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Antibacterial efficacy of rifampin loaded solid lipid nanoparticles against *Staphylococcus epidermidis* biofilm

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ABSTRACT

Objective: The aim of the present study was to assess the in vitro anti-biofilm activities of solid lipid nanoparticles (SLN) loaded with rifampin against biofilm-producing *Staphylococcus epidermidis*.
Methods: SLN were prepared and characterized for size, zeta potentials and encapsulation efficacy. The morphological and thermal properties of formulation were evaluated by TEM imagining and DSC analysis. The anti-biofilm activity of different formulations was assessed at different incubation times and concentrations by crystal violet (CV) and viable biofilm count methods.
Results: The zeta potentials, particle sizes and encapsulation efficiencies of final formulations were 17 ± 0.7 mV, 101 ± 4.7 nm and approximately 70%; respectively. Rifampin-SLN was able to reduce the biomass of biofilm at time- and concentration-dependent manner. According to biofilm count results, the Rifampin-SLN was more effective for removal of the bacteria with respect to free rifampin.
Conclusion: The results of this study highlight the advantages and efficiency of Rifampin-SLN in biofilm eradication.

1. Introduction

Biofilm is defined as a microbially derived sessile community characterized by cells that can adhere irreversibly to surfaces and interfaces and also to each other. In this structure the cells are embedded in a matrix of extracellular polymeric substances (EPS) and display unique properties which are related to alterations in growth rate or gene transcription. These structural and physiological properties of biofilm resulted in increased resistance of microorganisms to antimicrobial agents such as antibiotics, disinfectants and germicides. It is demonstrated that the living microorganisms in biofilms can be 1000 times more resistance to antibacterial agents than the planktonic counterparts [1]. The defense mechanisms, which lead to high levels of resistance, include the delayed penetration of antimicrobial agents and changes in the metabolism rate of the microorganism [2].The common biofilm producing organisms are: *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Moreover, Gram-positive cocci such as *S. epidermidis* are responsible to produce device related infections [3].

S. epidermidis often susceptible to rifampin, although emergence of rifampin resistance can be problematic. Rifampin can penetrate biofilm formed by *S. epidermidis*, but does not kill biofilm cell [4,5]. On the basis of these observations, enhancing the anti-biofilm activity of rifampin against *S. epidermidis* would be desirable. Recently, various strategies have been mentioned such as combination therapy, application of antibacterial enzyme, and employing nanotechnology [4,6,7].

Nanoparticles such as solid lipid nanoparticles (SLN), liposomes, and nanoemulsions have been developed for carrying antibacterial drugs. These carriers are playing an important role in bacterial eradication. They have been widely used for treatment of resistant infections caused by methicillin-resistant *S. aureus* (MRSA) or biofilms [8–10]. Novel drug delivery systems are able to concentrate antibacterial agents at biofilm interfaces and act against colonizing microorganisms [11].

Lipid based nanoparticles are desirable for drug delivery to

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bacterial biofilms [12]. Several studies show that the encapsulation of antibiotics in these vehicles resulted in better in vitro antibacterial activity against clinically relevant biofilm forming organisms compared to the free antibiotic [8,9].

SLN have the potential to deliver hydrophobic and hydrophilic drugs over long periods of time and also to decrease drug side effects by protecting the environment from direct contact with the drugs. SLN are stable when used as drug delivery systems in vivo, and have pronounced advantages over conventional drug delivery systems [13].

In the present study, SLN preparation was optimized in terms of percentage of surfactants and lipids, process variables such as homogenization time and speed. The lead formulations were characterized for encapsulation efficiency, DSC, TEM, and evaluated for in vitro anti-biofilm activity on biofilm formed by *S. epidermidis*.

2. Materials and methods

2.1. Materials

Glycerylmonostearate (GMS), Precirol® (glycerol palmitostearate) and stearic acid were gifted by Gattefossé (France). Tween 80 was purchased from Sigma–Aldrich (Deisenhofen, Germany). Poloxamer 188 was obtained from Uniqema (Everberg, Belgium). 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma (USA). Rifampin was obtained from Hakim Pharmaceutical Company (Iran). Chloroform, methanol, and trypticase soy broth (TSB) were provided by Merck (Germany). Muller Hinton broth (MHB) was purchased from Hi media (India). All of the original samples were used on arrival. Water used was double-distilled water.

2.2. Methods

2.2.1. Preparation of SLN

Free SLN formulations and Rifampin-SLN were prepared by high-shear homogenization and ultrasound, and high-pressure homogenization (HPH) methods. GMS, precirol, and stearic acid were the lipid phase, and Tween 80 and poloxamer 188 were used as surfactants. The lipid phase was melted by heating at 70 °C. The aqueous phase was prepared by dissolving Tween 80 or poloxamer 188 in double-distilled water to 10 ml, followed by heating to the melting point temperature of the lipid phase. Hot aqueous and molten lipid phases were mixed together and homogenized using a Diax 900 homogenizer (Heidolph, Germany) for 2.5 min at 12,000 rpm, 1 min at 21,000 rpm, and 1 min at 23,000 rpm. The temperature was kept at 5 °C above the melting point of the lipid. The obtained emulsion was ultrasonicated by probe sonicator (Bransonic, USA). The probe sonication was performed over 6 cycles, with 30 s of sonication separated by intervals of 15 s. The final obtained nanoemulsions were cooled to room temperature. SLN formulations were also prepared by the HPH method. Briefly, emulsions were provided as mentioned above and homogenized with T 25 Ultra Turrax (IKA T10, Germany) for 4 min at 11,500 rpm, 2 min at 14,500 rpm, and 2 min at 20,500 rpm. The pre-emulsion was processed at 1000 bar for 5 cycles using a high pressure homogenizer (EmulsiFlex-C5® Avestin Inc., Canada). Samples were then cooled at room temperature to form SLN. Cationic formulations were prepared by adding stearylamine to the lipid phase (Table 1).

Due to the hydrophobic properties of rifampin, for preparation of drug loaded SLN, rifampin in concentration 0.013% w/v (13 mg) was dissolved in the lipid phase. Normal saline 0.9% (NS) was also used as the isotonic agent.

Table 1
Composition of free SLN containing different types of lipids and surfactants. GMS: Glycerylmonostearate, P: Precirol as the solid lipid, SA: Stearylamine as the cationic lipid, SLN: Solid Lipid Nanoparticles and Conc: concentration. The formulations are without Rifampin.

SLN formulation	Lipid		Surfactant	
	Type	Conc (%)	Type	Conc (%)
SLN-GMS	GMS	5	Tween 80	2.5
SLN-Stearic acid	Stearic acid	5	Tween 80	2.5
SLN-P	Precirol	5	Poloxamer 188	2.5
SLN-P-SA1	Precirol	5	Poloxamer 188	2.5
	SA	3		
SLN-P-SA2	Precirol	5	Poloxamer 188	2.5
	SA	1		
SLN-P-SA3	Precirol	5	Poloxamer 188	2.5
	SA	0.3		
SLN-P-SA4	Precirol	3	Poloxamer 188	1.5
	SA	0.3		
SLN-P-SA5	Precirol	1	Poloxamer 188	0.5
	SA	0.3		
SLN-P-SA6	Precirol	1	Poloxamer 188	0.5
	SA	0.3	Tween 80	2

2.2.2. Characterization of SLN

Particle size and zeta potentials. The mean particle sizes, polydispersity index (PDI), and zeta potentials of the SLN formulations were assessed by the Dynamic light scattering (DLS) method (ZetaSizer Nano-ZS; Malvern Instruments Ltd., United Kingdom). All measurements were performed in triplicate [14].

Transmission electron microscopy (TEM). The morphological properties of the SLN formulations were characterized with TEM assessment as previously described. Briefly, the SLN were diluted 50 times with water and placed on a carbon-coated copper grid for 30 s, and the excess water was wiped off using filter paper. Then, 20 μl of uranyl acetate 2% in water was placed on the SLN and after 30 s was wiped off by another filter paper. The grid was dried at room temperature and assessed by TEM (TEM; CEM 902A; Zeiss, Oberkochen, Germany) [15].

Differential scanning calorimetry (DSC). DSC studies were performed using a Mettler DSC 821e (Mettler Toledo, Gieβen, Germany). An empty aluminum pan was used as reference. Samples were scanned from 25 °C to 200 °C at a rate of 5 °C/min under nitrogen atmosphere (20 ml/min). The melting point of SLN formulations was compared to the bulk lipid. Prior to the DSC measurements, the bulk lipids were heated up to 75 °C and cooled to room temperature to imitate the production conditions. Analysis was performed under nitrogen purge [15].

Entrapment efficiency. Encapsulation efficacy (EE) of Rifampin-SLN formulations was determined by a validated HPLC method.

HPLC analysis was performed on an analytical Knauer HPLC system equipped with k-1001 Knauer pump, C18 hypersil column, and k-2600 Knauer UV detector ($\lambda_{max} = 254$ nm). Chromatographic analysis was accomplished by injecting the sample into a C18 hypersil column (4.6 × 150 mm). An isocratic elution was performed with the solvent system using 0.05 M phosphate buffer: acetonitrile (55:45 v/v) at a flow rate of 1 ml/min.

The entrapment efficiency (%) was determined by measuring the concentration of entrapped rifampin after purification. To purify the Rifampin-SLN, 500 μl of the SLN dispersion was transferred to the upper chamber of an ultrafilter (Amicon Ultra-15, PLHK Ultracel-PL Membrane, 100 kDa, Millipore). Amicon tubes were centrifuged at 10,000 rpm for 30 min. The filtrate was analyzed for encapsulated rifampin using a valid HPLC method after suitable