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Preparation and Characterization of DMPC Liposomal-Gentamicin; Antibacterial Time-Kill Study on *Escherichia coli* ATCC 8739

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Abstract

Escherichia coli are gram –ve bacteria which can cause so many severe infections if not properly treated. A lot of aminoglycosides such as gentamicin are used in treating common gram -ve bacteria. However, it might become inactive if subjected to a resistant species of bacteria. Liposomes are used to encapsulate the drugs and deliver them efficiently to the desired tissues. In this study, liposomal-gentamicin antibacterial activity was tested against *E.coli* ATCC 8739. It proved its efficacy compared to the free antibiotic at the same concentration. The obtained results indicated an average size of liposomal-gentamicin equals to 69.72 ± 0.08 nm and a zeta potential equals to -3.89 ± 0.01 mV. FTIR spectroscopy indicated that most of the interactions between the antibiotic and the liposomal bilayer were in the phospholipid's fatty acid chain and phosphate head group regions. As a conclusion, the liposomal-gentamicin could be used as a better drug delivery system and treatment if compared with the free antibiotic by itself.

Keywords: *Escherichia coli*, liposomes, liposomal-gentamicin, drug delivery.

1. Introduction

Escherichia coli are the most commonly used experimental model organisms in laboratories. Most *E. coli* strains do not usually cause severe diseases. However, contagious strains can still cause many infections including urinary tract infections, gastroenteritis, meningitis and pneumonia [1]. The susceptibility of host individuals to these infections is usually affected by malnutrition, pre-existing disease, immunosuppressive drug therapy or any genetic factors.

Gentamicin is a broad - spectrum antibiotic. It is administrated in many serious bacterial infections that require hospitalization. However, some of the main problems associated with gentamicin include drug resistant pathogens carrying aminoglycoside-modifying enzymes as well as severe renal and neuromuscular toxicity [2]. As like all aminoglycosides, gentamicin is freely soluble in water, and after intravenous or intramuscular injection, the majority of the free drug remains in extracellular locations. Thus, it might be active in-vitro, while it often becomes inactive against intracellular bacteria in-vivo due to its poor penetration into those cells in the appropriate doses or even its full inactivation by the lysosomal enzymes.

Therefore, the development of novel antibacterial formulations or drug-carriers such as liposomes that are capable of intracellular drug delivery improves therapy against infections that are currently considered difficult to treat [3]. Liposomes are small spherical envelopes that mimic the cell membrane and consist of one or more lipid bilayers. It can be fused or engulfed by the plasma membranes of bacteria. These modern liposomal-antibiotics reduce the drug toxicity while enhancing its efficiency [4, 5]. In particular, liposomal-gentamicin showed an enhanced activity against intracellular and extracellular Gram-negative bacteria as the *E. coli* [6].

In this study, the antibacterial activity of liposomal-gentamicin was compared with the free antibiotic on identified isolate of *E. coli*. Liposomal size and zeta potential were also measured. FTIR spectroscopy was conducted to study the conformational changes induced into the liposomal bilayer after the incorporation of gentamicin. Results were positive and showed the antibacterial efficacy of liposomal-gentamicin over the drug in its free state.

2. Materials and Methods

2.1 Chemical products

1, 2-dimyrisitoyl-sn-glycero-3-phosphocoline (DMPC) was obtained from Avanti Polar Lipids Inc. as a dry powder of scientific grade purity ≥99%. Gentamicin was purchased from Memphis Co. for Pharmaceuticals and Chemical Ind.

2.2 Bacterial cultures

Escherichia coli ATCC 8739 isolated from feces, was kindly gifted as actively growing cultures from the Microbiological Resources Centre, Cairo MIRCEN. Bacteria was cultured on Mueller-Hinton agar and Mueller-Hinton broth, nutrient agar, 0.5 McFarland Turbidity Standard (BBL ™, Becton, Dickinson company, USA).

2.3 Preparation of liposomes

Liposomes were prepared according to the ethanol injection method [7]. They were prepared in a phospholipid: antibiotic ratio of 10:1. Initially, 10 mg/ml stock solution of DMPC was prepared in ethanol. Then a stock solution of gentamicin was prepared in Milli-Q® Gradient A10® water filtered with a Millipak® Express 20, 0.22 µm filter. 1 ml of gentamicin solution was stirred in a flat bottomed glass vial with a 0.5 cm Teflon-coated bar in a 30°C water bath on a magnetic hot stirrer plate. DMPC solution was then quickly injected with a fine tip under the surface of the heated antibiotic solution. Mixture was kept stirring at a moderate speed for 60 mins then sonicated for 15 mins at 5°C. After that, samples were stored at 5°C.

2.4 Physical characterization of liposomes

Dynamic light scattering technique was used to measure the size distributions, the polydispersity index (PDI), and the zeta potentials of the samples. Results were obtained 3 times then averaged using the Malvern Zetasizer Nano ZS in the Institute of Complex Systems ICS7 in Forchungszentrum Jülich, Germany.

2.5 Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy was conducted to study the changes that occurred on the bilayer membrane of the liposomes after incorporating gentamicin. 100 mg of potassium bromide (KBr) was pressed as a fine disc in a mechanical pressor for 5 mins. Then 5 µl of the empty control DMPC liposome and liposomal-gentamicin were evenly spread separately on different KBr discs and put in a vacuum pump to evaporate extra solvents from the disc surface [8]. FTIR records were obtained using a Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer in the research institute of ophthalmology, Giza, Egypt.

2.6 Determination of the susceptibility of E.coli to free gentamicin

Minimum inhibitory concentration and minimum bactericidal concentration (MIC and MBC respectively) of free gentamicin had to be firstly measured. The MIC is the minimum inhibitory concentration of the antibiotic enough to prevent the bacterial growth in-vitro. MIC was obtained by broth microdilution method according to CLSI guidelines using Muller-Hinton broth in 96 wells round bottomed microtiter plates. Bacterial inoculum was standardized to 10^5 CFU/ml. Plates were prepared in triplicates and incubated for 24h at 37°C. The MIC was defined visually as having the lowest growth concentration compared with the corresponding growth controls, equivalent to about >75% inhibition [9]. On the other hand, the MBC is the lowest concentration of antibiotic that resulted in more than 99.9% reduction of the initial inoculum. MBC was obtained by inoculating 10 µl from the wells with concentrations equal to and higher than the MIC on nutrient agar plates and incubating them overnight at 37°C. Results were obtained as the concentrations that totally inhibited the growth of bacterial colonies.

2.7 Time-Kill study

A bacterial inoculum standardized to 10^5 CFU/ml (i.e. CFU: colony forming unit) was used in the time–kill experiment. Micro-well plates containing Mueller–Hinton bacterial broth cultures with either free or liposomal-gentamicin at the MIC concentration vs. *E.coli* bacterial controls were incubated at 37°C for 1, 2, 3, 4 and 24h. At the end of each time period, 10µL of each well was evenly spread on Mueller–Hinton agar plates. The CFU at different time points were counted after 18h of incubation [10]. Plates with 30-250 colonies were used for the CFU counts.

3. Results and Discussion

3.1 Characteristics of liposomes

The average size of the empty control DMPC liposomes prepared in this study was 63.48 ± 2.39 nm. They had a quite homogeneous size distribution indicated by the polydispersity index (PDI) which was equal to 0.21 ± 0.03 and a slightly negative zeta potential equals to -6.40 ± 0.98 mV.

The introduction of gentamicin into the liposomal preparation in a low concentration equal to the MIC of the free antibiotic had slightly increased the average size of the liposomes and their PDI to be 69.72 ± 0.08 nm and 0.30 ± 0.01 respectively. On the contrary, gentamicin reduced the negativity of the zeta potential of the liposomes to be -3.89 ± 0.01 mV.

3.2 FTIR spectroscopy

Fig. 1 shows the full FTIR spectra of both the empty DMPC liposomes vs. the liposomal-gentamicin. The conformational changes induced by gentamicin into the bilayer were mostly noticeable in 2 main regions; the fatty acid chain region (3000-2800 cm^{-1}) and the phosphate group region (1300-900 cm^{-1}). In the fatty acid chain region there was a broadening in the bandwidth of stretching C-H_2 symmetric band and a narrowing in the stretching C-H_2 asymmetric and C-H_3 asymmetric bands. In the phosphate group region there was a broadening in the P-O_2 asymmetric band and a slight narrowing in the bandwidth of P-O_2 symmetric band. These conformational changes prove that gentamicin was successfully encapsulated in the interior aqueous moiety of the liposomes as well as in its bilayer membrane.

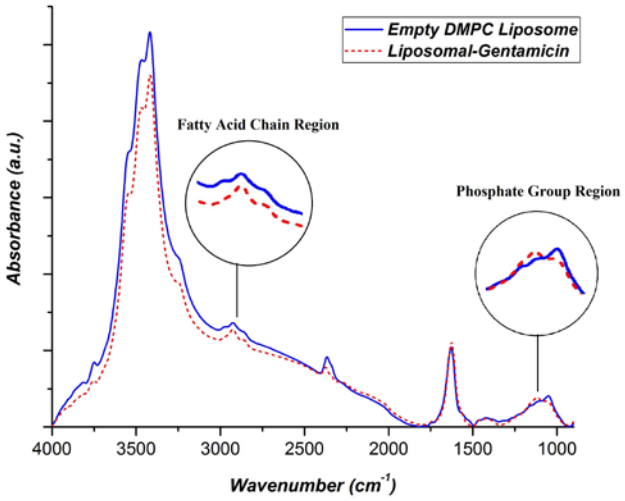

Fig. 1: The full FTIR spectra of the empty DMPC liposomes vs. the liposomal-gentamicin

3.3 MIC and MBC of free gentamicin

The measured MIC of free gentamicin for *E.coli* ATCC 8739 was equal to 0.008 mg/mL, while the MBC was equal to 0.016 mg/mL.

3.4 Time-Kill study

The time–kill study was performed over periods of 1, 2, 3, 4 and 24h with bacteria at the MIC concentration of free and liposomal gentamicin. Fig. 2 shows the obtained time–kill curves and it proves the efficacy of liposomal-gentamicin over free gentamicin. The figure indicates the sharp decrease in the bacterial growth rate after 1hr of treatment in both cases. This was followed by a steady state of growth for the next hour then with another sharp decrease in growth rate. After the 4th hour of treatment, there was a moderate bacterial re-growth again and there was no difference between the two applied treatments.
However, after 24h of treatment, there was a significant difference between free and liposomal-gentamicin. There was a 0.4 log CFU/mL drop between the two treatments and a 0.8 log CFU/mL drop between the bacterial control and the bacteria treated with liposomal-gentamicin. This result confirms the potency of liposomal-gentamicin against *E.coli* ATCC 8739.

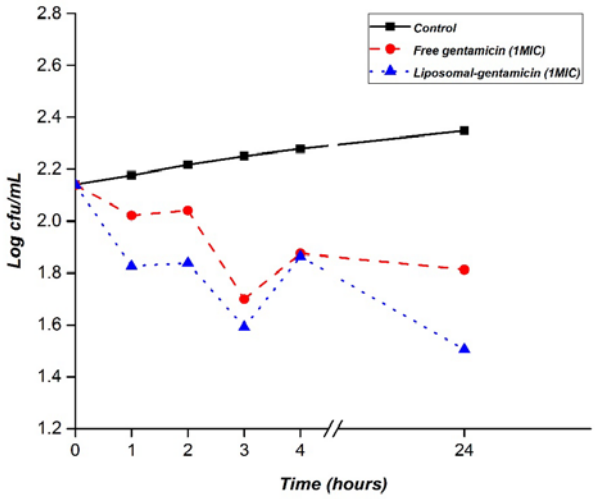

Fig. 2: Time-kill curves of free gentamicin vs. liposomal-gentamicin at 1 MIC for the E.coli ATCC 8739

4. Conclusions

The aim of this study was to prepare and characterize the DMPC liposomal-gentamicin. It was also important to use FTIR to study the conformational changes which were induced into the liposomal bilayer membrane after the addition of gentamicin into the liposomal system. This was followed by testing the antibacterial effect of this formulation on *E.coli* ATCC 8739 with a time–kill study over a period of 24h.
The addition of gentamicin had a slight increase on the average size and the PDI of the prepared liposomes. However, it reduced the negativity of the zeta potential of the liposome. FTIR spectroscopy indicated that gentamicin was equally incorporated into the aqueous moiety and the fatty acid chain region of the DMPC liposome.
The time–kill study furtherly proved the efficacy of liposomal-gentamicin against *E.coli* ATCC 8739 compared to the free gentamicin. Hence, this liposomal formulation could be furtherly used to deliver this antibiotic and others into the bacteria more efficiently.

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References

[1] Todar, K (2007). "Pathogenic *E. coli*". *Online Textbook of Bacteriology*. University of Wisconsin–Madison Department of Bacteriology.
[2] Ali BH (2003). Agents ameliorating or augmenting experimental gentamicin nephrotoxicity: some recent research. Food Chem Toxicol; 41: 1447–52.
[3] Bejjani RA, Jeanny JC, Bochot A, Behar-Cohen F (2003). The use of liposomes as intravitreal drug delivery system. J Fr Ophthalmol; 26:981–5 [in French].
[4] Liu, J., Wang, Z., Li, F., Gao, J., Wang, L., & Huang, G. (2015). Liposomes for systematic delivery of vancomycin hydrochloride to decrease nephrotoxicity: Characterization and evaluation. *asian journal of pharmaceutical sciences*, *10*(3), 212 222.
[5] Cipolla, D., Blanchard, J., & Gonda, I. (2016). Development of liposomal ciprofloxacin to treat lung infections. *Pharmaceutics*, *8*(1), 6.
[6] Lutwyche P, Cordeiro C, Wiseman DJ, et al. (1998). Intracellular delivery and antibacterial activity of gentamicin encapsulated in pH-sensitive liposomes. Antimicrob Agents Chemother; 42:2511–20.
[7] Rongen, H., Bult, A., & Van Bennekom, W. (1997). Liposomes and immunoassays. *Journal of immunological methods*, *204*(2), 105-133.
[8] Gamal, E. M., Aly, E. M., Mahmoud, S. S., Talaat, M. S., & Sallam, A. S. M. (2011). FTIR assessment of the effect of Ginkgo biloba leave extract (EGb 761) on mammalian retina. *Cell biochemistry and biophysics*, *61*(1), 169-177.
[9] Favre, B., Hofbauer, B., Hildering, K.-S., & Ryder, N. S. (2003). Comparison of in vitro activities of 17 antifungal drugs against a panel of 20 dermatophytes by using a microdilution assay. *Journal of clinical microbiology*, *41*(10), 4817- 4819.
[10] Rukholm, G., Mugabe, C., Azghani, A. O., & Omri, A. (2006). Antibacterial activity of liposomal gentamicin against Pseudomonas aeruginosa: a time–kill study. *International journal of antimicrobial agents*, *27*(3), 247-252.

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