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Liposomal nanoformulations of rhodamine for targeted photodynamic inactivation of multidrug resistant gram negative bacteria in sewage treatment plant

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ABSTRACT

The antimicrobial photodynamic therapy is an alternative method for killing bacterial cells in view of the rising problem of antibiotic resistance microorganisms. The present study examined the effect of a water soluble photosensitizer, Rhodamine 6G (R6G) in stealth liposomes on multidrug resistant *Pseudomonas aeruginosa* in the presence of visible light. Liposomes were prepared with cholesterol and phospholipids that extracted from hen eggs in a cost effective way and characterized by light microscopy, particle size analyzer, electron microscopy, steady state spectrophotometry and spectrofluorometry. The photoefficacies of R6G in polymer encapsulated liposomes and positively charged liposomes are much higher compared to the free R6G (R6G in water) in terms of singlet oxygen quantum yield. This high potential of producing more reactive oxygen species (ROS) by liposomal nanoformulated R6G leads to efficient photodynamic inactivation of multidrug resistant gram negative bacteria in waste water. Though the singlet oxygen quantum yield of polymer coated liposomal R6G was higher than the cationic liposomal formulation, a faster decrease in bacterial survival was observed for positively charged liposomal R6G treated bacteria due to electrostatic charge interactions. Therefore, it can be concluded that the positively charged liposomal nanoformulations of laser dyes are efficient for photodynamic inactivation of multiple drug resistant gram negative microorganisms.

1. Introduction

Microbial infection remains a major human health hazard, describing one of the primary causes of the mortality with lesions, surgical wounds and so many major clinical conditions [1]. Due to the excessive usage of antibiotics or improper dosage, microbes adopt a large variety of mechanisms to increase their resistance towards the antibiotics [2]. Therefore, it is necessary to develop alternative antimicrobial methods which can prevent the progression of microbial drug resistance. Photodynamic therapy (PDT) is one of the potential approaches for the treatment of microbial infections which was initially developed as a therapeutic modality for cancer treatment.

PDT involves the potential use of a photosensitizer, oxygen and light. Activated photosensitizers in the excited state generate ROS through two different pathways. The transfer of electrons to oxygen or other molecules to form a radical which reacts with molecular oxygen to produce superoxide anion is the primary (Type I) photochemical reaction. This superoxide can react to produce hydrogen peroxide that causes cellular damage. Secondary (Type II) photochemical reaction, the dominant pathway involves the transfer of energy to molecular oxygen to form ROS. The products of these reactions destroy the cells either through apoptosis or necrosis [3]. Both the mechanisms can happen simultaneously and the proportion of these two reactions depends on the type of photosensitizer used, concentration of substrate and presence of oxygen. Selective accumulation of the photosensitizer at the target site and deliverance of a focused light will help to reduce the damage caused to normal cells and thus increasing the efficiency of PDT. Antimicrobial photodynamic therapy (aPDT) has so many advantages over traditional antibiotics therapy. It has ability to inactivate wide range of antibiotic-resistant strains generating ROS which is toxic to almost all microbial cells. Since ROS is involved, microbes would never develop resistance mechanism against PDT therapy. Another significance of PDT is localized phototoxicity that happens only in presence of appropriate wavelength application [4,5]. Therefore, the increasing world-wide antibiotic resistance and new development of photosensitizers have renewed interest in aPDT as a modality for antibiotic treatment [6].

Most intravenously administered photosensitizers for PDT or aPDT are rapidly cleared from the circulation. The hydrophobic nature of most photosensitizers makes them to aggregate in aqueous medium and thus reduces PDT efficacy. This drawback not only prevents their delivery in the body but also causes reduction in singlet oxygen formation by self-quenching effect at the excited state [7]. Therefore, to maintain the monomeric state of photosensitizer, protect them from aqueous

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environment and to enhance the safety and efficacy of photodynamic treatment, various pharmaceutical carriers and delivery systems have been developed. These photosensitizer delivery systems include micelles, liposome, oil-based emulsions and polymeric nanoparticles etc.

One such delivery system is liposomes that came into spotlight because of their high loading capacity, biodegradability and size. In fact, these liposomal vesicles have two advantages in antimicrobial PDT. First, they can accommodate both hydrophobic and hydrophilic photosensitizers and thus can be used to kill microbial cells after irradiation with light. In particular, the hydrophobic photosensitizer encapsulated in the lipid bilayers could facilitate its release to the microbial cell membrane. Second, the synergistic effect of positively charged and highly fluid components of the liposomes optimizes the photosensitizer uptake by microbial cells, as well as the overall phototoxicity [8–10]. In the present work, we report the photosensitised inactivation of a well-known multi drug resistant gram-negative bacterium by using a liposomal nanoformulation of xanthene dye R6G.

2. Materials and methods

2.1. Materials

Ammonium molybdate, ammonium thiocyanate, boric acid, cetrimide agar, chloroform, cholesterol, ferric chloride hexahydrate, methanol, potassium iodide and sodium chloride were supplied by Himedia, India. Polyvinyl alcohol was obtained from Loba Chemie, India. Cetrimonium bromide (CTAB) and pyridine were received from Merck, India. Rhodamine 6G was obtained from Sigma-Aldrich, USA. All chemicals were of analytical grade and used without further purification.

Shimadzu UV-1800 absorption spectrophotometer was used for the absorption studies. Hydrodynamic diameter and zeta potential were determined by dynamic light scattering measurements using Nano - ZS (Malvern) at 25 °C. Optical images were taken in Leica DMIL LED inverted fluorescence microscope under 40× magnification. The morphologies of liposomes were characterized by field emission scanning electron microscopy (Su6600, Hitachi, Japan) using low energy secondary electron and transmission electron microscopy (JEOL JEM 2100 HRTEM), accelerating voltage 200 kV.

2.2. Isolation and confirmation of multi drug resistant Pseudomonas aeruginosa

Multi drug resistant *P. aeruginosa* was isolated from water sample which was collected from sewage treatment plant, Perungudi, Chennai, India. The samples were inoculated in cetrimide agar (selective media for *Pseudomonas*) for growth which was confirmed by oxidase and biochemical assays. Antimicrobial susceptibility testing was carried out for different classes of antibiotics – ßlactams (cefepime, piperacillin-tazobactam, cefotaxime, ticarcillin, ceftazidime, ampicillin), carbapenems (imipenem), aminoglycosides (gentamicin, tobramycin, amikacin), fluoroquinolones (ciprofloxacin) using disk diffusion method following CLSI Guidelines.

2.3. Genomic DNA isolation

Bacterial DNA was isolated by standard phenol-chloroform method. Briefly, 1 ml of lysis buffer (50 mM tris, 100 mM EDTA, 1% SDS, pH 8) and 1 mg/ml of proteinase-K were added to the bacterial culture pellet. It was then incubated at 56 °C for 2 h in the water bath.1 ml of phenol-chloroform (1:1) was added and centrifuged at 12,000 rpm at 4 °C for 15 min. Finally DNA was precipitated using 95% ethanol. DNA pellet was air dried and resuspended in 30 μl nuclease free water.

2.4. PCR amplifications

Conventional PCR was carried out for amplification of AmpC, Bla-CTXM1 and OXA-2 genes of multi drug resistant *P. aeruginosa* isolates in Eppendorf thermocycler for 20 μl reaction volume using gene-specific primers (Table 1). The reaction conditions were adjusted following the protocol of Takara Bio Inc., Japan. The reaction tubes were heated at 94 °C for 5 min followed by 35 cycles of 60 s at 94 °C, 60 s at corresponding annealing temperature and 60 s at 72 °C. The reaction completed with 5 min extension at 72 °C and then cooling at 4 °C for preservation.

2.5. Isolation of phospholipid from egg yolk

Phospholipids were isolated from egg yolk to prepare liposomes. The major lipid components present in hen egg yolk are phospholipids and triglycerides with cholesterol being a minor part. Phospholipids were extracted by our lab standardized method [11]. Briefly, to the egg yolk, 1 M sodium chloride was added to three times of its volume followed by vigorous vortexing. Methanol: chloroform (2:1) solvent mixture, sodium chloride and chloroform were added and centrifuged at 2000 rpm for 5 min. Separations of three phases were observed. Chloroform aids the phospholipids to settle down which was purified by chromatography and concentration was determined by Stewart assay [11]. The assay was performed colorimetrically by forming a complex with ammonium ferrothiocyanate. Volumes of 1 mg/ml phosphatidyl choline between 0.1 and 1 ml were added to test tubes containing 2 ml of ammonium ferrothiocyanate solution and suitable amount of chloroform to make up the final volume to 5 ml. It was then vigorously mixed and left undisturbed for 15 min. The lower chloroform phase was isolated for all standard samples by using a swing rotor centrifuge at 3000 rpm for 5 min and its optical density was read at 488 nm to draw a calibration graph.

2.6. Preparation of liposomes

Liposomes composed of egg phospholipid and cholesterol was formulated by thin film hydration method. 5 mg cholesterol was added to a round bottom flask containing 100 μl extracted phospholipid solution, chloroform/methanol solvent mixture followed by vigorous mixing. The solvents were then evaporated to yield a thin film which was thoroughly vacuum dried overnight to remove residual organic solvent. The dried thin film was dissolved in PBS followed by sonication at 40 kHz for 1 h at room temperature to facilitate the formation of liposomes.

Table 1
Primer information.

S. No.	Primer ID	Primer sequence	Size of amplicons (bp)
1	OXA-2 (FP)	5′-GCC AAA GGC ACG ATA GTT GT -3′	510 bp
	OXA-2 (RP)	5′- GCG TCC GAG TTG ACT GCC GG 3′	
2	BlaCTX-M-1 (FP)	5′ – ACC GTC GGT GAC GAT TTT AG – 3′	823 bp
	BlaCTX-M-1 (RP)	5′- CGT CAC GCT GTT GTT AGG AA – 3′	
3	AmpC (FP)	5′- CGG CTC GGT GAG CAA GAC CTT C 3′	218 bp
	AmpC (RP)	5′- AGT CGC GGA TCT GTG CCT GGT C-3′	