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Synergistic interactions between antimicrobial peptides derived from plectasin and lipid nanocapsules containing monolaurin as a cosurfactant against *Staphylococcus aureus*

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Abstract: Development of effective antibacterial agents for the treatment of infections caused by Gram-positive bacteria resistant to existing antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA), is an area of intensive research. In this work, the antibacterial efficacy of two antimicrobial peptides derived from plectasin, AP114 and AP138, used alone and in combination with monolaurin-lipid nanocapsules (ML-LNCs) was evaluated. Several interesting findings emerged from the present study. First, ML-LNCs and both plectasin derivatives showed potent activity against all 14 tested strains of *S. aureus*, independent of their resistance phenotype. Both peptides displayed a considerable adsorption (33%–62%) onto ML-LNCs without having an important impact on the particle properties such as size. The combinations of peptide with ML-LNC displayed synergistic effect against *S. aureus*, as confirmed by two methods: checkerboard and time-kill assays. This synergistic interaction enables a dose reduction and consequently decreases the risk of toxicity and has the potential of minimizing the development of resistance. Together, these results suggest that ML-LNCs loaded with a plectasin derivative may be a very promising drug delivery system for further development as a novel antibacterial agent against *S. aureus*, including MRSA.

Keywords: nanoparticles, antimicrobial peptides, glycerol monolaurate, synergy, antibiotic resistance, MRSA, methicillin-resistant *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a Gram-positive bacterium with spherical shape (cocci) that grows in clusters resembling grapes. This facultative anaerobe can frequently be found in the nose, respiratory tract, and on the skin. Although *S. aureus* is usually not pathogenic, it can cause skin infections such as abscesses or scalded skin syndrome, pyogenic infections (eg, endocarditis, septic arthritis), respiratory infections such as sinusitis or hospital-acquired pneumonia, toxic shock syndrome, and food poisoning.¹ The emergence of *S. aureus* strains such as methicillin-resistant *S. aureus* (MRSA) that have developed multiresistance to beta-lactam antibiotics including the penicillins (methicillin, nafcillin, and so on) and the cephalosporins is a worldwide problem in clinical medicine.² Strains unable to resist these antibiotics are referred to as methicillin-susceptible *S. aureus* (MSSA). Although MRSA strains are not necessarily more virulent than MSSA strains, some MRSA strains contain factors or genetic backgrounds that may enhance their virulence or may enable them to cause particular clinical syndromes.³ Moreover, the resistance makes the MRSA infections more difficult to

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treat with standard antibiotics and therefore more dangerous.⁴ MRSA infections are particularly problematic in places such as hospitals and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of infection than the general public.⁵ Hence, there is an urgent need to develop new antibacterial agents for the prevention and treatment of the infections caused by *S. aureus*, particularly by the MRSA strains.

Plectasin is a cationic antimicrobial peptide, which belongs to the class called defensins. It was obtained from the saprophytic ascomycete *Pseudoplectania nigrella*. It is composed of 40 amino acids and exhibits a potent activity against Gram-positive bacteria in vitro and in vivo.^{6–8} AP114 (formerly known as NZ2114) is a plectasin derivative with an improved activity against staphylococci and streptococci, including strains resistant to standard antibiotics.^{6,7,9–12} AP138 is the product of an extensive lead optimization program based on a set of in silico generated plectasin variants.¹³

In most cases, the single best antimicrobial agent should be selected for use because this minimizes side effects. However, there are several instances in which two or more drugs are commonly given, such as treatment of serious infections before the identity of the organism is known or achievement of a synergistic inhibitory effect against certain organisms. Using drug combinations also offers the advantage of prevention of the emergence of drug-resistant organisms – if bacteria become resistant to one drug, the second drug will kill them thereby preventing the emergence of resistant strains.¹

Monolaurin, also known as glycerol monolaurate or 1-lauroyl-glycerol, is a monoester formed from lauric acid and glycerol. This compound exhibits a potent activity against Gram-positive cocci and *Bacillus anthracis*.¹⁴ Monolaurin has been proven to be effective against both susceptible and resistant strains of *S. aureus*.¹⁵ Glycerol monolaurate is generally recognized as safe for human use by Food and Drug Administration, and this compound is commonly used in the cosmetic and food industries.^{16,17} Unlike most antibiotics that have single targets for antibacterial activity, monolaurin appears to target nonspecifically many signal transduction systems present at bacterial surface through interactions with plasma membranes.¹⁴ The main disadvantage of monolaurin is poor solubility in water, which may cause problems with its administration via different routes. One of the strategies to resolve the problem of poor solubility in water is by using nanocarriers, such as lipid nanocapsules (LNCs).¹⁸

LNCs are biomimetic nanocarriers obtained by the phase inversion temperature method.¹⁹ LNCs have been widely studied as carriers for lipophilic compounds.^{20,21} Our recent study showed that LNCs are capable of effectively adsorbing

peptides, including AP114.²² Recently, we proposed using fatty acids or monoglycerides as cosurfactants to obtain LNCs with antibacterial properties.¹⁸ Monolaurin proved to be the most promising cosurfactant among all tested fatty acids and monoglycerides. Monolaurin-LNCs (ML-LNCs) exhibited the lowest minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) against two strains of *S. aureus*: one MSSA and one MRSA. Moreover, they showed the lowest toxicity among the tested LNCs, with the bactericidal concentration being 32- or 64-fold higher than the hemolytic concentration.¹⁸

The aim of the present work was to study the antibacterial activity of plectasin derivatives, AP114 and AP138, against seven MSSA and seven MRSA strains and to further investigate the antibacterial activity of ML-LNCs. To date, no studies have been performed to analyze the interactions between the peptides and ML-LNCs. Therefore, another purpose of this paper was to evaluate the adsorption of plectasin derivatives onto these nanocarriers. Because ML-LNCs have antibacterial properties, it is very important to investigate their influence on the activity of the peptide. Hence, the last important aim of this work was to investigate in vitro interactions between ML-LNCs and plectasin derivatives using time-kill assay and checkerboard method.

Materials and methods

Materials

Antimicrobial peptide AP114 was synthesized and provided by Adenium Biotech (Denmark) and AP138 was synthesized and provided by PolyPeptide Laboratories (Limhamn, Sweden). Monolaurin was purchased from Sigma Aldrich (France). Labrafac® WL1349 (caprylic/capric acid triglycerides) was kindly provided by Gattefossé S.A. (Saint-Priest, France). Lipoid® S75–3 (soybean lecithin) was kindly provided by Lipoïd GmbH (Ludwigshafen, Germany). Solutol® HS15 (macrogol 15 hydroxystearate, polyoxyl 15 hydroxystearate; CAS number: 70,142–34-6; molecular weight 963.24 g/mol; HLB 14–16) was kindly provided by BASF (Ludwigshafen, Germany). Brain–heart infusion (BHI) broth was obtained from bioMérieux (France). Plates with Columbia agar supplemented with sheep blood were purchased from Oxoid (France). All other chemicals and solvents were of analytical grade.

Preparation of LNCs

ML-LNCs were prepared at a concentration of 200 mg/mL as described by Umerska et al.¹⁸ The components of the LNCs (polyoxyl 15 hydroxystearate 0.77 g, triglycerides 0.93 g, and monolaurin 0.3 g) and NaCl (0.089 g) were

weighed, mixed with 3 mL of water, and heated to 60°C and then cooled to 20°C. The sample was treated with three heating–cooling cycles; during the last cooling cycle, at the phase inversion temperature (37°C), the system was diluted with water to a final volume of 10 mL (Figure 1). The LNC dispersions were diluted with an aqueous solution of the plectasin derivative or water (control) and incubated at 37°C for 2 hours (sufficient for peptide molecules to achieve a dynamic equilibrium between the LNCs and the surrounding medium).

Characterization of LNCs

The Z-average particle diameter (“cumulants mean”) and the polydispersity index of the LNCs were determined by dynamic light scattering (DLS) using 173 degree-angle backscatter detection. DLS measurements were conducted on a Zetasizer nano series Nano-ZS fitted with a 633 nm laser (Malvern Instruments, UK) as described previously.²⁰

Rheological properties of ML-LNCs

Non-steady flow properties of the aliquots of ML-LNCs (concentration of 200 mg/mL) were studied using a Kinexus[®] rheometer (Malvern Instruments S.A., UK), with cone plate geometry (diameter 40 mm, angle: 2°) and with controlled shear rates ranging from 10 to 1,000 s⁻¹ at 37°C (2 minute of equilibration time before the measurement). The profiles of shear viscosity versus shear rate were registered in triplicate. Viscosity results were expressed as a mean.

Peptide-loading studies

Separation of nonadsorbed peptide and extraction of adsorbed peptide

Nonadsorbed peptide was separated from the LNCs using a combined ultrafiltration–centrifugation technique as described previously.²²

The adsorption efficiency (AE) and drug loading (DL) were calculated using the following equations:

$$AE = [(A - B)/A] * 100\% \quad (1)$$

where A is the total amount (mass) of the peptide (AP114 or AP138), and B is the mass of the nonadsorbed peptide.

$$DL = [(A - B)/C] * 100\% \quad (2)$$

where C is the total weight of all components of the nanocarriers (the associated peptide and the mass of surfactants and oil used for the preparation of the LNCs).

Quantification of peptides by high-performance liquid chromatography

The AP114 and AP138 contents were analyzed using a high-performance liquid chromatography system described previously.²² Briefly, standard solutions of the APIs (0.1–1,000 µg/mL) were prepared in deionized water, and 20 µL of the standard or sample was injected into the SymmetryShield[™] RP₁₈ 5 µm 4.6×250 mm column (Waters, USA). A flow rate of 1.0 mL/min was employed using mobile phase A composed of 0.1% trifluoroacetic acid (TFA) in water and mobile phase B composed of 0.1% TFA in acetonitrile. A linear gradient was run: 20% B for 25 minutes, 45% B for 0.1 minute, 100% B for 4.9 minutes, 20% B for 0.1 minute, and 20% B for 14.9 minutes. The API peaks had retention times of ~12 minutes for AP114 and 13 minutes for AP138, respectively. The UV detection was performed at 200 nm. Data collection and integration were accomplished using Empower[®]3 software.

The method showed good linearity for both molecules, AP114 and AP138 ($R^2 \geq 0.9999$). The quantification limits were 0.5 µg/mL for AP114 and 1 µg/mL for AP138, whereas the detection limits were 0.25 and 0.5 µg/mL for AP114 and AP138, respectively. More than 90% of peptides were recovered each time.

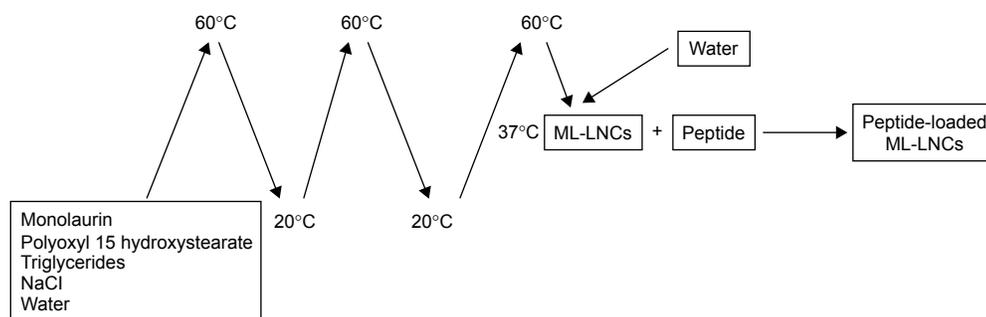


Figure 1 Schematic representation of preparation of ML-LNCs and peptide-loaded ML-LNCs.

Abbreviation: ML-LNCs, monolaurin-lipid nanocapsules.

Microorganisms

The strains used in this study were seven methicillin-resistant clinical isolates of *S. aureus*, six methicillin-susceptible clinical isolates, and one reference strain of *S. aureus* (ATCC 25923).

Preparation of inoculum

One to two colonies were taken directly from the Columbia agar plate into 2 mL of 0.85% NaCl solution, and the density of the microorganism suspension was adjusted to 1.1 McFarland. The bacterial suspensions were then further diluted 100 times with BHI medium.

MIC determination

MIC was determined via a broth microdilution method, as described previously^{18,23} with slight modifications. Serial two-fold dilutions of the samples in BHI were prepared in order to obtain the desired concentration range. An amount of 50 μ L of a bacterial suspension in BHI broth was added into each well of a sterile 96-well plate already containing 50 μ L of the tested sample or control. The positive control wells contained BHI and the bacterial suspension without the tested sample, whereas the negative control wells contained BHI and the tested sample without the bacterial suspension. The samples were incubated for 24 hours at 37°C. The MIC was the lowest concentration that completely inhibited the growth of the bacteria as detected by the unaided eye.

Checkerboard titration

Combinations of ML-LNCs and plectasin derivatives were tested via a checkerboard titration method. Two-fold dilutions of ML-LNCs and peptide were prepared before mixing. The concentration range of each antimicrobial agent in combination ranged from 1/32 \times MIC to 4 \times MIC. The fractional inhibitory concentration (FIC) index was calculated using the following equation:

$$FIC = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}$$

where MIC_A is the MIC of the compound A alone, $MIC_{A/B}$ is the MIC of compound A in combination, and MIC_B and $MIC_{B/A}$ are the MIC of the compound B alone and in combination, respectively.

Synergy was defined as an FIC index of ≤ 0.5 , additivity/indifference as an FIC index of > 0.5 but of ≤ 4 . Antagonism was defined as an FIC index of > 4 .²⁴

Time-kill assay

Samples were diluted with BHI medium to a volume of 1.98 mL, and 20 μ L of bacterial suspension (see "Preparation of inoculum" section) was added into each tube. Samples were then incubated at 37°C. An amount of 100 μ L was withdrawn from each tube after 0, 3, 6, 18, and 24 hours and serial 100-fold dilutions were prepared in distilled water. A total of 100 μ L of the diluted and/or undiluted sample were delivered onto the surface of the agar, spread, and allowed to be absorbed into the agar. After overnight incubation of the agar plates at 37°C, the colonies were counted.

Transmission electron microscopy

Twenty microliters of inoculum was added to 0.98 mL of BHI broth and incubated for 10 hours at 37°C. One milliliter of sample dispersed in BHI or BHI on its own (control) was then added followed by incubation for 1 hour. The bacteria were collected by centrifugation (10,000 \times g, 5 minutes) and suspended in 0.1 M phosphate-buffered saline (PBS) (pH=7.4). After centrifugation, the bacteria were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS (pH=7.4) and left overnight under vacuum. The bacteria were centrifuged, suspended in PBS, centrifuged, resuspended in water, centrifuged, resuspended in 1% osmium tetroxide in water, and incubated for 1 hour at room temperature. Samples were washed and dehydrated with graded ethanol solutions (twice with deionized water for 10 minutes, 50% ethanol for 20 minutes, 70% ethanol for 20 minutes, 95% ethanol for 20 minutes, and three times with 100% ethanol for 30 minutes). Samples were then embedded in Epon 812 resin, which was then left to polymerize for a few days. Thin slices (~60 nm) were cut from each sample using Leica UC7 ultramicrotome and deposited onto copper grids. The samples were stained with 3% uranyl acetate in 50% ethanol for 15 minutes and washed with deionized water. The samples were examined using JEOL JEM-1400 electron microscope.

Statistical analysis

The statistical significance of the differences between samples was determined using one-way analysis of variance. Differences were considered significant at $P < 0.05$.

Results and discussion

Properties of ML-LNCs and peptide-loaded ML-LNCs

LNCs are composed of an oily core of capric/caprylic acid triglycerides that is surrounded by a shell composed of a

hydrophilic surfactant, for example, macrogol 15 hydroxystearate. Although it is possible to obtain LNCs using solely macrogol 15 hydroxystearate as a surfactant, usually a cosurfactant is used to increase the LNC stability and decrease their size. The most common cosurfactant is lecithin.^{18–20,25} The macrogol 15 hydroxystearate/triglycerides mass mixing ratio (MMR) of 0.82 was selected as this is the ratio present in the most commonly described LNCs.^{18,21} A previous study has shown that it is the monolaurin that makes the LNCs active against Gram-positive bacteria. It is important to use a high concentration of monolaurin to reduce the amount of the nanocarrier required to achieve a desired effect. LNCs composed of monolaurin (15.0%), macrogol 15 hydroxystearate (38.4%), and triglycerides (46.6%) were characterized by a hydrodynamic diameter of 36 ± 0.3 nm and homogeneous size distribution (polydispersity index below 0.1). The zeta potential of ML-LNCs was close to neutral (-4.77 ± 2.19).¹⁸ This is not surprising, as ML-LNCs do not contain ionizable molecules. The incorporation of monolaurin resulted in an important decrease in phase inversion temperature from 80 to 85°C (LNCs composed solely of macrogol 15 hydroxystearate and triglycerides or lecithin-LNCs) to 37°C (ML-LNCs). The concentration of surfactants and oil in ML-LNCs at 200 mg/mL was sufficiently high for the system to undergo phase inversion on heating. Because monolaurin is not soluble in water, the use of LNCs offers the advantage of obtaining pharmaceutically acceptable dosage form without using organic solvents or cosolvents.

The shape of the flow curves (Figure 2) indicates that ML-LNC dispersion at a concentration of 200 mg/mL exhibits a shear thickening flow. In this non-Newtonian flow phenomenon, shear viscosity decreases (about 10-fold) as the shear rate is increased (about 100-fold). The non-Newtonian

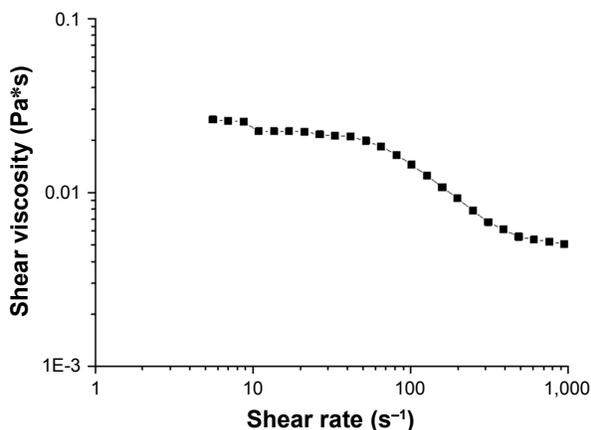


Figure 2 Flow curve of monolaurin-lipid nanocapsules.

behavior can be observed in concentrated particle systems because of asymmetrical microstructure caused by particle–particle flow field interactions.²⁶

In addition to covalent attachment and encapsulation within the nanocarrier, surface adsorption is one approach for incorporating drug molecules into drug delivery systems.²⁷ Due to their small size and large surface area, nanocarriers may interact with biomolecules such as peptides. Such interaction is a multifactorial process that depends on the properties of both, the peptide and the nanocarrier, and also on experimental conditions such as concentration and the medium.^{22,28} Both plectasin derivatives, AP114 and AP138, are peptides composed of 40 amino acids with very similar amino acid sequence and molecular weight (Table 1). They differ only by 3 amino acids (positions 9, 17, and 26 from the amino end). The net charge at pH 7 is +3 and +4, and the theoretical isoelectric points are 8.62 and 8.87 for AP114 and AP138, respectively. The cationic property of the antimicrobial peptides is very important, because the electrostatic forces between the positively charged peptide and the negatively charged bacterial surface are critical determinants for their interactions, which is a key factor for antibacterial activity whether the membrane itself is targeted or when an intracellular target must be reached by means of translocation.²⁹

It is very unlikely that the antimicrobial peptides showing good solubility in water, for example, plectasin derivatives, would penetrate into the oily core of the LNCs. Instead, they are expected to be localized within or on the particle shell.²² The adsorption of the peptide did not cause an important change in the properties of the LNCs (Table 2). The size distribution remained unaffected, and the increase in the nanocapsule diameter, although statistically significant at the lowest LNC/peptide MMR, can be considered as negligible, because the difference in particle size was considerably small (<1 nm). It is in agreement with previously reported data that the adsorption of peptide onto LNCs without ionizable surfactants did not change the properties of the nanocarrier.²² The adsorption yield ranged from 34% to 62%. The percentage of adsorbed peptide increased corresponding to an increasing LNC/peptide MMR. AP114 and AP138 showed comparable adsorption yield, which can likely be attributed to their similar composition and physical properties. Although the interactions between the peptides and the nanocarriers are governed mainly by electrostatic forces,^{30,31} it is possible to achieve significant adsorption of peptide onto LNCs even without the presence of an ionizable surfactant because of the enormous surface area of the nanocarriers and of the

Table 1 Structure and properties of antimicrobial peptides

Peptide	API14 = NZ2114	API38
Sequence	GFGCNGPWNEDDLRCNHCKSIKGYKGGYC AKGGFVCKCY Cyclic Cys4–Cys30, Cys15–Cys37, Cys19–Cys39	GFGCNGPWEDDLRCRHCKSIKGYRGGYC AKGGFVCKCY Cyclic Cys4–Cys30, Cys15–Cys37, Cys19–Cys39
Molecular weight	4,417 Da	4,460.3 Da
Theoretical pI	8.62	8.87
Number of amino acids	40	40
Number of cationic amino acids	6	7
Number of anionic amino acids	3	3
Net charge at pH 7	+3	+4

Note: The information was obtained from the manufacturer (API14: Adenium Biotech, Denmark; API38: PolyPeptide Laboratories, Sweden).

peptide–carrier interactions (such as dipole–ion interactions between the cationic groups of peptides and PEG dipoles on macrogol 15 hydroxystearate molecules).²² A peptide loading of 1.5% or even 2.7% can be considered as high for lipidic nanocarriers – they are usually characterized by smaller loading of molecules than the nanocarriers made of hydrophilic polymers encapsulating the drug based on electrostatic interactions.^{30,31} The DL is a key parameter when developing an effective nanoparticulate delivery system, because low loading may limit the use of nanoparticulate systems because a substantial amount of the formulation must be administered to achieve the therapeutic effect.³²

Antibacterial properties of ML-LNCs

The MICs of ML-LNCs against different strains of *S. aureus* are shown in Table 3. The MICs of ML-LNCs were 100 or 200 or 400 µg/mL depending on the strain (corresponding to 15, 30, and 60 µg/mL of ML, respectively). MSSA strains seemed to be slightly more sensitive to ML-LNCs – five strains were inhibited by ML-LNCs at 100 µg/mL (15 µg/mL of ML), whereas among MRSA strains only one strain was inhibited by 100 µg/mL of ML-LNCs; most of the strains were inhibited by ML-LNCs at 200 µg/mL (30 µg/mL). The highest MIC (400 µg/mL of ML-LNCs, which contains 60 µg/mL of monolaurin) was observed for MSSA.

Time-kill curves revealed that ML-LNCs were bactericidal (Figure 3). In the absence of ML-LNCs, bacterial counts reached their maximum 18–24 hours after inoculation. The killing was time dependent rather than concentration dependent. The killing occurred slowly – even at such a high concentration as 50 mg/mL (500× MIC) the reduction in the colony-forming unit (CFU) number after 3 hours was relatively small (~2 Log). This is in good agreement with the previously reported data.¹⁸

Because ML-LNCs were characterized by low killing rates even at very high concentrations (500× MIC), it can be concluded that they do not solubilize the bacterial membrane as do other detergents, including monoglycerides or fatty acids with shorter chains.¹⁸ It is very likely that monolaurin decreases the fluidity of the membrane, because it has a melting point of 63°C and, therefore, is solid at 37°C.¹⁸ This may modulate the activity of membrane proteins thereby playing an important role in cellular functions and homeostasis.³³ Monolaurin has been shown to inhibit the production of toxic shock syndrome toxin 1 and staphylococcal enterotoxins. The production of exotoxins by *S. aureus* was inhibited at monolaurin concentration that did not inhibit bacterial growth.^{14,17,34,35} Monolaurin has 12 carbon molecules in its fatty acid backbone chain, which makes it span exactly one-half the width of a lipid bilayer.³⁶ Interestingly, it has

Table 2 Composition and properties of ML-LNCs and peptide-loaded ML-LNCs

Peptide	LNC/peptide MMR	Adsorption efficiency (%)	Peptide loading (%)	Particle diameter (nm)	Polydispersity index
–	N/A	N/A	N/A	36.0±0.30	0.026±0.020
API38	100	61.96±0.94	0.6196±0.0094	36.2±0.18	0.024±0.009
API38	50	46.15±0.62	0.9230±0.0125	36.4±0.13	0.037±0.017
API38	25	37.39±0.81	1.4955±0.0322	36.8±0.17*	0.051±0.011
API14	50	47.80±0.47	0.9560±0.0094	36.3±0.13	0.021±0.009
API14	25	42.71±1.58	1.7802±0.0631	36.6±0.25	0.023±0.010
API14	12.5	33.91±2.88	2.7130±0.2305	36.8±0.25*	0.036±0.011

Notes: Peptide concentration was 1,000 µg/mL. *P<0.05 versus blank ML-LNCs.

Abbreviations: MMR, mass mixing ratio; ML-LNCs, monolaurin-lipid nanocapsules; N/A, not applicable.

Table 3 MICs of ML-LNCs and plectasin derivatives against different *Staphylococcus aureus* isolates

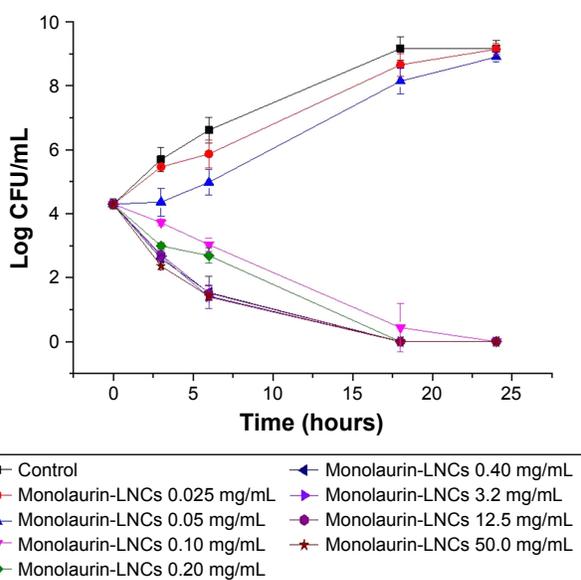
Strain	Strain ID	MIC AP114	MIC AP138	MIC ML-LNCs
MRSA	0706C0025	4	2	200
MRSA	11004533801	4	2	200
MRSA	11004691801	4	2	200
MRSA	11004787401	8	2	100
MRSA	11006153901	8	4	200
MRSA	070170095	4	2	200
MRSA	0702E0196	4	2	200
MSSA	11004327701	1	0.125	100
MSSA	11004480701	8	2	400
MSSA	11004697301	4	1	100
MSSA	11004010401	4	2	100
MSSA	0703H0036	4	1	100
MSSA	0701A0095	4	2	200
MSSA	ATCC 25923	8	4	100

Notes: MICs are expressed in $\mu\text{g/mL}$. ML-LNCs contain 15% of monolaurin.

Abbreviations: MICs, minimum inhibitory concentrations; ML-LNC, monolaurin-lipid nanocapsules; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*.

been reported that the optimum chain length of fatty acids and their corresponding monoglycerides to achieve the maximum antibacterial activity is 12 carbons.^{18,37,38} Because of its size and lipophilic nature, it has been postulated that glycerol monolaurate is inserted into cytoplasmic membrane and exerts its antibacterial effect by interfering with many transmembrane signal transduction systems.^{14,39}

The influence of ML-LNCs on *S. aureus* morphology was studied by transmission electron microscopy (Figure 4).

**Figure 3** Time-kill curves of ML-LNCs against MSSA ATCC strain.

Abbreviations: ML-LNCs, monolaurin-lipid nanocapsules; MSSA, methicillin-susceptible *Staphylococcus aureus*.

TE micrographs of control (untreated bacteria – Figure 4A) showed proliferating cells that were uniformly shaped with intact cell walls and well-defined cell membranes. In ML-LNC-treated cells, disappearance of plasma membrane was observed (Figure 4B). ML-LNCs also exerted the effects on the cell wall – in some cells, the disappearance of cell wall was observed. This is in agreement with the effects of monocaprin on group B streptococci observed by Bergsson et al.⁴⁰ Interestingly, ML-LNCs affected the shape of bacteria – characteristic concave deformations were observed in numerous cells. Some lysed cells were observed with the leakage of cytoplasm contents. Proliferating cells were observed, therefore suggesting that ML-LNCs do not stop cell division, but numerous abnormalities described above occurred during cell division.

Antibacterial activity of plectasin derivatives

The MICs of plectasin derivatives against 14 studied strains are shown in Table 3. The MICs of AP114 varied eightfold (range 1–8 $\mu\text{g/mL}$), whereas those of AP138 varied 16-fold (range 0.125–4 $\mu\text{g/mL}$). One of the MSSA strains was very sensitive to plectasin derivatives (MICs of 0.125 and 1 $\mu\text{g/mL}$ for AP138 and AP114, respectively). The existence of *S. aureus* mutants that are more sensitive to host defense peptides such as plectasin has been described previously.⁴¹ It can be concluded that AP138 was more effective than AP114. Both plectasin derivatives effectively inhibited all tested strains of *S. aureus*, independent of their resistance pattern. This is in good agreement with the previously reported data indicating that the antibacterial spectrum of NZ2114 or AP138 includes staphylococci such as MRSA.^{7,9,13,42}

Time-kill assay of plectasin derivatives was performed to determine the effect of an increasing peptide concentration on the extent of bacteria killing. Time-kill curves of ATCC 25923 (MSSA) exposed to AP114 are shown in Figure 5A. In the presence of 1 or 2 $\mu\text{g/mL}$ of AP114 (0.125 \times or 0.25 \times MIC, respectively), the bacterial counts grew more slowly than with the control. At 4 $\mu\text{g/mL}$ (0.5 \times MIC), an evident growth inhibition was observed – the effect can be considered as bacteriostatic; however, after 18–24 hours of incubation, large standard deviations were observed and it was possible to observe visually slight bacterial growth. At 8 $\mu\text{g/mL}$ of AP114 (1 \times MIC), a reduction by ~ 1.6 Log in the number of CFU was observed after 3 hours, 2.5 Log after 6 hours, and 4.3 Log after 18 hours. At 16 $\mu\text{g/mL}$ of AP114 (2 \times MIC), a dramatic reduction of bacterial density by 3.3 Log was observed after 3 hours, and the bactericidal effect was

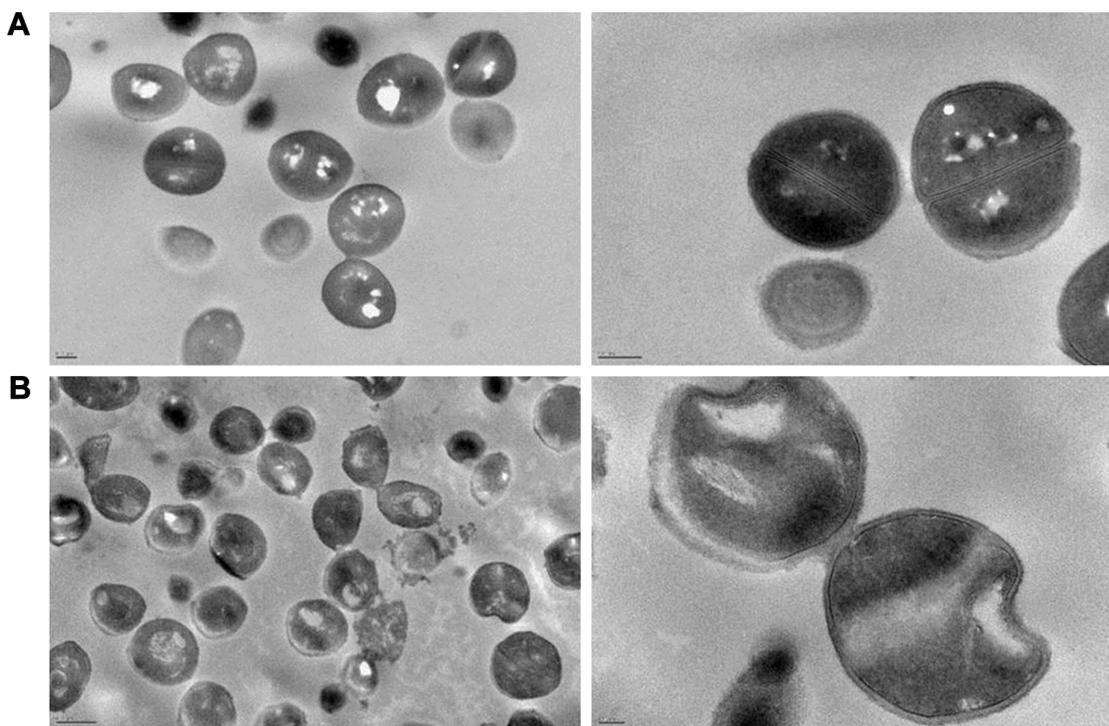


Figure 4 Transmission electron microscope images of MSSA ATCC strain untreated control (A) and treated with 3.2 mg/mL (32x MIC) of ML-LNCs (B) for 1 hour. Scale bars: (A) left: 0.2 μm, right: 0.2 μm; (B) left: 0.5 μm, right: 100 nm.

Abbreviations: MIC, minimum inhibitory concentration; ML-LNCs, monolaurin-lipid nanocapsules; MSSA, methicillin-susceptible *Staphylococcus aureus*.

maintained for at least 24 hours. Time-kill curves of ATCC 25293 (MSSA) exposed to AP138 are shown in Figure 5B. At 1 μg/mL of AP138 (0.25x MIC), the bacterial count grew more slowly than that of control. At 2 μg/mL (0.5x MIC),

an inhibition of bacterial growth was observed; the effect can be considered as bacteriostatic with the increase in the number of CFU by 1 Log compared with the initial inoculum. At 4 μg/mL (1x MIC), a gradual decrease in the

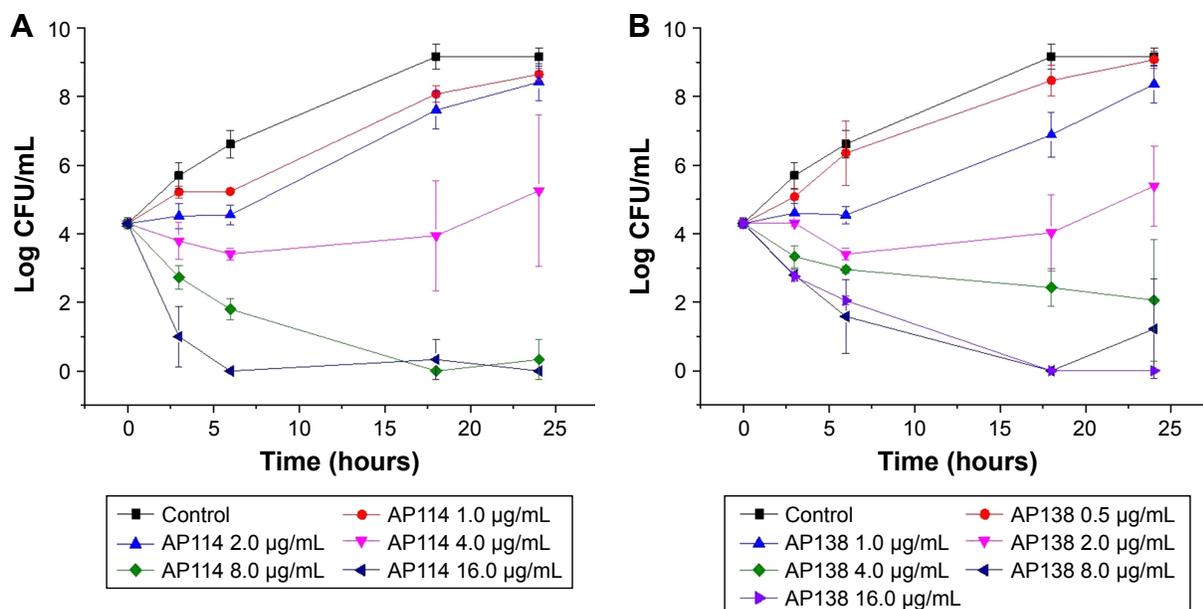


Figure 5 Time-kill curves of plectasin derivatives API 14 (A) and API 138 (B) against MSSA ATCC 25923 strain. The MIC values were 8 and 4 μg/mL for API 14 and API 138, respectively.

Abbreviations: MIC, minimum inhibitory concentration; MSSA, methicillin-susceptible *Staphylococcus aureus*.

CFU number by 2.3 was observed within 24 hours – at this concentration, the action can be considered as bacteriostatic. At 8 and 16 $\mu\text{g}/\text{mL}$ ($2\times$ and $4\times$ MIC, respectively), a reduction by 1.5 Log was observed after 3 hours, by 2.3–2.7 Log after 6 hours, and the bactericidal effect was reached after 18 hours. Time-kill results confirmed that AP138 was more potent than AP114. However, AP114 killed bacteria more rapidly than AP138. At 16 $\mu\text{g}/\text{mL}$, the bactericidal effect, that is, a reduction of more than 3 Log in CFU number compared with the starting inoculum, was observed after 3 hours for AP114, whereas AP138 was not bactericidal even after 6 hours of incubation.

Although many antimicrobial peptides act on and disintegrate bacterial cytoplasmic membrane, this is not the case for plectasin. In spite of its amphipathic structure, this peptide does not compromise membrane integrity, thereby reducing the risk of unspecific toxicity.⁷ Indeed, AP114 (NZ2114) showed very low hemolytic activity ($<0.1\%$ of hemolysis at 128 $\mu\text{g}/\text{mL}$).⁴² Another study has confirmed that plectasin is neither hemolytic for human erythrocytes (half maximal effective concentration $[\text{EC}_{50}] >400 \mu\text{g}/\text{mL}$) nor cytotoxic for human epidermal keratinocytes ($\text{EC}_{50} >400 \mu\text{g}/\text{mL}$) or for murine L929 fibroblasts ($\text{EC}_{50} >400 \mu\text{g}/\text{mL}$).⁶ Plectasin and its derivatives exhibit their antibacterial activity by interfering with cell wall biosynthesis. These peptides directly bind to bacterial cell wall precursor lipid II forming an equimolar stoichiometric complex.⁷ It has been

demonstrated that AP114 shows bactericidal mode of action, with the MBC/MIC ratios ranging from 1 to 2 for either MSSA or MRSA strains.^{41,42} These results are in a good agreement with our time-kill data. Time-kill studies of plectasin performed by Schneider et al⁷ demonstrated that the time-kill behavior of plectasin was similar to that of agents interfering with cell wall synthesis (such as vancomycin or penicillin). Plectasin did not exhibit similar time-kill profile characteristic as the rapidly lytic membrane-active agents such as polymyxin B.⁷

Figure 6 shows influence of AP114 (Figure 6A) and AP138 (Figure 6B) on the morphology of *S. aureus*. Incubation with plectasin derivatives resulted in a destruction of cell walls. It was possible to observe cell walls that have separated from the cells, disappearance of fragments of cell membrane, and the leakage of cytoplasmic contents. Shape deformations and abnormalities in shape division were also observed. These observations are in agreement with the fact that plectasin derivatives exert their antibacterial effect by interfering with cell wall synthesis.

Synergistic interactions between plectasin derivatives and ML-LNCs

Combinations containing a plectasin derivative and the ML-LNCs were tested on one MSSA reference strain and one MRSA strain to evaluate whether they can still enhance SA killing. The efficacies of these combinations were

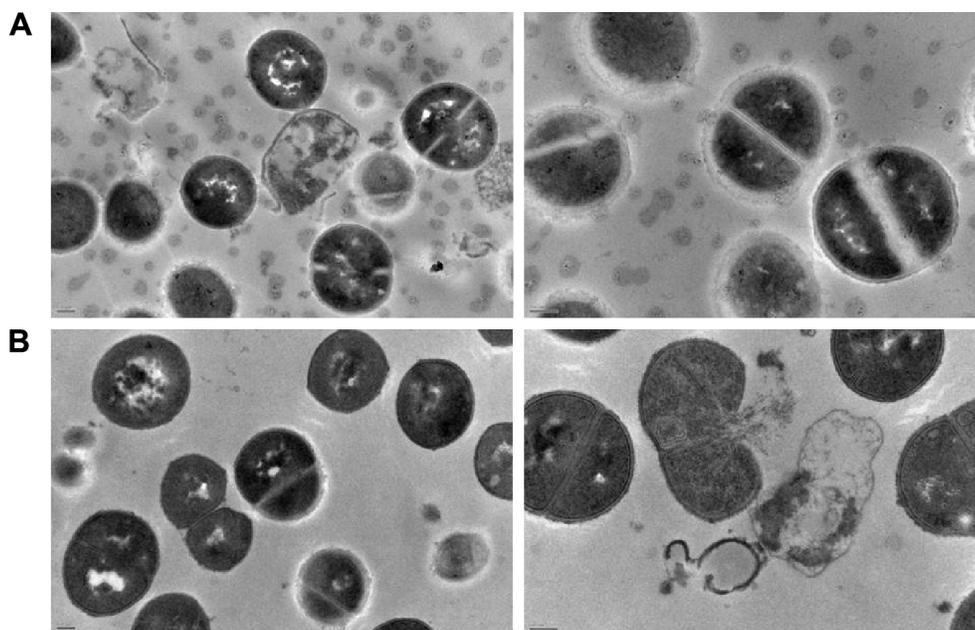


Figure 6 Transmission electron microscope images of MSSA ATCC 25923 strain treated with 128 $\mu\text{g}/\text{mL}$ ($32\times$ MIC) of AP114 (A) and 64 $\mu\text{g}/\text{mL}$ ($32\times$ MIC) of AP138 (B) for 1 hour. Scale bars: (A) left: 0.2 μm , right: 100 nm; (B) left: 0.2 μm , right: 200 nm.

Abbreviations: MIC, minimum inhibitory concentration; MSSA, methicillin-susceptible *Staphylococcus aureus*.

assessed using checkerboard method and time-kill assay. The antibacterial activity of peptide-loaded ML-LNCs was the same as the activity of combinations containing the same concentrations of ML-LNCs and a plectasin derivative that were prepared by mixing of the components in the 96-well plate just before the addition of bacteria (not shown). In the checkerboard method, the values of FIC index were equal to 0.375 or 0.5, indicating synergistic interactions between each plectasin derivative (AP114 or AP138) and ML-LNCs against both MSSA and MRSA strains (Table 4). By using the combinations, it was possible to reduce the concentrations of both, peptide and LNCs, fourfold.

Time-kill study showed that none of the ingredients used alone was capable of effectively inhibiting bacterial growth at tested concentrations (Figure 7). The combination of ML-LNCs at 25 µg/mL and AP114 at 1 µg/mL can be considered as synergistic (the CFU number was ~3 Log smaller than that of each of ingredients alone), but after 24 hours an increase in CFU number by 1.2 Log was observed compared with the initial inoculum (Figure 7A). An increase in the concentration of either AP114 or ML-LNCs or both of them yielded synergistic and bactericidal combinations. The reduction in CFU number was gradual (after 6 hours only the combination containing AP114 2 µg/mL and ML-LNCs 50 µg/mL proved to be bactericidal). The combinations showed at least an 8 Log reduction in the CFU number compared with the ingredients alone after 24 hours of incubation.

All tested combinations containing AP138 and ML-LNCs showed synergistic effects (Figure 7B). Both samples containing 0.5 µg/mL AP138 reduced the CFU number by 2.1 Log after 3 hours and 2.6–2.8 Log after 6 hours. The samples containing more AP138 (1 µg/mL) showed larger reductions in CFU number: by 2.6–2.7 Log after 3 hours and after 6 hours they showed bactericidal effect reducing CFU number by 3.6–3.9 Log. All combinations showed bactericidal effect after 18 and 24 hours with at least an 8 Log reduction in the CFU number compared with the ingredients alone.

Figure 8 shows influence of synergistic combinations containing ML-LNCs and AP114 (Figure 8A) or AP138 (Figure 8B) on *S. aureus* morphology. The changes described for ML-LNCs and the plectasin derivatives were also observed in their combinations. These include deformations in shape of bacterial cells, disappearance of cell walls and cell membranes, fragments of cell walls that have separated from bacterial cells, and leakage of cytoplasmic contents from the cells.

Both plectasin derivatives have been shown to act in synergy with ML-LNCs against *S. aureus*. A synergistic effect can result from a variety of mechanisms. Synergistic antibacterial effect is often observed when between the compounds that attack different targets. A possible explanation of synergistic effect between ML-LNCs and plectasin derivatives is that the peptides damage cell wall sufficiently to enhance the entry of ML and/or ML-LNCs. Indeed, inhibitors of cell wall synthesis, for example, penicillin, show synergistic interactions with some antibiotics (eg, gentamicin) against enterococci by facilitating their entry as a result of cell wall damage.¹

Antimicrobial peptides represent one of the most promising and innovative groups of anti-infective agents.^{6,29,41} The in vitro emergence of resistance to antimicrobial peptides is less common and less rapid than that observed for conventional antibiotics.^{43,44} More recently, however, a number of resistance mechanisms against antimicrobial peptides have been discovered and investigated in bacteria. The examples of such mechanisms include the release of glycosaminoglycans and other polyanionic molecules to scavenge positively charged peptides or membrane modification resulting in a decrease of negative surface potential of bacterial membranes.^{45–47} The latter mechanisms have been described for *S. aureus*.⁴⁸ On the other hand, it has been demonstrated that the resistance to monolaurin does not develop even after a year of passaging of *S. aureus* on media containing a subinhibitory concentration of this compound.^{14,17} Moreover, it has been shown that glycerol monolaurate inhibits the induction of resistance to vancomycin in *Enterococcus faecalis*.⁴⁹ In conclusion, combined

Table 4 MICs of ML-LNCs and plectasin derivatives used alone and in combination against MSSA and MRSA

Strain	Peptide	MIC of peptide alone	MIC of peptide in mixture	MIN of ML-LNCs alone	MIC of ML-LNCs in mixture	FIC index
MRSA 0702E0196	API38	2	0.5	200	25	0.375
MRSA 0702E0196	API14	4	1	200	50	0.5
MSSA ATCC 25923	API38	4	1	100	25	0.5
MSSA ATCC 25923	API14	8	2	100	25	0.5

Note: MICs are expressed in µg/mL.

Abbreviations: FIC, fractional inhibitory concentration; MICs, minimum inhibitory concentrations; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*.

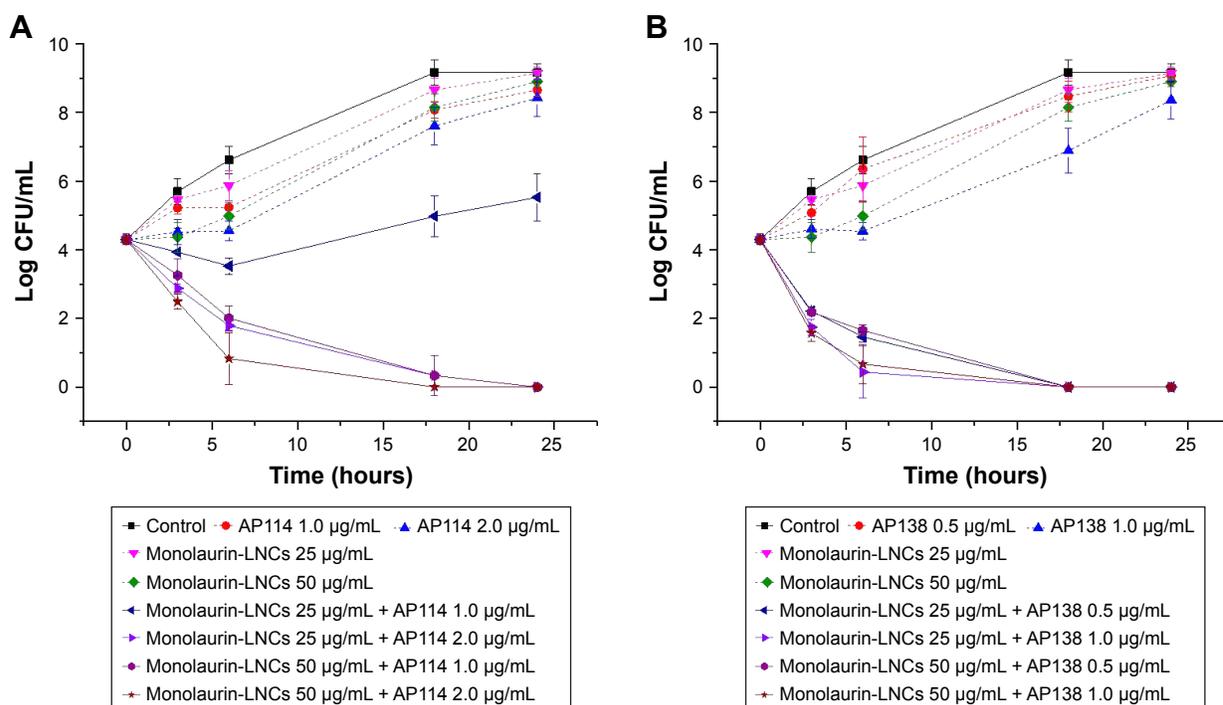


Figure 7 Time-kill curves of ML-LNCs and plectasin derivatives API 14 (A) and API 38 (B) used alone and in combination against MSSA ATCC strain.

Abbreviations: ML-LNCs, monolaurin-lipid nanocapsules; MSSA, methicillin-susceptible *Staphylococcus aureus*.

administration of ML-LNCs and plectasin derivatives could markedly decrease the probability of development of resistance to the latter. Therefore, ML-LNCs can be considered as a very promising carrier for plectasin derivatives and possibly for other antimicrobial peptides.

Conclusion

Several interesting findings emerged from the present study. First, plectasin derivatives were adsorbed on the LNCs with the efficiency between 34% and 62% without having an important impact on the nanocarrier properties such as size.

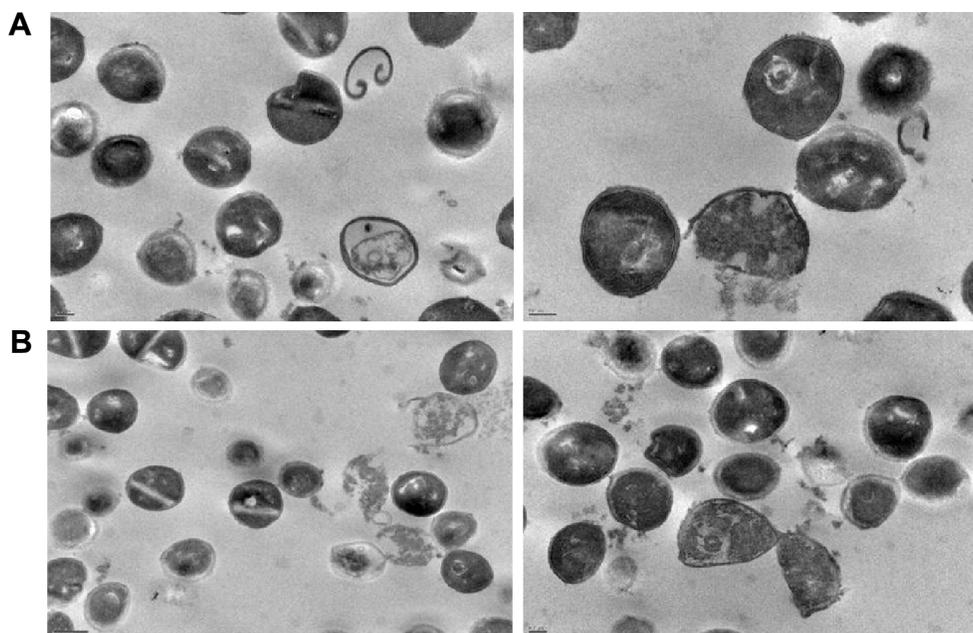


Figure 8 Transmission electron microscope images of MSSA ATCC strain treated with containing 1.6 mg/mL of ML-LNCs and plectasin derivative: 64 µg/mL of API 14 (A) or 32 µg/mL of API 38 for 1 hour. Scale bars: (A) left: 0.2 µm, right: 0.2 µm; (B) left: 0.5 µm, right: 0.2 µm.

Abbreviations: ML-LNCs, monolaurin-lipid nanocapsules; MSSA, methicillin-susceptible *Staphylococcus aureus*.

Second, when used alone, ML-LNCs and both plectasin derivatives were effective against all tested MSSA and MRSA strains with the MIC values of 100–400 µg/mL and 0.125–8 µg/mL for the nanocarriers and peptides, respectively. Both the peptides and the ML-LNCs showed bactericidal activity. Third, when used in combination, synergistic effect was observed between plectasin derivatives and ML-LNCs as confirmed by both, checkerboard and time-kill assay. This enables the dose reduction and consequently decreases the risk of toxic effects and minimizes the possibility of resistance development. Together, these results suggest that ML-LNCs loaded with a plectasin derivative may be a very promising drug delivery system for further development as a novel antibacterial agent against MRSA and MSSA.

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Disclosure

The authors report no conflicts of interest in this work.

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