Liposomes loaded with rifampicin and tobramycin for anti-*staphylococcus aureus* action

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ABSTRACT

Reducing patient recovery time and potential post surgical complications of warfighters after traumatic injury is of importance for the military. However, a current medical challenge in treating warfighters with traumatic injuries is bacterial infection due to multi-drug resistant bacteria. These infections can delay wound healing and increase the rate of mortality in severe cases of infections. We are developing a nanosystem that preserves functionality and controls delivery of antibiotics for an extended period of time. This system is expected to exert antibacterial activity for a broad spectrum of bacterial species. In this study, we investigated the particle size, entrapment efficiency, and antibacterial activities of two antibiotics, rifampicin and tobramycin, [active against *Staphylococcus aureus* (*S.a*, ATCC 12600) and Methicillin-resistant strain of *S.a* (MRSA, BAA-1720)] in liposomes synthesized by a modified dehydration–rehydration liposome method. Encapsulation efficiency of liposomes decreased with decrease in drug concentration, and was also affected by the type of drug encapsulated. For rifampicin, initial drug loadings of 20 mg, 10 mg, 5 mg and 2.5 mg resulted in entrapment efficiencies of 64.8 ± 17.3%, 49.6 ± 1.9, 39.2 ± 9.7%, and 36.9 ± 5.9%, respectively, and for tobramycin entrapment efficiencies were 26.7 ± 4.7%, 24.3 ± 5.3% and 22.5 ± 3.5% and 19.4 ± 3.7%, respectively. The average particle sizes of liposomes in the mixture was approximately 200-300 nm and 300-500 nm for tobramycin and rifampicin, respectively, and they also varied depending on the amount of drug that was used for liposome synthesis. *In vitro* antibiotic release studies were performed for 7-9 days in phosphate-buffered saline (PBS) at 37°C. The results demonstrated that liposomes released sufficient amount of antibiotics, and antibacterial activities were preserved over this period against *S.a.* Currently, we are investigating a variety of ways to incorporate these liposome systems into various antimicrobial agent delivery systems.

1. INTRODUCTION

Recently, antibiotic delivery to infection sites has been a major part of the treatment process for warfighters with burns and traumatic injury. Traditional methods of systemically delivering antibiotics have the difficulty of controlling exact dosage over a long period of time, and can cause severe side effects that include gastrointestinal upset, liver damage, cartilage damage, white blood cell reduction and neurological problems.

Nanotechnology has been applied to solving the problems associated with traditional drug delivery systems, and can be use for targeted and controlled delivery (1, 2). Liposomes, which are colloidal lipid-bilayer vesicles ranging from a few nanometers to several micrometers in diameter, safely entrap hydrophilic drugs in the center aqueous compartments and hydrophobic drugs in the lipid bilayer. They have been shown to promote local delivery of drugs to cells, and improve its efficacy by protecting drugs from enzymes that can cause degradation (3) .

In this study we have modified a method developed by Mugabe et al. (4) for encapsulating rifampicin and tobramycin in liposomes and tested their efficacy against *Staphylococcus aureus* and other bacteria commonly seen in infections in injured warfighters.

2. MATERIALS AND METHODS

2.1 Materials

Rifampicin, tobramycin, L-α-Phosphatidylcholine (PPC), cholesterol, chloroform, acetone, triton-X100, sucrose, and Phosphate Buffered Saline (PBS) were obtained from Sigma-Aldrich.

2.2 Bacteria

Staphylococcus aureus (*S.a*) 12600, *S. aureus* Methicillin-resistant strain (MRSA) BAA-

1720 (*S.a*-R), *Acinetobacter baumannii* BAA-1605, *Pseudomans aeruginosa* 10145, and *Proteus mirabilis* 4630 were from American Type Culture Collection (ATCC), Rockville, MD). Mueller-Hinton broth was used for overnight culture.

2.3 Preparation of dehydration-rehydration liposomes

A previously published method for making liposomes was modified to encapsulate antibiotics, rifampicin and tobramycin (4,5). Briefly, a 50 µmol of PPC and 25 µmol of cholesterol were dissolved in 1 ml of chloroform in 125 ml round-bottomed flask. The solution was dried to form a lipid film with a rotary evaporator at 50°C under controlled vacuum. The lipid film was flashed with nitrogen gas to eliminate traces of chloroform before hydration. In *Step 1 (hydrate)*, the lipid film was hydrated with 2 ml of sucrose/distilled water (1:1, w/w). The lipid suspension was vortexed for 2 min to form multilamellar vesicles, and then sonicated for 10 minutes in an ultrasonic bath (model 2510, Branson). The resulting mixtures were centrifuged at low speed (400g, 10 min at 4°C) to remove large vesicles. In *step 2 (dehydration-rehydration)*, the suspension of small unilamellar vesicles was mixed with 1 ml (2.5-40 mg/ml) of antibiotic. Tobramycin was dissolved in dH_2O and rifampicin dissolved in acetone, respectively. The mixture was then lyophilized overnight (Freeze Dryer, Labconco). For rehydration, 200 µl of distilled water was added, vortexed, and incubated for 30 min at 50°C. This step was repeated with 200 µl of PBS (pH 7.2). After incubation, 1.6 ml of PBS was added. The mixture was vortexed and incubated for another 30 min at 50°C. Excess unencapsulated drug was removed by washing with PBS three times (18300g for 15 min at 4°C). The encapsulation rate was quantified using an agar diffusion microbiological assay after lipid vesicles were lysed with 0.2% Triton X-100. Triton X-100 did not show inhibitory activity.

2.4 Particle size analysis

The mean diameter of liposomes were determined using a 90 Plus Size Analyzer (Brookhaven Instruments Corporation) and Transmission Emission Microscopy (TEM).

2.5 Encapsulation efficiency of antibiotics into liposomes

Encapsulation efficiency was determined as the percentage of antibiotic incorporated into vesicles relative to total amount of drug in solution and was calculated using the following equation:

$$\text{Encapsulation efficiency} = C_{vesicles}/(C_{vesicles}+C_{sol})$$

Where $C_{vesicles}$ is concentration of the antibiotic entrapped in vesicles and C_{sol} is the total concentration of antibiotic in solution.

2.6 *In vitro* release of drugs from liposomes.

One ml of liposomes loaded with 1 mg antibiotics (tobramycin or rifampicin) was placed in dialysis tubing and dialyzed over 100 ml of PBS buffer at 37°C with stirring. Free antibiotic solutions were used as controls. The 100 µl of PBS solution was taken at 0, 2, 4, 6, 12, 24, 48, 72, 96, 128, 156, 180, 204, 228 hrs. The released antibiotics were quantified using an agar diffusion microbiological assay.

2.7 Quantification of entrapped antibiotics

Concentration of encapsulated antibiotics were determined using an agar diffusion assay using laboratory strains of *S.a*. Briefly, bacterial suspensions were prepared in Trypticase soy broth (TSB). Bacterial density was adjusted to 0.2 at $OD_{620\ nm}$, and the bacterial solution was added into warm (50°C) Muller Hinton agar (2×10^7 organisms/ml). The bacterial agar was then poured into a sterile Petri dish and left to solidify for 1 h at room temperature. Wells of 5 mm diameter were made with a well puncher and filled with 25 µl of sample or standard solutions. The plates were incubated for 18 h at 37°C. The inhibition zones were measured and the average of duplicate measures was used in data analysis. A standard curve was constructed with known concentrations of free antibiotics (Rifampicin, 0.156-10 µg/ml; Tobramycin, 1.56-100 µg/ml) and was used to estimate concentrations of the entrapped antibiotics that were released from the liposomes. The minimum detection limit of the assay for rifampicin and tobramycin were 0.015 and 1.5 µg/ml, respectively.

2.8 Determination of minimum inhibitory concentration

Free antibiotics in solution and antibiotic-loaded liposomes were serially diluted and inoculated onto agar plates with the bacteria of interest (*S.a*, MRSA, *A.b, P.a*, and *P.m*). The minimum inhibitory

concentration needed to form growth inhibition rings were recorded. The further detail methods are described as section 2.7.

2.9 Statistical analysis:

All experiments were repeated at least three times. The data were analyzed by ANOVA and Paired Student's t-test to determine whether the differences between two groups were significant.

3. RESULTS AND DISCUSSION

3.1 Particle size analysis and encapsulation efficiency of antibiotic-loaded liposomes

Table 1 shows that the average particle sizes of liposomes were approximately 300-500 nm and 200-300 nm for rifampicin and tobramycin, respectively. The average size and encapsulation efficiency varied depending on the amount of drug used for liposome formation and the type of antibiotic. A decrease in amount of drug used for loading decreased both encapsulation efficiency and particle size. There is also a direct relationship between particle size and encapsulation efficiency, and this may explain why Tobramycin-loaded liposomes, which have lower encapsulation efficiency, have smaller particles.

Table 1. Particle size and encapsulation efficiency of antibiotic-loaded liposomes

Antibiotics	Concentration (mg/ml)	Encapsultion Efficiency (%)	Particle size (nm)
Rifampicin	20	64.8 ± 17	543.5 ± 26.1
	10	49.6 ± 1.9	533.5 ± 23.3
	5	39.2 ± 9.7	497.0 ± 5.7
	2.5	36.9 ± 5.9	351.5 ± 21.9
Tobramycin	20	26.7 ± 4.7	334.5 ± 65.8
	10	24.3 ± 5.3	311.5 ± 91.2
	5	22.5 ± 3.5	262.0 ± 73.5
	2.5	19.4 ± 3.7	209.0 ± 14.1

3.2 Quantification of entrapped antibiotics

The amounts of antibiotics released over the 7-9 day period were sufficient for inhibiting bacterial growth (data not shown). At day 9, released rifampicin and tobramycin both demonstrated antibacterial activity against *S. aureus.* The amount of antibiotics retained in liposomes in dialysis tubing compared to free antibiotics is shown in Figure 1.

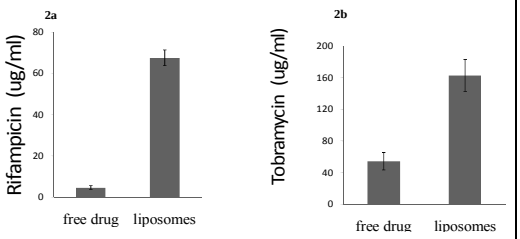
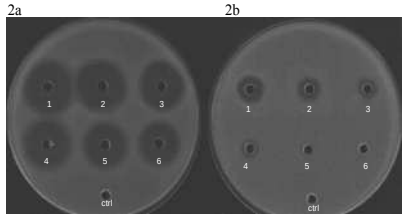


Figure 1. Amount of (1a) rifampicin and (1b) tobramycin retained in loaded liposomes after 9 days. The samples were located in dialysis tubing and dialyzed over 100 ml of PBS buffer at 37 C. The remaining antibiotics inside dialysis tubing were determined after 9days of dialysis. The results showed that more antibiotics were retained in dialysis tubing containing liposomes compared with dialysis tubing containing free drugs ($P < 0.01$ for each pair).

Results from this assay also show that there were no significant differences in the level of inhibition against *S.a* or MRSA by free rifampicin in solution or rifampicin released from liposomes (data not shown). However, tobramycin-loaded liposomes were much more effective against both *S.a* and MRSA than free tobromycin in solution (Figure 2).



1,2,3: Tob-liposoms with 1, 0.5, 0.25 mg/ml 4, 5, 6: Tob-free drug at 1, 0.5, 0.25mg/ml

Figure 2. Comparison of free tobramycin and tobramycin encapsulated-liposomes against (a) *S. aureus* (*Sa*) agar-plate and (b) *S. aureus* - resistent strain *(Sa*-R) agar-plate. As seen in figure 2b, tobramycin loaded–liposomes (well #1, 2, and 3) showed the stronger capacity to inhibit *Sa*-R strain compared to using free tobromycin (well #4, 5 and 6). Ctrl wells were loaded with 0.2% triton x-100.

3.3 Minimum inhibitory concentration

The minimum concentrations needed for inhibitory ring formation (μg/ml) on the agar plate was determine against five selected bacterial strains. As seen in table 2, *S.a* and *S.a*-R (MRSA) strains showed lower sensitivity to tobramycin compared to rifampicin. In contrast, *A.b, P.a, and P.m* showed higher sensitivity to tobramycin compared to rifampicin. Previously, we have shown that an antibiotic cocktail can be a way to inhibit a wide range of bacteria (6). Therefore, we envision a liposomal system comprising of both antibiotics

could provide a system with better inhibitory capacities against the five strains.

Table 2. Antimicrobial activities of rifampicin and tobromycin against *A. Baumannii* (*A.b*), *P. aeruginosa* (*P.a*), *P .mirabilis* (*P.m*), *Staphylococcus aureus* (*S.a*) and MRSA (*S.a*-R)

	Minimum concentration needed for each bacterial strain inhibition ring formation (μg/ml)				
Liposomes	A. b	P. a	P. m	S. a	S. aR
Rifampicin	5.1 ± 0.26	15.4 ± 1.1	15.1 ± 0.54	0.005 ± 0.001	0.005 ± 0.001
Tobramycin	0.67 ± 0.08	0.67 ± 0.07	2.0 ± 0.17	0.2 ± 0.11	>80

A.b = A. baumannii, P.a= aeruginosa. Pm = P .mirabilis
S.a = S. aureus, S.a-R = S. aureus Methicillin-resistant strain.

4. CONCLUSION

Multiple infections increase the degree of difficulty to treat wound infections, and *S. aureus,* Methicillin-resistant *S. aureus*, *A. baumannii*, *P. aeruginosa* and *P. mirabilis* were found in high frequencies in wound infections of military personnel returning from wars in Iraq and Afghanistan. Thus, more effective ways to deliver antibiotics that prolong their activities and reduce antibiotic resistance formation is needed. Liposomes as nanocarriers have been extensively studied because of their biocompatibility and lack of immune system activation or suppression. We have modified a dehydration-rehydration method (4,5) to encapsulate tobramycin, a hydrophilic antibiotic, and rifampicin, a hydrophobic antibiotic in liposomes to develop an antibiotic carrier system that prolongs antibacterial activities and reduces antibiotic resistance. Our present results demonstrate that liposomes released sufficient amount of antibiotics, and antibacterial activities were maintained over 9 days against *S.a* and MRSA. We have also seen that tobramycin is more effective when delivered as a liposomal formulation than as a free antibiotic solution. Currently, we are investigating the use of antibiotic cocktails encapsulated in liposomes as a treatment for multiple infections and for preventing biofilm formation.

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