hourly and overnight for their antibiofilm effect (Tables 3 and 4). For 100 nm (estimated size) liposomes, a significant difference was observed between the treatment in all tested times ($p < 0.01$). However, no difference was observed when comparing 3 to 4 h exposure, 6 and 8 h exposure, and 8 and 18 h exposure. However, we observed a tendency on the 18 h exposure to present the best results. For 200 nm liposomes, a significant difference was observed between the treatment in all tested times ($p < 0.01$). However, no difference was observed when comparing 6 h to 8 h exposure, and 8 to 18 h exposure. We also observed a tendency on the 18 h exposure to present the best results on vesicles of 200 nm (estimated size).

4. Discussion

Liposomes can target biofilms by direct attachment, allowing the release of the entrapped substances in the surrounding area of the microorganisms. The permeability of entrapped molecules across the lipid bilayers of the liposomes is the main point in the development of formulations in which controlled release is expected, thus, we have chosen the 100 and 200 nm sizes to improve the efficacy of the vesicles. Traditional methods such as film hydration provide a low yield of entrapment (Gregoriadis, 2007; Frézard et al., 2011), therefore, we used the dehydration-rehydration method, which provided a better entrapment efficiency of chloramphenicol. The entrapment efficiency was lower for 200 nm liposomes when compared to 100 nm extruded vesicles. Also, 200 nm extruded liposomes diffusion in the biofilms can be somehow lower when compared to 100 nm extruded vesicles, due to size influence. Taken together, these observations can help to explain our results regarding their efficiency on the control of the bacterial biofilms.

Conventional interventions with antimicrobials to eradicate biofilms are frequently ineffective. Production of EPS in biofilms is considered the most common mechanism of enhanced antimicrobial resistance (Epstein et al., 2011; Jiang et al., 2011; Ghosh et al., 2015). The EPS can both adsorb or react with antimicrobial molecules, decreasing their penetration in the biofilm, and thus, the interaction with the microorganisms (Jiang et al., 2011; Houry et al., 2012; Kovács et al., 2012). Horizontal resistance gene transferences amidst bacteria might happen in biofilms, as well (Hall-Stoodley et al., 2004).

Different studies described that varied strategies for water treatment for tower supply may influence antimicrobial resistance. For instance, bacteria can be exposed to sub-inhibitory concentrations of antimicrobial compounds, which are often not retained in filtration systems (Xi et al., 2009). Such exposure not only increases the expression of virulence factors associated to antimicrobial resistance mechanisms to antimicrobials, but also triggers biofilm formation (Xi et al., 2009; Liu et al., 2011). This becomes especially important when considering bacteria in cooling towers: although we explored here bacterial species isolated from environmental sources, they are associated to several diseases of clinical relevance such as meningitis, intra-abdominal infections, bacteremia and pneumonia, and may infect individuals nearby the towers by dissemination of the stream formed as the towers work (Liu et al., 2011).

The exploration of liposomes for antimicrobial purposes, especially for inhibition of biofilms, is very recent, and scarce published data is available. Most of the research is still focusing planktonic bacteria, although they live mostly as biofilms in natural aquatic or terrestrial environments, or even in living organisms (Hall-Stoodley et al., 2004). The aims of using liposome-entrapped antimicrobial substances replacing their free form include their use in more wide ranges of pH, temperature, and mainly, to obtain the desired effect with a lower concentration of the entrapped substance, when compared to its free (or non-entrapped) form (Santos and Dias-Souza, 2016). Loading liposomes with drugs can increase their effectiveness and overcome resistance mechanisms that involve drug degradation, for instance, due to enzymatic hydrolysis (Gregoriadis, 2007; Santos and Dias-Souza, 2016).

Studies on liposomes loaded with chloramphenicol remain scarce, what makes our data even more relevant. Pavelic et al. (2004) explored drug during an extended period of time. Liposomes were prepared by two different methods, and tested for *in vitro* simulating human vaginal conditions. They also used a proliposome such as Phosal 75 AS; however, the entrapment efficiency was lower than in the present study. Moreover, the liposome size used in their study is considerably higher than the sizes assessed in this study. Although the authors have not tested the efficiency of the formulations against bacterial strains, the release behavior of the formulation indicated that it could be used for the treatment of bacterial vaginosis.

Mahmoud et al. (2008) loaded dimyristoylphosphatidylcholine liposomes with chloramphenicol. The vesicles were prepared in different ways and their efficiency against *Staphylococcus aureus* was investigated using an agar plate methodology. Interestingly, three out of the six tested formulations were effective, but no statistical difference was observed when their antimicrobial activity was compared.

More recently, Ingebrigtsen et al. (2016) developed chloramphenicol liposomes by dual asymmetric centrifugation as phospholipid gels. Vesicles diameter ranged from 200 to 300 nm and an entrapment efficiency of near 50% was reached. Although the authors have not tested the efficiency of the formulations against bacterial isolates, the formulations were considered adequate for the treatment of skin infections, considering the pharmaceutical parameters assessed in their study.

5. Conclusion

Two sizes of liposomes were tested for chloramphenicol entrapment, and their size was correlated to the antibiofilm effect, possibly due to influences on interactions of the drug with bacteria. This strategy offers advantages such as prolonged activity due to sustained release of the drug and increased adaptability to the conditions on the cooling tower environment. Using liposome-entrapped antimicrobial molecules can be advantageous over free molecules to preclude biofilm formation. Our data open doors for exploring biocides entrapped in liposomes on the real scale cooling towers from where the bacteria were isolated.

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