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Nanoformulated Myristic Acid for Antimicrobial Applications

Abstract

Free fatty acids possess antimicrobial properties in the natural defense system of many eukaryotes. In this study, we synthesized nanoformulations of myristic acid for antimicrobial activity. The myristic acid nanoformulations (MN) were unstable due to crystallization. This problem was overcome by the addition of the liquid fatty acids and by using two surfactants of hydrophilic and lipophilic nature. MN exhibited a small size (100 nm), and their physical form was affected by the amount of the liquid fatty acid. MN with myristic acid and liquid fatty acid in 1:1 formed solid NLC and 2:3 formed liquid nanoemulsions. In antimicrobial studies, MN was effective against the Gram-positive bacteria, i.e., Staphylococcus aureus and Bacillus subtillis, and the Gram-negative bacteria, i.e., Pseudomonas eruginosa and Salmonella typhi. MN8 (Myristic acid and oleic acid in 2:3) showed the highest antimicrobial activity and should be explored as potential antimicrobial agents against more strains of microorganisms.

Key Words: Antimicrobial Resistance, MRSA, Fatty Acids, Algae, Nanoparticles, Antibiotics

Introduction

Antibiotics were introduced around 70 years ago and greatly reduced illness and mortality due to bacterial infections [1]. However, bacteria started to resist the action of antibiotics to reduce their efficacy in treating bacterial infections. Bacteria have developed defense systems to neutralize the effect of the antibiotics either by the expulsion out of the cell [2], the enzymatic degradation [3] or the modification of drug target [4]. The resistant strains of bacteria have been treated by the combination of different classes of antibiotics which, in turn, lead to the multi-drug resistant (MDR) strains of bacteria. In the United States alone, around 2 million people are infected by drug-resistant bacteria, and around 23,000 of them die each year [5].

Accordingly, recent research interest has been focused on the development of antibacterial drugs that are chemically and structurally different from current antibiotics. Antimicrobial peptides have been developed to inhibit cell wall and plasma membranes of bacteria or to synergize human immune response leading to cell death [6, 7]. Inorganic nanoparticles and ions have been used to target cell membrane or inhibit enzyme activity [8, 9]. However, bacteria are also reported to develop resistance to antimicrobial peptide and other new antimicrobial candidates. Therefore, new strategies for drug-free treatment of resistant bacteria are needed to prevent the increasing burden of bacterial resistance.

The natural defense system of some eukaryotes, such as algae, use free fatty acids for their antimicrobial properties [10]. These fatty acids can breakdown the plasma membrane or cell wall as well as disrupt intracellular processes such as energy production and oxidative phosphorylation [11]. Among these fatty acids, myristic acid (C14) is the most effective saturated fatty acid with broad-spectrum activity against different microorganisms. However, it is insoluble in water [12], and literature on the development of its drug delivery systems is scarce due to instability associated with crystalline nature [13]. Previously, we have reported lauric acid (C12) nanoformulations containing oleic acid and stearic acid possess antimicrobial properties. These nanoparticles, when coated on an endotracheal tube, were able to kill bacteria and reduce adhesion of dead

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bacterial mass [14]. In another paper, myristic acid (C14) nanoemulsions were prepared by dissolving the fatty acid in isopropanol and curcumin was also added as an antimicrobial agent. Although nanoemulsion showed marked antimicrobial properties, the role of myristic acid could not be justified due to the presence of curcumin and isopropanol [15]. In this paper, we synthesize myristic acid nanoformulations (NM) for the drug-free treatment of bacteria. Nanoformulation of myristic acid was achieved by the addition of oil or by using a double surfactant system. The antimicrobial activity of MN was evaluated against both Gram-positive and Gram-negative bacteria.

Materials and Methodology

Materials

Myristic acid and Brij 58 was purchased from Acros Organics in analytical grade. Oleic acid, linoleic acid (pharmaceutical grade) and Span 80 (analytical grade) were purchased from Sigma-Aldrich, USA. Pseudomonas aeruginosa (ATCC® 9027) and Staphylococcus aureus (ATCC® 6538) were purchased from Microbiologics Inc., Pakistan. Salmonella typhi (Accession No. 042) and Bacillus subtilis (Accession No. 174) were purchased from Fungal Culture Bank, University of Punjab, Pakistan.

Synthesis of Nanoparticles

Myristic acid was nano formulated as NLC by forming a heterogenous lipid mixture and/or use of two surfactant systems (Table 1). For heterogeneous lipid mixture formation, myristic acid was mixed with different proportions of either oleic acid or linoleic acid, as shown in table 1. The resulting lipid mixtures were melted to 60°C and stirred for 5 minutes to make a homogenous lipid mixture. Then, lipids were added dropwise to the aqueous solution of surfactant also heated to the same temperature as lipids. In an attempt to stabilize them, myristic acid nanoparticles were prepared with hydrophilic mono-surfactant (Brij 58) as well as a double surfactant system consisting of a hydrophobic surfactant (Brij 58) and a lipophilic surfactant (Span 80). After 5 minutes of stirring of aqueous and lipid phase, heating was switched off, and hot melt emulsion was allowed to cool down. NLC was treated with sonication for 5 min and separated by centrifugation for 10 min at 5°C. Nanoparticles were washed by resuspension of nanoparticles in water and centrifugation to remove additional surfactant.

Table 1. Chemical Composition of MN Prepared by Different Strategies

Formulation code	Myristic acid	Oil	Brij 58	Span 80
MM	1	–	300mg	–
MD	1	–	200mg	100 mg
Myristic acid + Oleic acid				
MOM21	2	1	300mg	–
MOD21	2	1	200mg	100 mg
MOM11	1	1	300mg	–
MOD11	1	1	200mg	100 mg
MOM23	2	3	300mg	–
MOD23	2	3	200mg	100 mg
Myristic acid + Linoleic acid				
MLM21	2	1	300mg	–
MLD21	2	1	200mg	100 mg
MLM11	1	1	300mg	–
MLD11	1	1	200mg	100 mg
MLM23	2	3	300mg	–
MLD23	2	3	200mg	100 mg

Study of Physical Form of Nanoformulations

The lipid nanoparticles, particularly those made from fatty acids, may undergo recrystallization leading to the formation of a gel-like phase. Therefore, all NLC formulations were observed for gel formation and, if gel formation occurred, its onset time was recorded. Next, a light transmittance experiment was done to evaluate physical form. Nanoformulations were suitably diluted, and light transmittance was measured at 500 nm. As compared to liquids, solid

particles would show less transmission due to opacity and scattering of light [16].

Size and Zeta Potential

Size and zeta potential are integral to the performance of nanomaterials in terms of safety and efficacy [17]. MNs stable after 1 hour were analyzed for size and zeta potential by zetasiizer (ZS90, Malvern, UK). Samples were suitable diluted with water and taken in cuvettes. Measurements were taken at an equilibration time of 2 minutes and a temperature of 25°C.

Compatibility Studies

The chemical compatibility of lipids and surfactants was evaluated by ATR-FTIR studies. The FTIR spectra of all formulation components and MN were taken. The chemically compatible MN would show characteristic peaks of surfactants and fatty acids. In case of incompatibility, some peaks of formulations components either decrease in intensity or disappear completely from the spectra of formulations [18].

Morphology studies

The morphology of NLC formulations was studied by using electron microscopy (FE-SEM, JSM 7500, Jeol, Japan). Samples were loaded into silicon wafers and negatively stained. After drying in air, samples were loaded into SEM and images were taken.

Antimicrobial Activity

The bacterial stock culture was prepared by the McFarland method. After 24 hours, the inoculum was prepared at a cell density of 1.5 X 10^8 CFU/ml. Antimicrobial activity was evaluated by the good diffusion method Dogruoz and Karagoz (2008) with slight modification [19]. 20 ml of sterilized Muller Hinton Agar was transferred into Petri dishes and solidified. Bacterial culture was streaked on the agar surface and allowed to dry. Then, four holes of 6 mm diameter were made by sterile corn borer in dried agar in each petri dish. 20 µl of standard ceftriaxone or nanoformulations were added to wells and incubated at 37°C. After 24 hours of incubation, Petri plates were taken out, and the zone of inhibition was measured. To elucidate the mechanism of antimicrobial activity, the good diffusion method was performed with slight modification. In this method, all steps were performed as described previously except that bacterium were streaked onto Petri plates and allowed to grow for 24 hours at 37°C. Then, samples were added to the wells and incubated for 24 hours at 37°C. Zone of inhibition was measured to evaluate antimicrobial activity.

Results

Synthesis of Myristic Acid Nanoparticles

Myristic acid did not form SLN due to crystallization as soon as it is cooled down to room temperature. Crystallization leads to the deformation of SLN and exposes new surfaces that are not coated with the surfactant. These hydrophobic surfaces stick to the other particles leading to the formation of a gel-like phase. In this study, we found that hot melt emulsion was cooled down to form a gel like phase, which meant that myristic acid SLN could not be formed at room temperature. To overcome this problem, we made a heterogeneous lipid mixture of myristic acid with oleic and linoleic acid at a different proportion. Nanoformulations made from heterogeneous lipid mixtures with a lower proportion of liquid fatty acid modestly improve stability, i.e., crystallize within a few hours to form a gel. However, nanoparticles with higher lipid proportion were stable. Nanoparticle's stabilization was also achieved by using a surfactant system consisting of hydrophilic and lipophilic surfactant. Although pure myristic acid nanoparticles with a double surfactant system were unstable, myristic acid heterogeneous lipid mixture was stable when nanoformulation with a double surfactant system. Although low liquid proportion nanoparticles remained stable for several hours, they formed gel after 12 hrs when stored overnight. Lipid nanoparticles prepared with a higher ratio of myristic acid and oleic or linoleic acid (1:1 and 2:3) were stable with both one and two surfactants.

The Physical Form of Nanoformulations

Light transmittance of nanoformulations was dependent upon liquid content (Table2). Light transmittance was
highest at higher liquid content of 2:3, and it decreased as the liquid proportion was lowered to 1:1. This suggests that nanoformulations exist as liquid nanoemulsions when liquid content is higher than solid content. However, they existed as solid NLC when the ration of the solid and liquid phase was 1:1 and light transmittance was higher in this case.

Table 2. Characteristics of as Prepared-Nanoformulations

Formulation code	Gelling time (hrs)	Light transmittance (%)	Physical form (37°C)
MM	0	–	Gel
MD	0	–	Gel
MOM21	1	–	Gel
MOD21	12	–	Gel
MOM11	–	63.6	NLC
MOD11	–	70.2	NLC
MOM23	–	80.1	Emulsion
MOD23	–	89.1	Emulsion
MLM21	1	–	Gel
MLD21	12	–	Gel
MLM11	–	59.6	NLC
MLD11	–	73.2	NLC
MLM23	–	83.4	Emulsion
MLD23	–	88.8	Emulsion

Size and Zeta Potential of Nanoformulations

Nanoformulations that showed instability and gel formation were excluded from further characterization. All stabilized nanoformulations were around 100 nm or less, although linoleic acid-containing nanoformulations were smaller in size than those containing oleic acid (Table 3). Similarly, double surfactant system nanoformulations were smaller and showed more negative zeta potential than mono-surfactant nanoformulations.

Table 3. Characteristics of as Prepared-Nanoformulations

Formulation code	Size (nm)	PDI	Zeta potential (mV)
MOM11	109	0.1	-37.5
MOD11	198	0.2	-34
MOM23	106	0.2	-40.5
MOD23	182	0.2	-32
MLM11	281.7	0.1	-34
MLD11	277	0.1	-27
MLM23	362.5	0.2	-36.5
MLD23	556	0.2	-31

Chemical Compatibility of Lipids and Surfactants

Nanoformulations are prepared by heating lipids and an aqueous solution of surfactants. Therefore, it was possible that lipids, consisting of saturated and unsaturated fatty acids, may react with surfactant.

The comparison of FTIR spectra of all materials and nanoformulations exhibited characteristic peaks of myristic acid, oleic acid, linoleic acid, Brij 58, and Span 80, and no new peak was observed (Figure 1). Hence, lipids and surfactant exist intact in nanoformulation without chemical reaction.

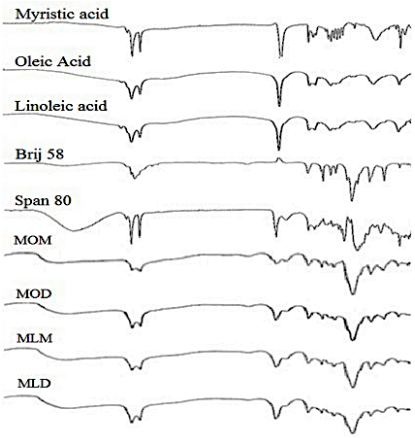
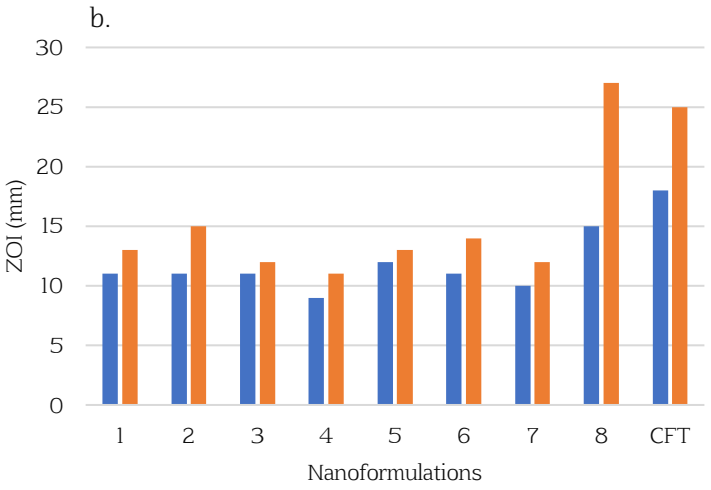


Figure 1: FTIR Spectra of MN Formulations Prepared at 1:1 of Myristic Acid and Oleic Acid

Antimicrobial Activity

Nanoformulations were evaluated against both Gram-positive and Gram-negative bacteria (Figure 2). MN was found to be effective against both tested strains of Gram-positive bacteria, i.e., *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*). Although MN showed a mixed degree of antimicrobial activity, MN containing linoleic acid were more effective against both Gram-negative bacteria. Overall, MN8 showed the highest activity against all tested bacteria.

In the second experiment, we found that all MN showed minimum to no effect on already grown bacterial colonies. This suggested that MN exert antimicrobial activity mainly by inhibiting the growth of the bacteria. Only MN6 showed ZOI against all tested bacteria. MN8 was active only against *B. subtilis,* and it was highest among all MNs.



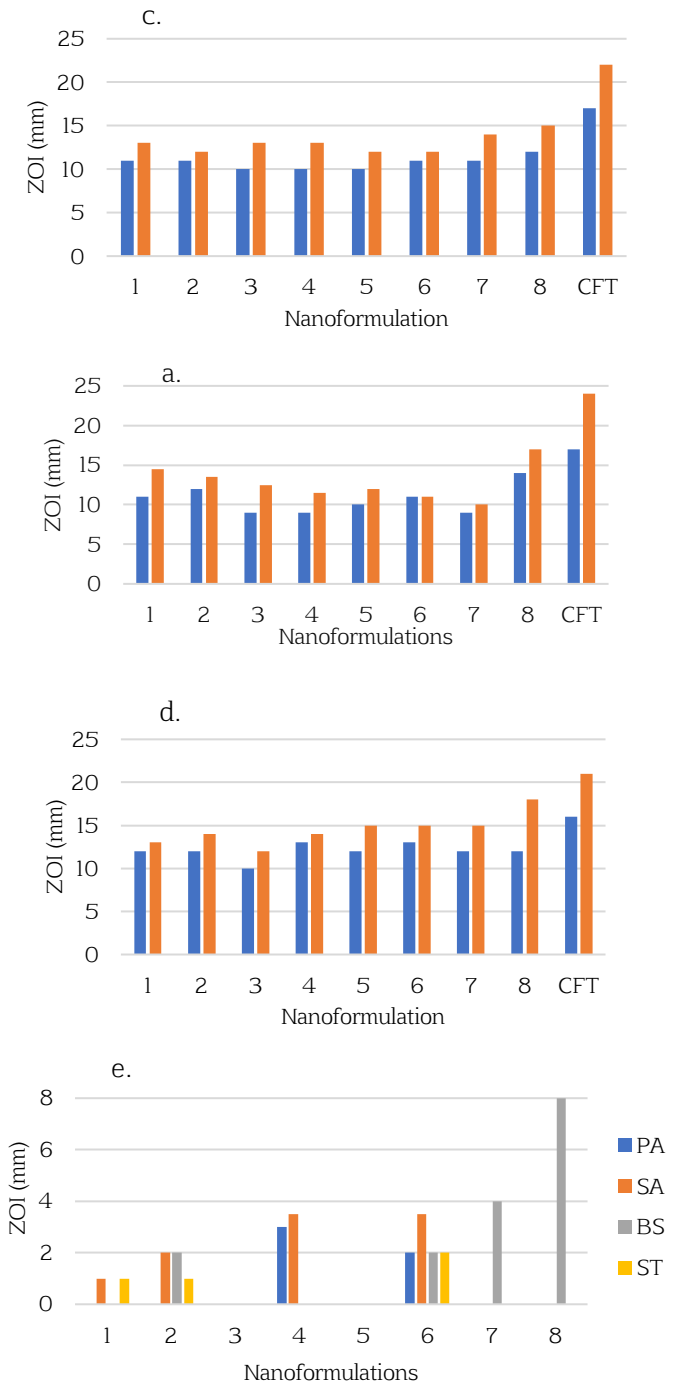


Figure 2: Antimicrobial activity of the 8 MN formulations against a) S. sureus, b) B. subtilis, c) S. Typhi, and d) P. aureginosa, by well diffusion method. e) exhibits antimicrobial activity against bacteria 24 hours grown cultures of bacteria. Scale bar is different for graphs.

Discussion

Antimicrobial properties of free fatty acids are widely reported and have been supported by studies conducted in different microbes such as bacteria and fungi [11, 20]. Although the literature on the comparative efficacy of FFA is controversial, it is believed that FFA of 12 and 14 carbon chains are most active. Previously, some papers have been published describing the antimicrobial activity of the nanoformulations of free fatty acids [14, 15]. However, these papers used a combination of different active antimicrobial materials in the nanoformulations, which made it difficult to define the role of free fatty acids. Therefore, this study was designed for the synthesis nanoformulation of myristic acid to evaluate their antimicrobial properties.

We found that nanoformulations of myristic acid cannot be prepared due to instability conferred by their crystalline structure. In this study, stability was improved by the addition of liquid fatty acids such as oleic acid and linoleic acid that also possess antimicrobial properties. Oleic acid and linoleic acid are C18 fatties with one and two double bonds, respectively [21]. Furthermore, stability was increased when two surfactants system was used. The stability enhancement effect of two surfactant systems has been reported previously, although the exact mechanism is still ambiguous. One proposed mechanism is that the hydrophilic and lipophilic surfactants would arrange themselves in the opposite direction at oil-water interphase. This improved stability of surfactant coating on the nanoparticles [22]. Another possible explanation is that the hydrophilic surfactants have larger polar head groups that are protruding into the aqueous phase. The smaller hydrophobic surfactants can fit in the spaces between hydrophilic surfactants and provide a more compact surfactant coating [23]. Both mechanisms would prevent the formation of hydrophobic patches upon recrystallization of lipid chains and prevent aggregation. Light transmission studies indicated that MN with myristic acid to liquid fatty acid ratio of 1:1 are solid NLC, whereas those with 2:3 are liquid nanoemulsions due to higher light transmission in later case [16].

Although MN containing myristic acid and liquid fatty acid in both 1:1 and 2:3 was stable, we selected the former due to the higher amount of myristic acid and higher stability of solid nanoformulations. These MN were <100 nm, which may also have been reported previously to prevent protein-mediated adhesion of bacterial biofilms [24]. FTIR spectrum of selected MN showed the integrity of fatty acids in MN without chemical modification. Availability of free fatty acids on MN surface was also confirmed by negative zeta potential, which would impart antimicrobial properties [14].

MN showed antimicrobial activity to varying extent against both Gram positive and Gram-negative bacteria. In MN, the predominant mechanism of antimicrobial activity is the damage to bacterial plasma membrane and cell wall. Fatty acid chain gets inserted into plasma membrane which leads to formation of pores. Ultimately, cell membrane is damaged which also compromises membrane associated processes and leakage of cell contents [11]. In biomedical research, myristic acid has also been used to attach enzymes to cell membranes. Myristic acid polar head group is chemically crosslinked with the enzyme by a process known as myristylation. The carbon chain would insert into plasma membrane of cell and anchor the enzyme in membrane [25]. Therefore, it could be assumed that the MN, once anchored on bacterial plasma membrane by the myristic acid carbon chains, might exert strong pull to the membrane due to large size of MN, hence, leading to disruption of the plasma membrane. MN8 was found most effective of the MNs against all tested bacteria and its activity was comparable to standard antibiotic in case of *B. subtilis* and *P. aureginosa*. With the exception of MN8, the MNs containing liquid oleic acid showed slightly superior activity than linoleic acid containing MNs in Gram positive bacteria ($p<0.05$ for *S. aureus*). It has been reported previously that unsaturated long chain fatty acid possesses antimicrobial activity as compared to most saturated long chain fatty acids with minimal antimicrobial activity. Therefore, oleic acid and linoleic may also be responsible for antimicrobial activity of MN. The higher activity of linoleic acid MN may be justified on the basis of presence of one double bonds more than oleic acid [26]. On the other hand, NE formulations were more active against Gram negative bacteria as compared to NLC formulations ($p<0.05$ for *P. aureginosa*). NE are liquid formulation, and they can show higher contact surface due to deformable nature. This will allow more fatty acid on MN surface to interact with bacterial membrane and more pronounced antimicrobial activity. The results of antimicrobial activity were variable in terms of surfactant system regardless of its remarkable effect on surface charge.

In the second antimicrobial activity experiment, MN showed low activity in terms of ZOI against 24 hours grown bacteria cultures. MN6 showed activity against all tested bacteria. MN8 showed activity only against *B. subtilis,* but it was the highest ZOI recorded in this experiment. This result is in agreement with the previous experiment. The lower bactericidal activity in this experiment may be due to a large number of bacteria per colony that are difficult to kill. Also, this study design is not commonly used due to complications in controlling the growth of bacteria and counting dead bacterial cells in a colony.

Conclusions

Myristic acid nanoformulations (MN) have been prepared by the addition of oil or the use of two surfactant system to overcome the problem of instability. MN with an equal proportion of myristic acid and oil, either oleic acid or linoleic acid, formed NLC, whereas those with higher oil proportion formed NE. Both size and zeta potential of MN were affected by the amount of and nature of the oil and surfactant system. All MN showed antimicrobial activity against both Gram-positive and Gram-negative bacteria. Oleic acid-containing MN was active against Gram-positive bacteria, whereas NE was more active against Gram-negative bacteria. MN8 containing myristic acid and oleic acid (2:3) and two surfactant system (Brij58 and Span 80) was most effective, and its ZOI was comparable to that of standard antibiotic. Hence, MN exhibits broad-spectrum antimicrobial activity and appear as a suitable candidate for drug-free treatment of infectious agents.

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