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Formulation and Characterization of Erythromycin–loaded Solid Lipid Nanoparticles

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ABSTRACT
The aim of this work was the preparation and characterization of cationic Solid Lipid Nanoparticles (cSLN) containing erythromycin (ERY), as a potential tool to improve the penetration and accumulation of the antibiotic into bacteria cells. Using a solvent injection method, homogeneous populations of lipid nanoparticles with an average size between 250 and 400 nm and with a polydispersity index lower than 0.3 were obtained; positive zeta potential values were granted by addition of the cationic lipid quaternary ammonium salt didecyldimethylammonium bromide (DDAB). Stability studies showed that the formulations were almost stable for up to one year, depending on the storage temperature. *In vitro* microbiological studies confirmed the antimicrobial activity of the SLN, providing Minimal Inhibitory Concentration (MIC) values for drug loaded cSLN comparable to unencapsulated ERY. In particular, the presence of increasing concentrations of DDAB in the SLN matrix contributed to the antimicrobial activity of the nanocarriers, although elucidation of the mechanism underlying this interaction would deserve supplementary investigation.
Keywords: Drug Delivery; Solid lipid nanoparticles (SLN); cationic SLN; Erythromcyin; *in vitro* activity; Minimum Inhibitory Concentration (MIC); MIC; *in vitro* antibacterial activity.

1. INTRODUCTION

Solid lipid nanoparticles (SLN) are an interesting alternative to traditional colloidal carriers such as emulsions, liposomes and polymeric nanoparticles [1, 2]. They can enhance the benefits of colloidal carriers in terms biocompatibility and biodegradability, as well as physical stability and easy production scaling-up. Lipid nanocarriers are finding large applications in various health-related fields, including pharmaceutical and cosmetic ones.

SLN are nano-sized (colloidal) systems consisting of a mixture of one or more lipids, that are in the solid state at room temperature, water and a layer of stabilizing surfactant(s). The most frequently used lipids are triglycerides, partial glycerides, fatty acids, steroids, and waxes; using these physiological lipids reduces the risk of acute or chronic toxicity phenomena. However, also synthetic lipid components are often chosen, since they are expected to ensure more constant physico-chemical properties and thus a higher reproducibility of the characteristics of the produced nanocarriers [3].

Cationic SLN (cSLN) are innovative nanopharmaceutical formulations, in which one of the solid lipids bears cationic groups and thus a fixed positive charge at physiological conditions. This feature would offer various possibilities for their pharmaceutical application, from targeting properties [4] to a bioadhesive performance at on the eye surface [5], to an easier loading, protection and transfection efficiency of biotech products, such as DNA oligomers and siRNA [6, 7].

In the microbiological field, where lipid nanocarriers are acquiring a potential role as effective nanomedicine tools [8], the positive charge on nanoparticle surface could drive the adhesion to and penetration through negatively charged bacterial membranes [8, 9], thus ensuring an accumulation of the encapsulated antibiotics in the bacteria cells and thus enhancing their activity and/or enlarging their spectrum of activity.

Erythromycin (ERY) is the founder of the macrolide class of antibiotics containing 14 carbon atoms. It is widely used to treat infections of the upper and lower airways, skin and soft tissues caused by susceptible gram-positive bacteria, such as *S. aureus*, pneumococci and streptococci, as well as gram-negative bacteria, including *B. pertussis*, or other organisms such as *M. pneumoniae* and *Rickettsieae* [10, 11].

ERY shows a limited solubility in water and an unpleasant taste, is instable at gastric pH, and possess a low half-life (<1.5 h) and low oral bioavailability (about 35%) [12]. All these features strongly limit its oral administration and prompted many researchers to find alternative application routes, such as topical one, and to develop innovative nanotechnological delivery systems [13]. Sometimes resistance to ERY may be developed by many bacterial strains due to different mechanisms, among which an impaired permeability of the bacterial cells, resulting in a reduction of the drug concentration in the cytoplasm to insufficient (subactive) levels. Topical administration of drug might be beneficial for the treatment of skin diseases because it reduced the systemic side effects and improves the patient compliance, but topical administration of drug is still a challenge in drug delivery due to the difficulties in controlling the fate of drug within the skin [4].

For overcoming the above difficulties lipid nanoparticles have shown a great potential as a carrier for topical administration of active pharmaceutical ingredients. In this study, ERY was loaded in cSLN made of Softisan® 100 (S100), a mixture of coconut hydrogenated triglycerides as the main lipid matrix,

combined with the cationic lipid didecyldimethylammonium bromide (DDAB) at different percentages, to achieve the wished nanoparticle surface charge. The SLN were produced using a hot solvent injection (SI), a method largely used to produce this type of systems [14]. The microbiological activity of the nanocarriers was assessed *in vitro* against a range of gram-positive and gram-negative bacterial strains of pathological significance.

2. EXPERIMENTAL SECTION

2.1. Materials. Softisan 100® was kindly gifted by IOI Oleo GmbH (Hamburg, Germany). DDAB, Tween® 80 (Polysorbate 80) and acetone (purity ≥ 99%) were purchased from Sigma-Aldrich srl (Milan, Italy); Sulfuric acid (d=1.82 g/ml and 98% of H2SO4 in weight) and HPLC-grade water were purchased from Merck (VWR International srl, Milan, Italy).

2.2. SLN preparation method (SI method). An organic phase consisting of 100 mg S100 and DDAB (at the concentrations reported in Table 1) in 2.6 ml of acetone, and containing the antibiotic (5 mg), and an aqueous phase consisting of a solution of Tween 80 at 0.25% (w/v) in 10 ml HPLC-grade water were separately obtained. The two phases were warmed at the same temperature (33-35 °C, approximately corresponding to the melting temperature of S100) and the organic phase was added dropwise through the use of a plastic syringe equipped with a G-23 needle in the water solution, under constant magnetic stirring at about 700 rpm. The mixture was further left stirring at room temperature for 24 h to allow the evaporation of acetone. The dispersion was then sonicated for 20 min at room temperature at 20W (Branson 5002, USA) to enhance the homogeneity of the system and the samples (**E1**-**E4**, Table 1) were finally stored in closed glass vials.

Blank SLN, not containing ERY, were similarly prepared and were labeled as **BL1**, **BL2**, **BL3**, and **BL4** (Table 2). For the sake of in vitro microbiological evaluation, a further composition of unloaded SLN was produced, labeled as **BL5** (Table 2) and containing a higher concentration of DDAB (6 mg/ml; 0.6%, w/v).

2.3. SLN characterization.

2.3.1. Dynamic light scattering. The SLN batches were analyzed using a Nanosizer ZS90 (Malvern Instruments, UK) connected to a PC (running the PCS v1.27 software, Malvern Instruments) to collect and process the data obtained, according to Dynamic Light scattering (DLS) principles. To measure the mean size (Z-ave) and polydispersity index (PDI), an aliquot of each sample was diluted ten-fold with HPLC-grade water and introduced into a glass cuvette and subjected to the action of a laser beam having a wavelength of 633 nm. The reported values are the mean ± SD of 90 measurements (three sets of 10 measurements in triplicate). The Zeta potential (ZP) was determined by Electrophoretic Light Scattering with the same instrument. Up to 100 measurements on each sample were registered at room temperature, to calculate the electrophoretic mobility and, using the Smoluchowski constant (Ka) with a value of 1.5, the corresponding ZP values.

2.3.2. Determination of drug loading. An 1-ml amount of each formulation was transferred into a Whatman Vectaspin® 20 tube, equipped with a 0.45 µm- pore size polypropylene membrane filter (Sigma-Aldrich Chimica srl, Milan, Italy). The tubes were ultracentrifuged (IEC CENTRA MP4R) at 10 °C and 4,400 rpm for 20 min. Aliquots of the supernatant from the bottom of the device, containing the amount of drug that had not been incorporated in the lipid particles, were withdrawn and analyzed for ERY concentration by a spectrofluorimetry technique reported in the literature [15]. The procedure consists in a preliminary acid-induced photo-derivatization of the drug, which shows a very low absorbance in the UV-Vis light spectrum. In brief, 4 ml of the above supernatant or of a control solution of ERY in water (containing from 1 to 10 mg/ml of the drug) were mixed with 3.5 ml of concentrated sulfuric acid and adjusted to 10 ml with HPLC-grade water (the final concentration of sulfuric acid was approximately 6.3 mol/l) [15]. Three-ml aliquots of the acidified solution were placed in quartz tubes that were closed and poured for 5 min in water at 65 ºC. The tubes were then exposed to UV radiation (60 min). Blank solutions (containing water and sulfuric acid) were submitted to the same procedure.

Fluorescence measurements were made on a Perkin-Elmer LS-55 luminescence spectrometer using a 10 nm spectral bandpass, 800 nm/mm scan rate and quartz cuvettes (1 cm optical pathlength), with λ_{exc} = 412 nm and λ_{em} = 465 nm [15].

2.3.3. Stability studies. The SLN samples were closed in glass vials and kept away from direct light at different temperature conditions: in a refrigerator (2-4 °C), at room temperature (25-30 °C), and in an oven at 40 °C. At predetermined intervals, the mean size, PDI and ZP values of the stored samples were measured and compared with those registered within 24 h from their production.

2.3.4. Microbiological assay. The blank and ERY-loaded SLN samples were subjected to a microbiological evaluation against the following bacterial strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Lactobacillus casei* ATCC 393, and *Streptococcus pyogenes* ATCC 700294. Moreover a strain of *Acinetobacter baumannii* (clinical isolated) was also added. To assess the quality control of experiment the activity of formulations was compared to that of free drug. For this reason, selected strains as suggested by CLSI were used to evaluate MIC determination. Because MIC Quality Control Ranges are unavailable for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 those suggested by Fass and Barnishan were used [16].

The microdilution method was performed according to CLSI M100S [17] and ISO-Sensitest broth was used alternatively to CAMHB (Cationic Adjusted Muller Hinton Broth) as suggested by Furneri et al, [18]. Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of erythromycin that inhibited visible bacterial growth after overnight incubation. Briefly, the method involves the preparation of stock solutions at a concentration of 5120 µg/ml, from which serial dilutions in ISO-Sensitest broth in a range between 8 to 0.015 µg/ml were obtained. Then, a bacterial suspension of 0.5 McFarland (concentration was

verified with spectrophotometric reading at an absorbance of 600 nm) was made for each strain and the dilutions in ISO-Sensitest were prepared so as to obtain a final concentration of 103-104 CFU/ml. Each plate was set up by including a positive control for growth (C+) and negative control of sterility (C-). Finally, the microplates were incubated at 37°C overnight under aerobic conditions. To ensure reproducibility, the strains were tested six times and, on a separate day, six more times.

3. RESULTS SECTION

3.1. Preparation and characterization of solid lipid nanoparticles. Four batches of SLN loaded with ERY, along with the corresponding unloaded (blank) systems were produced using a warm solvent injection procedure. The organic phase was selected upon preliminary solubility experiments of the drug and lipids in acetone, ethanol, methanol, and isopropanol, after which acetone was chosen as the more suitable solvent, also considering its low toxicological and eco-toxicological level of risk, in the light of a future scaling-up of the method. Using this technique, homogeneous populations (as confirmed by the PDI values below 0.3) of lipid nanoparticles with a mean size between 250 and 400 nm were produced, without a clear relationship with the composition of the lipid matrix (percentage of DDAB) (Table 4).

The SLN dispersions showed a pH value around 5-5.7; in these conditions, the surface charge measured for the systems was positive, in the order of +60 mV, except for sample **E1** which did not contain the cationic lipid and gave a Zeta potential value of -15.0 mV, typical of triglycerides dispersions. The positive Zeta potential values of the SLN looked to increase linearly with the concentration of DDAB used for their production (**E4**> **E3**> **E2**).

The corresponding blank SLN showed mean sizes, PDI and Zeta potential in an analogous range of values (not shown), suggesting that ERY (free base) did not exert a decisive influence on the agglomeration of the lipids and on the surface charge of the resulting nanoparticles. The latter observation, in particular, would reinforce the hypothesis that the majority of the drug was dispersed within the lipid matrix of the SLN.

Using a photo-derivatization method [15], the concentration of ERY in the SLN samples was determined. It was expressed as percentage encapsulation efficiency (EE%), i.e., the % ratio between the initially added amount of drug and that one found in the lipid matrix, calculated in turn by difference from the drug concentration measured in the supernatant derived from SLN ultracentrifugation. As Table 4 reports, all the samples showed similar EE% values, between 65 and 80%, figures that are not far from those found in the literature for ERY-loaded glyceryl monostearate SLN [19]. These results would suggest that the addition of the cationic lipid exerts a positive, but limited effect on the encapsulation of ERY, as a free base.

3.2. Stability studies. The SLN were stored at different temperatures (4, 25 and 40 °C) and checked monthly for any change in Z-ave, PDI and Zeta potential values (Figs. 1-4). It was observed that samples **E1** and **E4** maintained a constant particle size along 12 months when stored at +4 °C. The **E2** batch showed relatively constant values of size for the first 5 months of storage, while after one year the particle size was almost doubled, as also demonstrated by the raise in the PDI values. The **E3** sample was the less stable under these conditions, with the formation of 500-600 nm aggregates already after 3 months of storage.

In general the PDI of the samples stored at 4 °C showed a tendency to increase with time; it could be explained by a phenomenon of coagulation and aggregation of the lipid particles due to the low temperature. All samples showed a constancy in the Zeta potential (data not shown), with values not far from those measured at the production time (Table 4). This would suggest that the chemical composition of the lipid matrix was maintained, otherwise the loss of DDAB would have progressively reduced the nanoparticle surface charge.

At 25 °C all samples were stable up to 4-5 months, thereafter tended to form larger nanoparticles (500-660 nm). The **E4** SLN remained highly stable in terms of Z-ave along the whole 12-month analysis, accompanied by an excellent size homogeneity, shown by the low and constant PDI values. All the SLN batches showed a certain physical instability when stored at 40 °C, giving fluctuating Z-ave and PDI values along the measuring times. The only exception was the batch **E4**, which showed to be relatively stable, in terms of particle mean size, also at these temperature conditions.

By comparing the behaviour of the four SLN systems, a general conclusion that can be drawn is that the presence of the cationic DDAB positively affected the stability of the colloidal suspensions, most probably by creating a repulsive electrostatic force and thus hindering the approach and coalescence of the lipidic particles.

3.3. Microbiological evaluation. Experimental data confirmed the typical profile of activity for ERY, with variable MIC values against the different bacterial strains (Table 5). A greater antibacterial activity against E. coli, as demonstrated by a significant reduction of the MIC, was registered for ERY-loaded cSLN, compared to the neat drug. It should be however also noticed the value of 8 µg/ml against *P. aeruginosa*, with a MIC reduction compared to free ERY (Table 5).

The other bacterial strains showed a sensitivity to cSLN almost similar to the free drug, although in some cases concentrations were slightly reduced in confirmation of the best antibacterial activity of nano-encapsulated ERY. This was more evident for DDAB-containing cationic nanoparticles, in which the positive surface charge probably allowed a better penetration of the carrier through the bacterial cell wall. Such enhanced antibacterial activity appeared in fact to be proportional with increasing concentrations of DDAB, and was more evident against Gram-negative bacteria, whose cells possess a pouter phospholipid membrane, compared to Gram-positive ones.

To stress up this aspect, blank (unloaded) cSLN prepared with 6 mg/ml of DDAB (batch **BL5**, Table 2) were assessed, showing a clear inhibitory activity on bacterial growth (Table 5). Therefore, albeit direct cytotoxic effect of DDAB towards the bacteria cannot be excluded, since a similar phenomenon has been documented [20], it is presumable that at the concentrations of

DDAB used for the production of cSLN **E2-E4** a real benefit on the antibacterial potency of ERY was, also, obtained from the resulting positive charge.

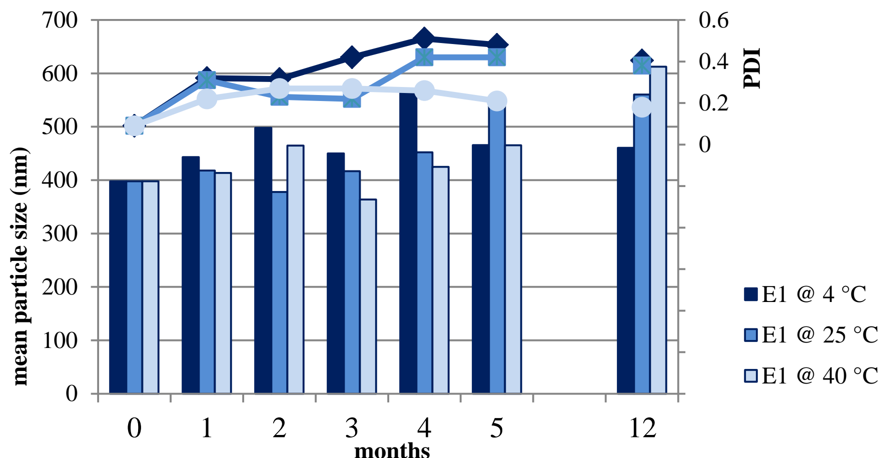

Figure 1. Size (bars) and PDI changes (lines) of SLN batch **E1** at different storage temperatures.

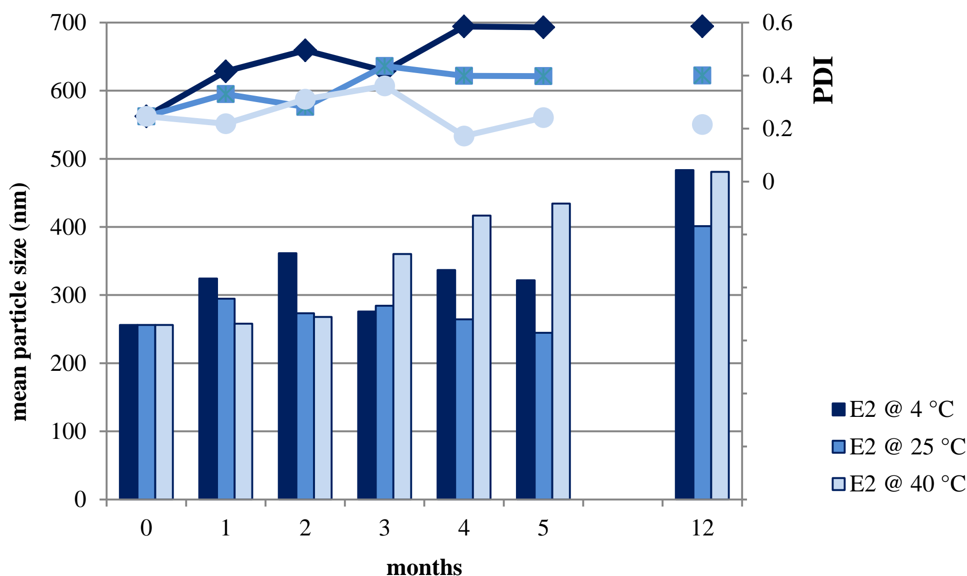

Figure 2. Size (bars) and PDI changes (lines) of SLN batch **E2** at different storage temperatures.

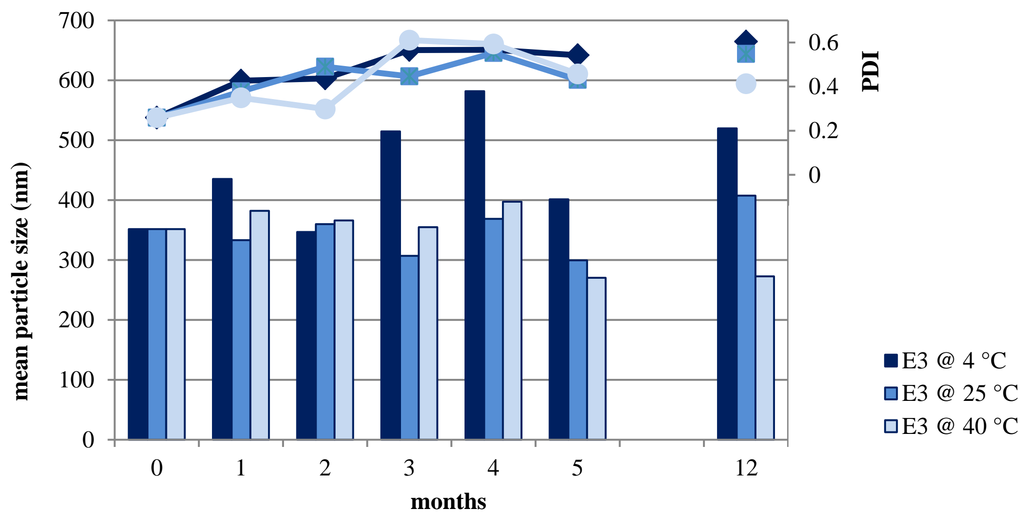

Figure 3. Size (bars) and PDI changes (lines) of SLN batch **E3** at different storage temperatures.

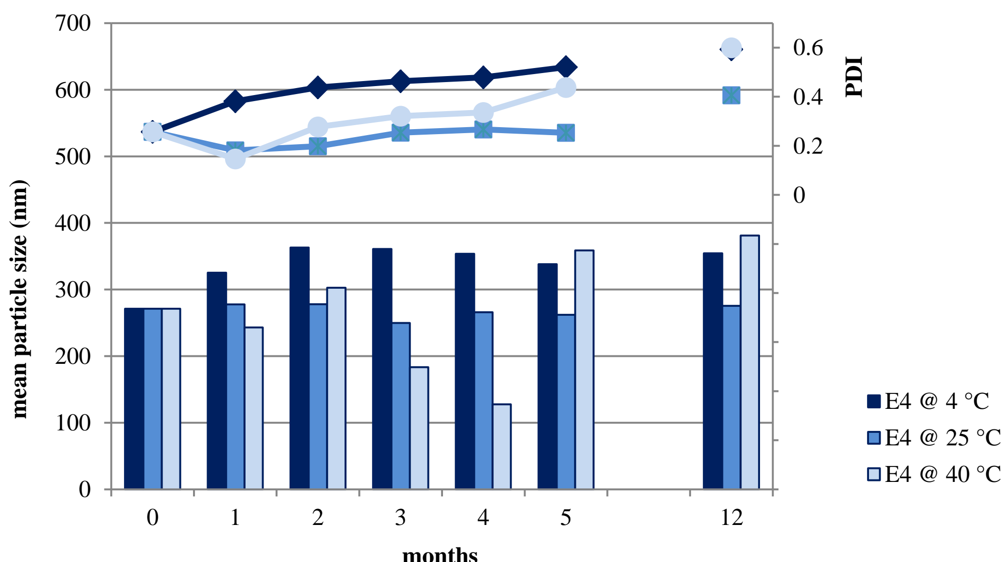

Figure 4. Size (bars) and PDI changes (lines) of SLN batch **E4** at different storage temperatures.

Table 1. Composition of ERY-loaded SLN (concentrations are expressed as %, w/v).

SAMPLE	ERY	Softisan 100	DDAB	Tween 80
E1	0.05	1	0	0.25
E2	0.05	1	0.05	0.25
E3	0.05	1	0.10	0.25
E4	0.05	1	0.15	0.25

Table 2. Composition of blank SLN (%, w/v).

SAMPLE	Softisan100	DDAB	Tween 80
BL1	1	0	0.25
BL2	1	0.05	0.25
BL3	1	0.10	0.25
BL4	1	0.15	0.25
BL5	1	0.60	0.25

Table 3. Quality Control Ranges for ERY (μg/ml) [16-17].

BACTERIAL STRAIN	MIC (μg/ml)
E. coli ATCC 25922	64
P. aeruginosa ATCC 27853	>64
E. faecalis ATCC 29212	0.25-2
S. aureus ATCC 29213	0.12-0.5

Table 4. Mean size (Z-ave), polydispersity index (PDI), ZP and encapsulation efficiency (EE%) values of ERY-loaded SLN.

SAMPLE	Z-ave (nm)	PDI	Zeta potential (mV)	EE%
E1	398.0 ± 2.57	0.100 ± 0.094	-15.0 ± 0.49	63.4 ± 4.44
E2	256.1 ± 6.31	0.246 ± 0.050	+56.0 ± 0.90	73.4 ± 6.12
E3	351.6 ± 5.53	0.259 ± 0.078	+60.1 ± 1.15	74.1 ± 4.98
E4	271.2 ± 4.58	0.277 ± 0.007	+61.2 ± 0.65	77.1 ± 8.02

Table 5. In vitro MIC values (μg/ml) of ERY-loaded SLN compared to the free antibiotic. The **BL5** batch consisted of blank (unloaded) SLN formulated with a higher amount of DDAB (6 mg/ml, cf. Table 2).

BACTERIAL STRAIN	MIC (μg/ml)					
	ERY	E1	E2	E3	E4	BL5
E. coli ATCC 25922	>32	>32	2	1	1	0.156
P. aeruginosa ATCC 27853	>32	>32	>32	16	8	2.5
E. faecalis ATCC 29212	1	1	0.5	0.25	0.25	0.156
S. aureus ATCC 29213	1	1	0.5	0.25	0.25	0.08
L. casei ATCC 393	0.25	0.25	0.25	0.25	0.25	-
S. pyogenes ATCC 700294	0.12	0.25	0.25	0.25	0.25	-

4. CONCLUSIONS

Positively-charged lipid nanoparticles, produced by using a solvent injection method, offer a potential strategy to improve the growth inhibitory activity of ERY on different microorganisms, and in particular against Gram-negative bacteria.

Stability studies demonstrated that these dispersions are fairly stable over time and at different storage conditions, although samples that have preserved a greater size homogeneity were those kept at room temperature. The *in vitro* microbiological assays showed for the sample not containing the cationic lipid DDAB MIC values comparable to free ERY. The cationic cSLN, instead, showed similar or slightly lower MIC values compared to those of the free antibiotic.

Microbiological experiments indicated an antimicrobial activity also for an unloaded cSLN batch: this observation would deserve further experiments, at present in course, to evaluate how the two main components of the SLN, ERY and DDAB, can synergistically interact in affecting the antimicrobial activity of the final formulations [20].

Moreover, it would be interesting to study the intracellular increase of ERY using cSLN, because ERY belongs to the class of lysosomotropic drugs and has thus the peculiarity of concentrating intracellularly [21].

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