

Development of Biodegradable/Biocompatible Nanoliposome-Encapsulated Antimicrobial Essential Oils for Topical Creams and Gels

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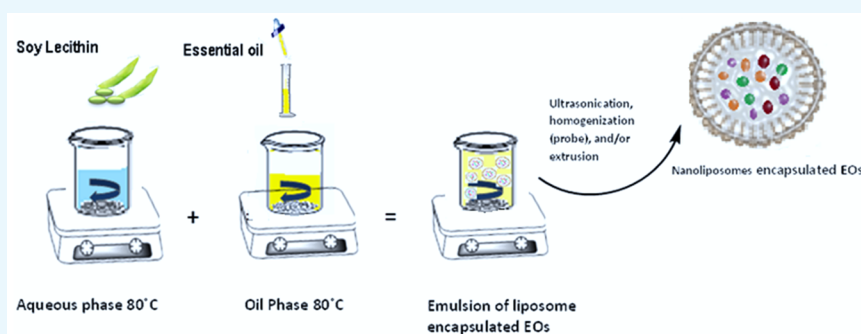


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ABSTRACT: Nanoencapsulation with safe materials improves delivery, stability, and activity of bioactive components. We report a novel safe, and effective method for the development of encapsulated antimicrobial essential oils (EO) for topical creams and gels. The method developed features three aspects that, to our knowledge, had not been previously demonstrated: (1) use of novel liposomes (LPs) to encapsulate EOs, (2) use of the EOs to replace synthetic organic solvents that are potentially toxic and/or leave harmful residues, and (3) an encapsulation process at temperatures below the boiling point of water. The LPs were made from soy lecithin, phytosterol, and α -tocopherol (vitamin E) that were synthesized using the EOs as the solvent. The liposomes were converted to nanoliposomes (NLPs) through a series of sonication, homogenization, and extrusion steps. Transmission electron microscopy indicated that the NLPs alone and nanoliposome encapsulated EOs (NLP-EOs) were spherical in shape with sizes ranging between 50 and 115 nm diameter and with negative zeta potentials ranging from -34 to -43 mV. There was no significant heavy metal contamination [As, Pb, Cd, Hg] based on inductively coupled plasma (ICP) mass spectrometry MS analyses. Nearly complete EO encapsulation (95% encapsulation efficiency) was achieved and confirmed by GC/MS. Three of the NLP-EOs made of various essential oils were used to make topical formulations (cream and gel) which exhibited antimicrobial activities against *Escherichia coli* (Gram negative) and *Bacillus subtilis* (Gram positive) bacteria. The creams with NLP-EOs were as active against the two bacteria in the antimicrobial assays as the conventional antibiotic Kanamycin that was used as positive control.

INTRODUCTION

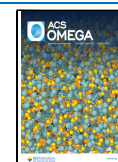
The growth in the use of nanoparticles (NPs) in life science applications has been uphill for more than a decade, with new applications in medicinal, biological, and industrial areas appearing regularly.¹ However, health and environmental concerns associated with the use of NPs have also arisen.² Thus, there is a need for biocompatible/biodegradable (BD/BC) nanoparticles that could provide the desirable properties associated with nanomaterials which are free from health and environmental concerns. Generally, BD/BC NPs can be made from natural compounds, typically those that are commonly found in plants, humans, and animals that are generally regarded as safe for such use. For example, the lactic/glycolic copolymers proposed for use in BD/BC NP drug delivery^{3–5} are derived from glycolic and lactic acids. The use of such

materials, which are generally regarded as safe or “natural”, can come with some trade-offs, however. Nontoxic, biodegradable natural materials are often less effective in applications than are synthetic materials. For example, for pharmaceuticals, natural plant materials are generally less active on a per unit-mass basis than synthetics.⁶ One potentially powerful avenue for dealing with the tendency of natural products to have lower bioactivity than synthetic compounds is improved delivery. NPs offer an

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avenue for potentially improving delivery of bioactive compounds based on modification of membrane transport, solubility, stability, or other properties that could make a smaller dose more effective. For example, an increase in the antimicrobial activity of plant components upon encapsulation in liposomes, albeit not necessarily in nanoparticles, has already been reported.⁷ Delivery of plant-derived bioactive compounds using NPs may represent an ideal combination of a delivery vehicle and the bioactive compound if both are safe, effective, biocompatible, and biodegradable. The use of nanoenhanced plant-derived antimicrobials with activity suitable for food preservation has been reviewed recently.⁸ While the simple inhibition of bacterial growth reported in their study may represent a less challenging application than more complex therapeutic uses, the enhancement of EO activity based on nanodelivery has clear implications.

Essential oils (EOs) are important potential sources of compounds having biological or therapeutic activities, including antimicrobial activity. EOs are plant distillates or extracts having defined (sometimes loosely) combinations and concentrations of specific low molecular weight compounds which are typically hydrophobic and volatile. Most of these bioactive compounds are present in small percentages of the overall composition, but a few may dominate, sometimes with a single compound representing more than half of the total material in the extract. The biological activity of these plant EO extracts has been described in traditional medicine or in alternative remedies based on the properties of the mixture rather than individual components. Reports date back to ancient times with texts describing Greek herbal medicine having been translated and re-edited for more than two millennia.⁹ Because these mixtures have been used for long periods of time, the activity has become associated with the complex EO mixture rather than individual components. In some cases, the activity of EO extracts is not well understood. Differences between the efficacy of the extracts and that predicted based on individual components therein may reflect synergistic action. In some cases, activity contributions or synergisms from minor components may be important. In the absence of a clear understanding of the specific individual components responsible for activity, or the possible synergistic effects, it has been easier to study the EOs themselves rather than their isolated or purified individual components, especially if a hypothesis is associated with a traditional or alternative medicine.¹⁰

EOs are generally considered to be at least somewhat antimicrobial based on published studies. For example, it has been reported that vapors from EOs can impart antifungal activity to edible films.¹¹ Another recent study has focused on applications involving more general antibacterial activity.¹² Because EOs have been protecting their plant sources against microbes over the millennia, their lower activity is perhaps offset by the notion that this activity will likely last and is independent of the mechanisms used by traditional antibiotics which are rendered ineffective quickly by microbial evolution. The potential of the EOs for use as antimicrobials is such that many new methods have been proposed recently to test these plant-based materials for their activity. New and reconsidered methods specifically for evaluation of plant-derived antimicrobials were recently reviewed.¹³ It is perhaps also noteworthy that plant EOs could simultaneously exhibit antimicrobial activity and other beneficial bioactive properties such as antioxidant activity.¹⁴

Some of the drawbacks of EOs include instability with respect to environmental factors (pH, O₂, moisture, temperature, and light), difficulty of administration, and loss of material by evaporation.¹⁵ These problems might be mitigated with the right delivery vehicle. This has led to an interest in encapsulation as a method for modulating the release of EOs, increasing their stability, and perhaps even mediating issues with solubility, delivery, or volatility. Encapsulation techniques have been used to provide the same sorts of benefits when used for delivery of bioactive proteins and peptides.¹⁶ Liposomes have been shown to be highly adaptable for encapsulation of active materials for medical, food, and other applications for both synthetics and natural materials.^{8,16} Their biological and technical advantages over other forms of delivery for actives are so profound that it was claimed in a recent review that they might well be considered the most successful drug-carrier yet developed.¹⁷ They can improve the distribution and the effectiveness of the encapsulated bioactive agents, reduce their toxicity, and even increase selectivity. Moreover, because they have a unique structure, containing an aqueous central core and an amphiphilic lipid bilayer, they can encapsulate amphiphilic, hydrophobic, and hydrophilic molecules.¹⁸ In a few studies, liposomes have already been combined with EOs or their components to access the effectiveness of anticipated benefits. For example, Liolios et al.¹⁹ reported that carvacrol and thymol showed increased antimicrobial activity when encapsulated with liposomes for delivery. They provided evidence of the effectiveness of the liposome encapsulation using carvacrol and thymol as model preservative agents for pharmaceutical, cosmetic, and food manufacturing applications. In addition, Sinico et al.²⁰ reported that the antiviral activity of an EO from *Artemisia arborescens* L. was enhanced when delivered in liposomes to infected cells. Likewise, upon liposome encapsulation, eucalyptus EO from leaf tissue exhibited enhanced antifungal activity.²¹ It is likely that this enhanced activity is associated with improved delivery of the monoterpenes in the EOs. However, encapsulation could also potentially reduce loss of the active component and, hence, maintain its activity by minimizing evaporation or air oxidation. The antimicrobial activity of EOs themselves had been attributed to interactions between constituent monoterpenes and the microbial cell wall.²² In that study, monoterpenes induced leakage of model biomembranes as evidenced by fluorescent markers. This and other evidence led to the conclusion that major changes in the lipid components of the microbial cell wall was an important mechanism for antimicrobial activity of monoterpenes. We presume therefore that the enhanced activity after EO encapsulation is based at least in part upon improved delivery of antimicrobial monoterpenes in EOs into the microbial cell wall.

Nanoparticle delivery systems have been developed for skin care and dermal treatment. However, the specific NP interactions with the dermal barrier are not fully understood, and data from model animal systems regarding deep dermal penetration is still controversial at best.²³ In the simplest models it had been assumed that the small sizes of NPs would enhance penetration through skin layers. However, recent research has led to more complex models, even including concepts such as classical and deformable liposomes.²⁴ Skin-penetration properties of nanoparticles can also be different in individuals based on age or skin health.²⁵ Despite these uncertainties, nanoparticles have useful characteristics for topical applications. For example, in some applications it is

desirable to have a relatively constant concentration of the bioactive.^{26,27} Recent studies have looked at alternatives to simple or liposomal nanoparticles for delivery, including the use of nanocomposites.²⁸ We propose an alternative to improving delivery using biodegradable biocompatible nanoparticles rather than composite NPs. This approach combines the capabilities of biodegradable and biocompatible nanoparticle delivery approach with the simple traditional dermal delivery method, namely “classical” creams and emulsions prepared using generally regarded as safe (GRAS) compounds. The idea is to enhance the delivery of the active EOs by encapsulating in NLPs (nanoliposomes) and using it in cream and/or gel for the delivery of the actives as well as using the nonencapsulated EOs as a solvent in the formulation of the cream or gel. As noted below, we also demonstrated the use of the nonencapsulated EO as replacement for organic solvents which are critical chemical components usually needed in the manufacturing process. In this way, the EOs contributed some activity as a nonencapsulated component in the cream or gel because of its solvent role, enhancing the activity of the nanoencapsulated EOs while replacing expensive synthetic organic solvents that are potentially toxic. The elimination of expensive and potentially toxic materials from the manufacturing process by using the EOs which are also the bioactive antimicrobial components is a novel approach to enhancing activity and environmental and health safety, as well as reducing manufacturing costs.

Creams and emulsions, either oil in water (o/w) or water in oil (w/o), have been used to deliver or modulate the release of the active ingredients while at the same time improving skin moisturization.^{29,30} These simple delivery systems have some limitations, but these are well understood, and their use is now widely accepted. Many formulations exist whose efficacy and safety are both well established.²⁹ Moreover, they are also suitable for delivery of bioactives, either separately or using NLPs within the cream or emulsion. Our novel approach involves codelivery. Suspension of NLPs for delivery into a classical cream or emulsion topical formulation could combine the benefits of both systems; preserving the functionality (and other benefits) specific to the cream or emulsion while adding functionality from the NLPs delivered bioactive components as well. Such a dual delivery approach might mitigate some of the limitations of either delivery system when used alone, and this approach is also likely to be safer than some of the nano formulations that have been proposed.²⁶

Whether delivered independently or in a cream or gel, the size of a liposomal delivery vehicle will affect its functional characteristics as well as the accumulation of encapsulated active components on the skin. The useful size range available for efficient delivery of liposomal particles is affected by the immune system, namely the mononuclear phagocytic system.³¹ Interactions between foreign materials and macrophages are very much size dependent.³² Methods have been developed to mitigate the immune system response to liposomal delivery vehicles such as surface modification with biocompatible polymers.³³ An alternative approach for minimizing liposomal-particle interactions with the immune system is the use of components that mimic the skin. Thus, one of the aims of this study was to produce BD/BC encapsulated EOs in all-natural NLPs to eliminate interactions with macrophages and avoid the need for surface modification.

The bioactive agent(s) selected for this study was (were) chosen for antimicrobial activity. An emerging therapeutic

challenge of almost pandemic proportions is the growing multidrug resistance of bacterial and fungal organisms as the anticipated pipeline of conventional antibiotics is exhausted.³⁴ While synthetic antimicrobials are dwindling in their effectiveness against microbial infections, EOs with antimicrobial, antiviral, and antifungal properties are emerging as possible alternatives.^{35,36} These antimicrobial properties of EOs are well-known and have been explored for many years, but a major limitation on their effectiveness has been their lower activity compared to standard antibiotics. As resistance to traditional antibiotics increases, EOs will continue to be evaluated as either supplements or replacements as traditional therapies. EOs could be produced in quantity, just like other agricultural products making them a sustainable resource. Various plants produce antimicrobial EOs through their leaves, flowers, fruits, roots, and even the stems and bark. These have been extracted into complex mixtures along with other components which could dilute the antimicrobial effectiveness. A balance between the low antimicrobial efficacy of EOs and the cost of isolating the individual bioactive components has probably the most compelling cause which impeded the more widespread use of EOs as antimicrobials.³⁷

As noted above, liposomes, including nanoliposomes, have been shown to have advantages for delivery of bioactive agents.²⁶ Nevertheless, problems with preparation and encapsulation remain to be fully worked out, and their preparation is not yet routine.³⁸ Some of the proposed methods are either inappropriate for human applications or are difficult to implement on an industrial scale. Many procedures rely on expensive organic solvents³⁹ or require using thin film techniques and freeze-drying to avoid aggregation and precipitation.⁴⁰ Expensive instruments are often needed to provide specific experimental conditions (pressure, temperature) or a supply of inert gas.^{41,42} In this study, we developed a novel, simple and inexpensive method for the preparation of biodegradable biocompatible NLPs that were used for encapsulating various blends of EOs. The NLP-EOs were used as ingredients of topical cream and gel formulations which were evaluated *in vitro* for antibacterial activity.

■ MATERIALS AND METHODS

Materials. Chemicals were obtained as follows: soy lecithin and phytosterol from Puritans Pride (Oakdale, NY); disodium hydrogen phosphate, sodium chloride, potassium chloride, and monopotassium phosphate from AMRESCO (Solon, OH); ethylenediaminetetraacetic acid disodium salt dihydrate and potassium sorbate from Lotion Crafters (East Sound, WA); olive oil, stearic acid, vegetable glycerin, tea tree oil, lemon oil, α -tocopherol (vitamin E), clove oil, and eucalyptus oil from JEdwards International (Braintree, MA); peppermint oil from Piping Rock (Ronkonkoma, NY); Oliwax from Hallstar (Chicago, IL); nitric acid, ethyl alcohol, and neutral phosphotungstic acid from VWR Scientific (Radnor, PA); cetearyl alcohol, xanthan gum, and coconut oil from Nature's Oil (Aurora, OH); kanamycin from TCI (Portland, OR); nutrient Agar from Home Science Tools (Billing, MT); and Cola Fax CPE-K from Colonial Chemical, Inc. (South Pittsburgh, TN). Reconstituted from concentrate 99.8% pure aloe vera juice from Fruit of the Earth Inc. (Fort Worth, TX) and CARMEX lip balm (Carmex Inc. Franklin, WI) were purchased from a local retail chain. A proprietary blend of essential oils, PERL, was obtained from Zystein (Fayetteville,

AR). Two new blends, PETL and PETC, were also prepared at Zystein for this study.

Nonpathogenic *Escherichia coli* was obtained from Carolina Biological Supply (Burlington, NC). Nonpathogenic *Bacillus subtilis* was gifted by Dr. Guillermo Tellez-Isaias from the JKS Poultry Health Laboratory, University of Arkansas (Fayetteville, AR).

Preparation of EO Blends. Three blends of EOs were prepared as follows: PETC contained peppermint, eucalyptus, tea tree, and clove oils (17:11:1:1, v/v) and was diluted with coconut oil (30:70) v/v, respectively; PETL containing peppermint, eucalyptus, tea tree, and lemon oil was prepared using the same ratios (v/v) in coconut oil. A commercial antimicrobial proprietary blend PERL (Zystein, AR, USA) containing peppermint, eucalyptus, rosemary, lavender, wintergreen, lemon, and limonene in coconut oil (30:70) was used as the standard of comparison for antimicrobial activity. Eucalyptus oil by itself (E) was used to test encapsulation efficiency at 30:70 v/v with coconut oil. The blends of essential oils were characterized for their volatile components by headspace GC/MS analysis.

Liposome Encapsulation. Liposome-encapsulated EO blends (PETC, PETL, PERL, and E) were prepared as follows: mixture A was made with soy lecithin (5%) added to 10 mM PBS buffer, (56% v/v) and heated to 80 °C with constant stirring. Mixture B was prepared from phytosterol (3%) and α -tocopherol vitamin E (0.5%) and enough PBS buffer to make a total of 100% when the 25–30% EO blends (PETC, PETL, PERL, and E, respectively) are finally added. When the temperature of mixture B reached 80 °C, the EOs were added respectively shortly before removal of the source of heat. When both mixtures reached 80 °C they were combined under vigorous stirring with the heat turned off. Sufficient olive oil (5 mL for PETC and for PETL; 10 mL for PERL) was added to produce a dense emulsion. At 40 °C, Na₂EDTA (0.5% w/w) and potassium sorbate (0.5% w/w) were added as preservatives. The resulting emulsion contained the liposome encapsulated PERL, PETL, PETC, or E.

The liposome encapsulated EOs in the microparticle size range based on observations under an optical microscope (data not shown) were subjected to ultrasonication, homogenization, and extrusion treatment to create the NLP-EOs of PETL (NLP-PETL), PETC (NLP-PETC), and the PERL (NLP-PERL). In brief, the procedures were as follows. Vials containing 10–20 mL of emulsion were placed in an ultrasonic water bath at 20 kHz for a total of 1 h at 37 °C using 10 min pulses with 3 min breaks. Homogenization (SCIOLOGEX, D-160 Homogenizer) was carried out at 14000 rpm for 3 min, at 18000 rpm for 2 min, at 26000 rpm for 1 min, and at 30000 rpm for 1 min with 1 min break between each step. The NLP-EOs were extruded through 100 nm pores using an Avestin Extruder (Ottawa, Canada) at 45 °C. Extrusion was repeated at least 4 times in both the forward and backward directions through the membrane at 600 psi extrusion pressure.

Encapsulation efficiency was estimated using NPs prepared with one of the oils present in all three blends, eucalyptus oil. The eucalyptus oil (E) was prepared at 30:70 v/v with coconut oil to mimic the preparation using the other more complex EO blends. Head-space GC/MS sampled above NLP-E after the release of the eucalyptus oil from the NLP was compared with a standard curve for headspace above nonencapsulated E dissolved in coconut oil at 150, 75, 37.5, 18.75, and 9.37 mg of E per mL. This range covered the expected concentration of

eucalyptus oil after release from the NLP-EOs which was about 65 mg E/mL assuming complete encapsulation and recovery of the NLP-EOs after synthesis. The nonencapsulated E in the NLP-EOs was removed beforehand by ultracentrifugation (Avanti J-E - Beckman Coulter, Inc. USA) which was used to precipitate the NLP-EOs at 20,000 rpm for 1 h at 4 °C. The supernatant was discarded, and the pellet (NLP-EOs) was dissolved in ethanol and then subjected to ultrasonication for 3 h. The resulting material was subjected to centrifugation again at 6000 rpm for 15 min on the assumption that this last step, in the presence of ethanol, would disrupt the NLP-EOs and release the free oil into the suspension for detection by headspace GC/MS. It was assumed that the released oil would equilibrate into the headspace in the same manner as the free oil in the standards.

NLPs Characterization. In order to establish the shape and the sizes of the NLP-EOs, they were subjected to transmission electron microscopy (TEM) (JEM-1011, Peabody, MA) at 100 kV and W/LN2. One drop of each diluted sample was placed onto a carbon-coated copper grid and stained with 1% neutral phosphotungstic acid solution for 2 min. Excess stain was removed by wicking with filter paper. The mean hydrodynamic diameter size and zeta potential of the NLP-EOs were measured using dynamic light scattering (DLS) (Brookhaven Instruments Corporation, Holtsville, NY) at 25 °C.

The NLP encapsulation of the essential oils was expected to reduce the instability of EOs when exposed to heat, air, and light oxidation. To assess the contribution of NLP encapsulation to the stability of the EOs, the stability of PETC was used as a model. The headspace GC/MS profile of the PETC coconut oil blend was obtained for reference and the profile for NLP-PETC after dilution (1:2) in coconut oil was measured on days 0 and 30.

Preparation of BD/BC Topical Cream and Gel with NLP-EOs. Three antimicrobial creams (ACs) plus control samples were prepared using EOs and NLP-EOs. These topical formulations were designed to model creams used for human use. Like the NLPs and the EOs, the creams were prepared using natural and biodegradable reagents. The general AC formulation process is described briefly as follows using % w/v solids or % v/v for liquids out of a total of 100% for the oil phase and the water phase, respectively. An oil phase was prepared using 7% of coconut oil and 3% of EO blend with constant stirring. To this was added 3% w/v cetearyl alcohol, 2% stearic acid, and 5% OLIWAX, sequentially. This oil phase was heated to 75–80 °C with constant stirring. An aqueous phase was prepared by mixing 3% of aloe vera, 3% glycerin, and 3% Cola Fax CPE-K with 63% distilled water and heated to 75 °C with constant stirring. When all solids in both phases were dissolved, the hot plate was turned off, and the two phases were combined with continuous stirring until a uniform emulsion was formed. The mixture was cooled at room temperature with constant stirring to 40 °C, after which 7% of NLP-EO, and 1% of preservative (0.5% each of Na₂EDTA and potassium sorbate) were added to produce the AC containing both free EOs (from the oil phase) and NLP-EOs. The ACs were transferred into plastic jars after cooling. Control ACs that did not contain any EOs (blank, negative control) or that contained EO without NLP encapsulation (same total amount of EO, positive control for EOs) were prepared similarly.

Three antimicrobial gels (AGs) were produced with each of the EOs and NLP-EOs as described for the ACs using % w/v

solids or % v/v for liquids, respectively. Xanthan gum (2%) was added to 92% of distilled water previously heated to 80 °C with constant stirring until complete dissolution. To this was added 2% glycerin before being cooled to 60 °C, after which the following were added: 2% EO blend, 1% NLP-EOs, and preservatives (0.5% each of Na₂EDTA and potassium sorbate) with constant stirring. The mixture was cooled to room temperature before being transferred to a plastic container. Note that the amount of NLP-EOs and EOs in the gel were less than for the ACs because of the inability to produce a gel from higher quantities of NLP-EOs.

Physical Characterization of the Topical Creams and Gels. The AC and AG formulations were evaluated using simple tests for texture uniformity, homogeneity, pH, phase separation, and viscosity. The preparations were first inspected visually for texture uniformity and homogeneity. Texture uniformity and homogeneity were evaluated further using a manual pressure as follows: pressing a small amount (about 0.25 mL) of the topical preparation (cream or gel) between the index finger and thumb. Proper formulations gave no sensory evidence of lumps, grit, or suspended solids. More quantitative evidence regarding the homogeneity of the preparations and presence of heterogeneous particles was obtained using an optical microscope at 10 X and 40X (American Scientific). A 1.0 g sample of each cream or gel was added to 25 mL of distilled water, and the pH was measured after shaking using a meter calibrated with buffer solutions at pH 4.0 and 6.8 (APERA, SX-610).

Stability of the Topical Creams and Gels. To assess the ease of phase separation, each AC and AG sample was exposed to centrifugation at 3000 rpm for 30 min, after which any visual separation between the oil and water layers was noted. The viscosity of each AC and AG was measured using a Brookfield viscometer model DV-I prime (TC-550, Middleboro, MA) to test the thixotropic (thinning) behavior of the AC and AG. Thixotropy is time dependent and under certain circumstances over time the cream and gel may become less viscous and thinner.⁴³ The concentric cylinder spindle size was #40 rotating at 2.5, 5, 10, 20 rpm for creams and 2.5, 5, and 10 rpm for gels at 25 °C.

Microbial Contamination Evaluation of the Topical Creams and Gels. Microbial contamination of the ACs and AGs were evaluated following protocols described in the [Methods](#) for cosmetics.⁴⁴ The ACs and AGs were diluted to 10⁻¹ to 10⁻³. The 0.1 mL samples of diluted ACs and AGs were spread onto nutrient agar plates and incubated at 37 °C. The plates were checked after 24 h and after 72 h. The number of colony-forming units in each sample was enumerated.

Biodegradability Evaluation of the Topical Creams and Gels. To establish the biodegradability of the ACs and AGs, a 1 g sample of each was diluted with 3 mL of sterilized distilled water. A 1 mL sample of this mixture was spread over nutrient agar on a Petri dish. This was immediately exposed to natural outdoor environment for about 1.5 h before the lid was replaced. The plates were kept at 37 °C for 24–48 h before inspection. This exposure to outdoor air was repeated daily until significant growth of opportunistic airborne bacteria and/or fungi (biodegradants) was observed in each plate.⁴⁴ Nutrient agar plates without ACs or AGs were used as control. In this test, the extent and speed of colony formation was taken as a measure of biodegradability.

Heavy Metal Contamination Evaluation of the Topical Creams and Gels. Potential heavy metals which are not

allowed in consumer products (Pb, As, Cd, Cr, and Hg) in the ACs and AGs were screened using inductively coupled plasma mass spectrometry (ICAP Q, Thermo Fisher Scientific, Bremen, Germany). About 0.5 g of sample (cream or gel) was freeze-dried to remove moisture at -47 °C under 0.1 mbar for 12 h. To this residue was added 1 mL of 30% hydrogen peroxide followed by 0.15 mL of concentrated nitric acid. After complete digestion, the samples were submitted for analysis by the Stable Isotope Laboratory at the University of Arkansas. The Pb, As, Cd, Cr, and Hg contents were measured based on a comparison of the responses obtained with reference standards prepared down to the levels of regulatory concern.

Antimicrobial Activities of the Topical Creams and Gels with NLP-EOs. Nutrient agar medium was made and sterilized according to the manufacturer's instructions. Briefly, premade sterile agar (60 mL) was melted and poured onto 100 × 15 mm diameter Petri dishes and allowed to cool to 25 °C before storage at 2–8 °C. To evaluate the antibacterial activity of the ACs and AGs containing NLP-EOs, free EOs, or the cream ingredients without any EO-s or NLP-EOs, *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) were used. One day before testing, the microorganisms were subcultured to facilitate logarithmic-phase growth at the time of testing. For antimicrobial testing a single colony was selected using a sterilized loop and suspended in normal saline with vortex until the suspension appeared uniform. The turbidity of the bacterial suspension was adjusted by dilution until the values were the same as the McFarland 0.5 standard value ($A = 0.09$) at 625 nm by UV-vis spectrophotometry (Spectro Visa TM sv1000, Azzota Corporation, Claymont, DE).

To evaluate zones of inhibition, inoculated plates were prepared by streaking sterilized cotton swabs over the surface of the prepared agar plates after immersion into the respective bacterial suspensions described above. Plates were air-dried for 5 min. Approximately 50 μ L wells were made using a sterile 11 mm cork bore to remove a plug of agar from the inoculated plates for placing samples. About 50 μ g of ACs and AGs were applied to fill each well. A small volume (50 μ L) of a solution containing 30 μ g Kanamycin was used as a positive control. Key components of the NLP-EOs and the AC or AG NLP-EO preparations were compared with each other and a commercial product, CARMEX lip balm containing nonencapsulated essential oils.

RESULTS AND DISCUSSION

A novel, safe, and effective method for encapsulating blends of antimicrobial EOs into BC/BD NLPs without the use of harmful organic solvents, expensive instrumentation, or extreme conditions (i.e., high temperatures and high pressure) is reported for the first time, to our knowledge, in this study. The resulting NLP-EOs were used as components of biocompatible biodegradable AC and AG topical formulations which exhibited antimicrobial activity against *E. coli* and *B. subtilis*.

Preparation of NLP-EOs. Organic solvents such as hexane, methanol, or chloroform, normally used in the preparation of BD/BC NLPs, were replaced with plant oils, specifically the same EOs as those encapsulated with NLPs. The method developed was simple and suitable for large-scale production. Typically, liposomes have been prepared previously by dissolving lipids in organic solvents^{45,46} at elevated temperatures. Eliminating the use of organic solvents facilitates use of these products in applications involving deliberate exposure to

humans or animals by minimizing safety or purification and clean up steps needed to remove undesirable materials. The materials used in this study are already regarded as safe for human consumption,⁴⁷ thus eliminating the need for purification after synthesis. Lack of any need for extreme/special conditions, expensive equipment, or removal of toxic precursors, ingredients, or byproducts greatly reduces the potential costs and increases the safety.

The primary structural component of the NLPs prepared in this study was food grade soy lecithin which is an inexpensive and safe source of phospholipid. Soy lecithin is also an excellent emulsifier, a nontoxic surfactant, and a stabilizer for vitamin E that was used in our application as one of the other structural components of the biodegradable biocompatible NLPs.⁴⁸ In place of cholesterol, another typically used component in liposome preparations, we used phytosterol, which provides the same functionality as cholesterol in the preparation of NLPs but also other functional benefits related to the proposed topical end use.^{49,50} Phytosterol is an agent used in skin care preparations supporting skin by improving moisture content and keeping the epidermis from drying while also stimulating the absorption of topical actives.^{51–53} Encapsulating liposomes and their contents has been reportedly degraded by oxidation or hydrolysis.⁵⁴ Adding vitamin E as one of the ingredients in the preparation of the NLP-EOs improved their stability. Vitamin E, an active skin agent and a safe antioxidant, also minimized oxidation of the BD/BC NLPs-EOs. Likewise, a small quantity of food-grade Na₂EDTA was added as a chelating agent^{54,55} and together with potassium sorbate served to prevent both unwanted microbial and fungal contamination.

In the optimization process, soy lecithin at final percentage concentrations of 2, 2.5, and 4%, phytosterol at 1.25, 1.5, and 2%, and α -tocopherol vitamin E concentrations at 0.2, 0.3, and 0.5% were tested. The percentage by volume of the EO blends in the NLP encapsulation steps was tested at 15, 20, and at 30% to produce the stable NLP-EOs % to produce the stable NLP-EOs and optimized (data not shown). Stable NLP-EOs were achieved using 25–30% EO depending on the EO used.

As in other topical skin care applications involving EOs, the blends of essential oils used in this study were diluted with a carrier oil. Some pure EOs have been reported to cause skin damage when exposed skin is subjected to UV.⁵⁶ Despite their health benefits, at high concentrations these materials can cause irritation or render strong and unacceptable aromas.⁵⁷ Therefore, the EOs were diluted with coconut oil at a ratio of 30/70 v/v. Peppermint, eucalyptus, clove, lemon, and tea tree oils were used in the blends because of their well-known antibacterial properties.^{58,59} Peppermint, eucalyptus, and lemon oils also have a pleasant aroma, while the tea tree oil may be repugnant to some.^{60,61} Additionally, tea tree oil is considered a relatively strong irritant⁶² so its contribution to the blends was deliberately kept low.

Headspace GC/MS was used to confirm the chemical composition of the EO blends and to screen for decomposition. While specific EO compositions from various sources or lots differ somewhat in composition, the primary volatile components in these EOs are well established and this method could be used to detect mislabeled, degraded, or fraudulent EOs. The major compounds in each blend, the comparative percentages of the total peak areas, and retention times resulting from headspace GC/MS are presented in Table 1. While these data represented only the volatile portion of the

Table 1. Retention Time (min), Identification, and Relative Peak Area (%) for Volatile Components Detected in EO Blends by Headspace GC–MS

t_R	compd	PETC	PETL	PERL
9.57	tricyclene	nd	nd	0.03
9.71	α -thujene	0.2	0.3	0.2
9.94	α -pinene	17.2	16.5	30.2
10.48	camphene	0.1	0.1	7.7
10.61	2,4-thujadine	0.05	nd	0.02
11.25	sabinene	nd	nd	0.2
11.40	β -pinene	1.7	4.5	6.2
11.81	β -myrcene	1.5	1.6	1.9
12.26	linalyl formate	nd	nd	0.1
12.35	α -phellandrene	1.4	1.4	0.6
12.44	3-carene	nd	nd	0.6
12.70	α -terpene	1.2	1.2	0.3
12.94	α -cymene	8.2	7.8	2.9
13.12	lymonene	6.0	9.8	23.8
13.21	eucalyptol	40.5	37.1	15.2
13.33	β -ocimene	0.9	0.1	0.9
14.04	γ -terpene	3.2	3.8	1.6
14.92	terpinolene	0.2	0.2	0.1
15.34	linalool	nd	nd	0.6
15.68	rose oxide	nd	nd	0.03
16.82	camphor	nd	nd	1.02
17.10	l-methone	8.5	6.8	1.12
17.23	menthone	0.3	nd	nd
17.37	iso-menthone	3.4	2.6	nd
17.54	menthol	0.5	0.5	nd
17.61	borneol	nd	nd	0.02
17.68	isopulegone	0.1	0.07	0.01
17.78	neo-menthol	2.6	2.9	0.3
17.85	terpinen-4-ol	1.19	0.9	0.2
18.18	methyl salicylate	0.04	nd	3.17
18.28	α -terpineol	0.12	0.1	0.03
19.53	pulegone	0.11	0.06	0.01
19.83	linalyl acetate	nd	nd	0.19
19.99	piperitone	0.18	0.12	0.02
20.50	citronellyl formate	nd	nd	0.003
20.86	bornyl acetate	nd	nd	0.01
20.93	isobornyl acetate	nd	nd	0.003
21.00	menthyl acetate	nd	nd	0.07

EOs in the headspace, the 70% coconut oil in the blends prevented direct GC/MS analysis after simple dissolution and liquid injection. The coconut oil was not compatible with the GC interface; however, testing the EO after dilution with coconut oil and just prior to use in NLP synthesis allowed us to confirm that decomposition or oxidation had occurred.

For the proprietary blend, PERL, 35 volatile EO compounds were identified by headspace GC/MS (Table 1) based on relative retention and comparison of spectra with the NIST mass spectral database. The major compounds were α -pinene, limonene, eucalyptol, camphene, and β -pinene, representing 30, 23, 15, 7, and 6%, respectively, of the total peak area in the headspace. The compounds found in PETC and PETL were very similar to each other as shown in Table 1. This was expected because they were 96% identical in EO composition as blended. PETC and PETL data showed 25 and 22 components, respectively, the five main components being eucalyptol, α -pinene, limonene, cymene, and 1-menthone at 40, 17, 8, 8, and 6% (in PETC) and 37, 16, 9, 7, and 6% (in

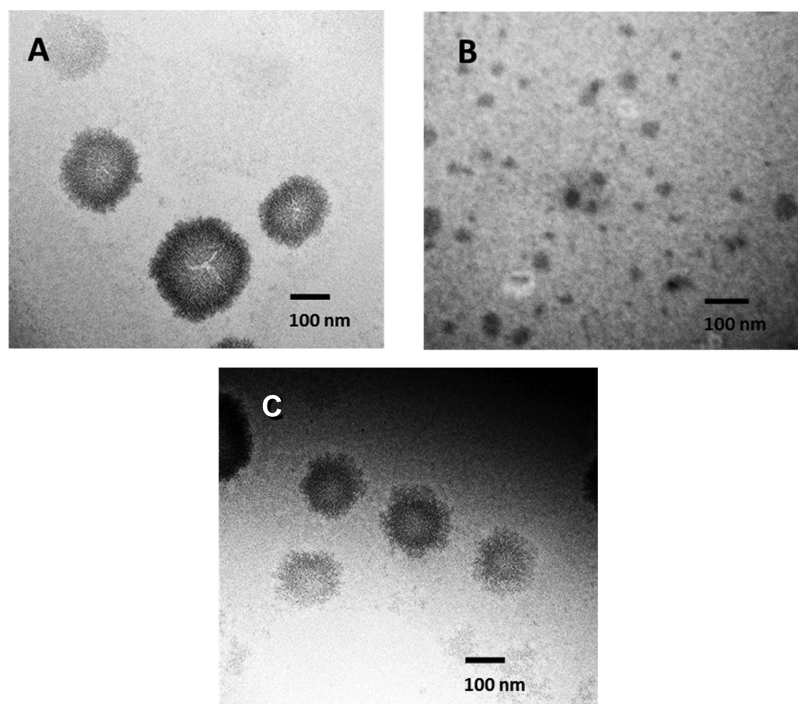


Figure 1. TEM images of EO-loaded NLPs: (a) NLP-PETC, (b) NLP-PERL, and (c) NLP-PETL.

PETL), respectively. The components were consistent with the use of eucalyptus oil as a major EO component in all three blends. Eucalyptol and α -pinene were the dominant compounds reported in eucalyptus oil (typically 72 and 9% respectively) by GC/MS.⁶³ The other major ingredient in PETC and PETL, peppermint oil, contained significant levels of menthol and menthone which was also observed by headspace GC/MS. The blends gave a significant signal for the more volatile menthone, while the higher boiling and more polar menthol was detected only at trace levels. This likely represented the difference in sampling between liquid injection and headspace sampling rather than a problem with the EOs. The presence of cymene in the PETC and PETL blends was attributed to tea tree oil.⁶⁴ Limonene was expected in lemon oil and in the other oils at lower concentrations as well.⁶⁵

Characterization of the NLPs. After synthesis of the NLP-EOs, morphology analysis was done using TEM, which provided the most accurate estimate of nanoparticle size. The results showed that the NLP-EOs were spherical with sizes ranging from 50 to 115 nm as shown in Figure 1. The NLPs produced using our simple approach (sonication, homogenization, and extrusion) were smaller than those previously reported (163.37–259.83 nm) by synthesis using more complex methods (modifying dehydration–rehydration vesicles) and more expensive ingredients (cholesterol 1,2-dipalmitoyl-sn-glycero-3-phosphocholine).⁶⁶

Dynamic light scattering (DLS) was used to characterize the average hydrodynamic size of the NLP EOs and to measure their zeta potential values (Table 2, Figure 2). The DLS derived sizes were 241, 229, and 210 nm for NLP-PETC, NLP-PETL and NLP-PERL, respectively. Observation of the apparently larger sizes by DLS compared to TEM has been previously noted,⁶⁷ which could be attributed to the water of hydration that is used in DLS sample preparation. The NLP-EOs showed negative zeta potential values ranging between -34 and -43 mV which provided evidence regarding NLP-

Table 2. DLS, Zeta Potential, and Polydispersity of NLPs and NLPs-EOs

name	size	zeta potential	polydispersity
NLP-PERL	210 \pm 2.48	-38 ± 6	0.190 \pm 0.027
NLP-PETC	241 \pm 3.30	-43 ± 5	0.153 \pm 0.030
NLP-PETL	229 \pm 2.43	-34 ± 8	0.144 \pm 0.042
empty NLP	768 \pm 18.53	-24 ± 4	0.300 \pm 0.016

EOs expected long-term stability in a dispersed form. Highly negative zeta potential values (-30) have been associated with diminished aggregation, and zeta potentials less than -30 mV or greater than $+30$ mV generally reflect stable materials.⁶⁸

The NLPs were analyzed with and without EO encapsulation (Table 2). The NLPs without EOs had average sizes that were somewhat bigger than the NLP-EOs. This finding was consistent with other studies where an encapsulated material caused a reduction in the size of the NLPs.^{69,70} It has been postulated that presence of EOs inside the NLPs enhanced stability and caused greater cohesion and packing between the polar chains in the NLPs membrane vesicles.⁷¹ The specific ability of monoterpenes to reduce the size of liposomes has also been attributed to another mechanism involving increased interaction with phosphatidylcholine vesicles.⁷² Either mechanism could explain the decrease in size going from NLPs to NLP-EOs observed in this study. The polydispersity values, which are indicators of aggregation, ranged between 0.109 and 0.150 for the NLP-EOs and 0.300 for the NLPs alone. Polydispersity values in this range have been reported to be associated with mono dispersive behavior and minimum tendency for aggregation.⁷³

The measurement of encapsulation efficiency was based on a comparison of the EO released from an NLP-EO with free EO suspended in the same sample matrix. This was done using a single EO to simply quantification. Eucalyptus oil, E, was selected for testing as an EO common to all three blends used

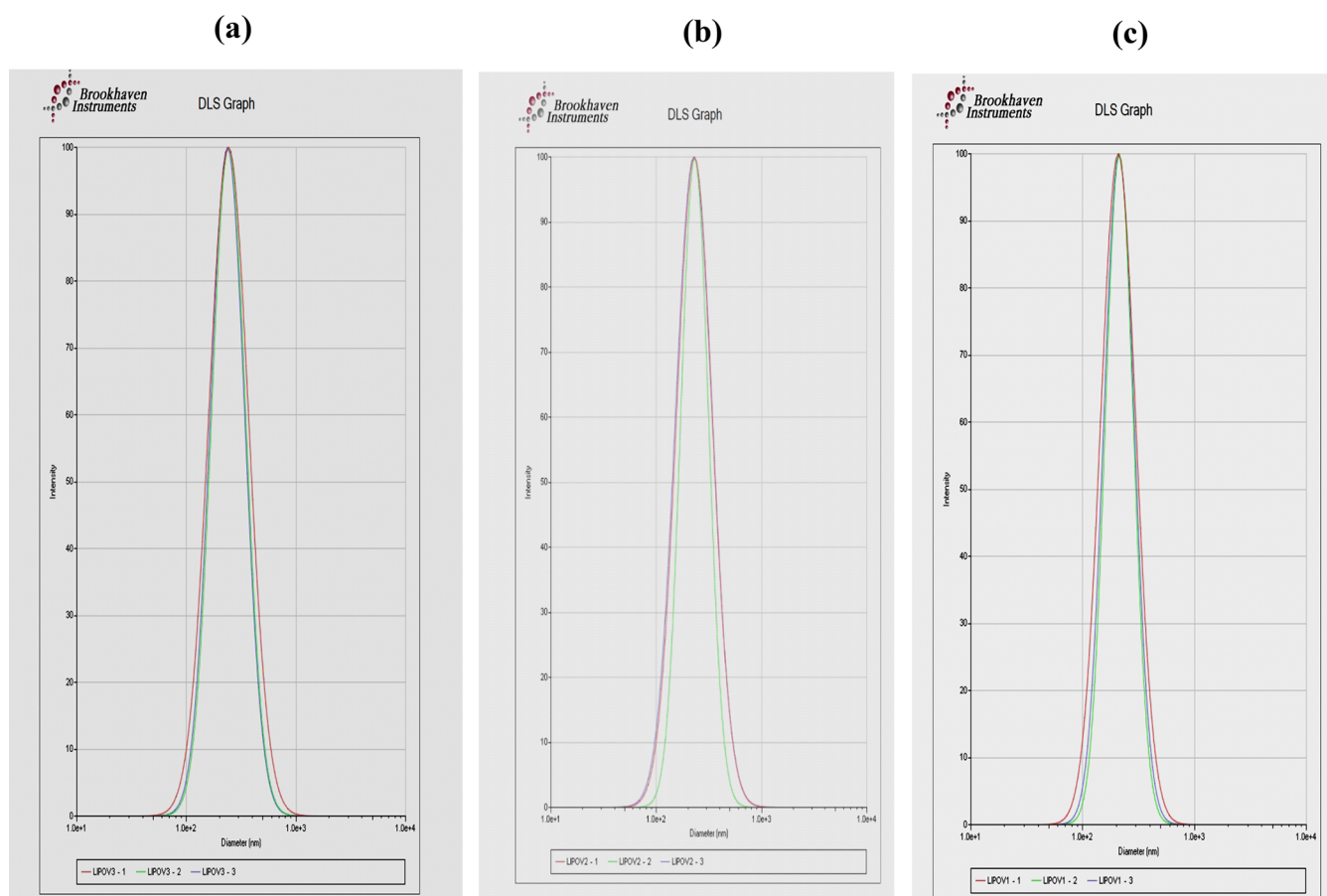


Figure 2. DLS of nanoliposome encapsulated EOs (a) PETC, (b) PETL, and (c) PERL.

in this study and one giving a strong signal for a major component, eucalyptol, which was used for quantification and measurement of encapsulation efficiency. It should be noted that the EO encapsulation efficiency was measured from ruptured NLP-EOs that were washed, isolated, collected, and recovered from newly synthesized NLP-EOs to eliminate signals from potential nonencapsulated EO residues. Thus, only EOs released from the NLP-EOs were, theoretically, present and detected for the encapsulation efficiency evaluation. A calibration curve was obtained using standards of EO spiked into the same matrix as the NLP-EOs. A linear regression for the association between peak area (y) and concentration (x) (Figure 3) gave $y = 0.3193x + 0.843$ with an $R^2 = 0.9964$. To establish the actual capsulation efficiency, 65 mg of eucalyptus oil was encapsulated in 1 mL of nano-

liposomes to form NLP-E. If the recovery was 100%, 65 mg would have been released from the NLP-E after washing, isolation, collection, and disruption of the EOs. Headspace analysis of the released EO measured 62 mg corresponding to a 95% encapsulation efficiency. This was substantially better than values reported in earlier studies which were typically around 40%.⁷⁴ The higher degree of encapsulation in this study, compared to other methods, could be attributed to our synthesis approach, but such a claim would be tentative without further studies. Differences in the measured values could also be associated with the different methodologies used in the various studies. Nevertheless, the novel method in this study resulted in nearly complete encapsulation of the bioactive EO.

The NLP encapsulation of the EOs was expected to reduce EO instability to heat, air, and light. Significant losses of the oil components due to decomposition and volatility have been reported in other studies.⁷⁵ We tested for such changes by monitoring the headspace GC/MS profiles on days 0 and 30 using NLP-PETC to model potential decomposition of itself and the other NLP-EOs which were not individually tested. The headspace GC/MS profiles of a reference material (PETC) as well as NLP-PETC diluted in coconut oil were measured on days 0 and 30. The reference material, PETC, was used to establish a reference composition based on relative abundance (RA) and the reproducibility of the experiment. The measured RA (% peak areas) were reproducible to within 0.2% (mean error 0.1%) from run to run over the time period of the experiment (data not shown). Peaks with area values

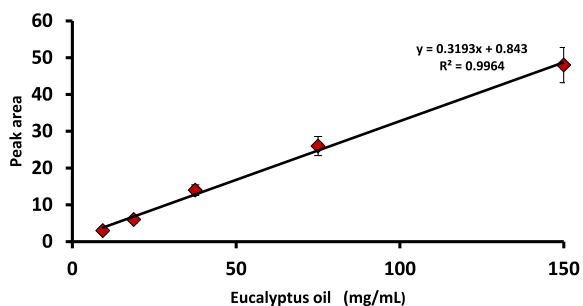


Figure 3. Standard curve for encapsulation efficiency evaluation using eucalyptus oil in coconut oil by headspace GCMS.

below 0.3% RA were excluded from Table 3 as their measurement was considered unreliable. Thirteen compounds

Table 3. Headspace GCMS for Significant Volatile Components (>0.3% Relative Abundance) from Nano-encapsulated PETC on Days 0 and 30 (% Peak Area)

compd	day 0	day 30
α -pinene	13.1	12.8
β -pinene	1.6	1.7
β -myrcene	0.7	1.2
α -phellandrene	1.1	1.1
α -terpinene	0.9	0.9
cymene	9.2	9.1
limonene	5.7	5.8
eucalyptol	51.7	50.9
γ -terpinene	2.5	2.5
menthone	5.9	6.1
menthol	3.0	2.6
terpinen-4-ol	0.7	0.8
menthyl acetate	0.7	0.7

were detected in NLP-PETC diluted into coconut oil on day zero above 0.2% RA. On day 30, the same compounds were detected again with very little change in relative abundance. The changes in the values reported in Table 2 do not reflect a significant level of evaporation or decomposition over the 30 days of the test, as no new peaks were observed above the 0.2% relative abundance threshold and the abundances did not change significantly. The only peak that changed significantly was the most abundant component (eucalyptol) which changed from 51.7% RA on day one to 50.9% RA on day 30. This change from 51.7% to 50.9% RA might reflect small changes in the main component based on decomposition or evaporation, but it should be pointed out that in the worst case a loss of 0.8% RA of the signal for this component represented a net change of only about 1.5% with respect to the overall composition. This indicated that 98.5% of the original composition appeared unchanged on a relative abundance basis.

Preparation of BD BC Topical Cream and Gel with NLP-EOs. Antimicrobial creams or ACs were prepared in an oil-in-water emulsion for each of the three EOs blends (PETC, PETL, and PERL). These creams were prepared with the same components that would be necessary in a commercial product for human or animal use. The oil phase was primarily food grade oil (coconut oil) and free EOs with the addition of vegetable-derived cetearyl alcohol as a thickener, vegetable stearic acid as a surfactant, and Oliwax as a coemulsifier. Aloe vera was added as a topical moisturizer and to minimize skin allergic response,⁷⁶ while vegetable glycerin was added as a humectant and moisturizer.⁷⁷ Na₂EDTA and potassium sorbate were both added as preservatives. The free EOs and NLP-EOs were the active antimicrobial components of the ACs formulations.

Compared with the creams, all of the gels were constrained to lower amounts of EOs and especially NLP-EOs because higher levels resulted in loss of stability causing the product to separate into two liquid layers, no longer meeting the criteria for a gel. The AC formulation had about 7% NLP-EOs, while the AG only had 1% NLP-EOs. As in the cream, inactive components were used to create the gel and to serve as moisturizers, antidrying agents, and preservatives.

Physical Characterization of the Topical Creams and Gels. All of the ACs and AGs, with and without NLPs, exhibited smooth texture, homogeneous texture, and colors that ranged from very light cream to ivory white. When inspected under an optical microscope at 20 \times and 40 \times magnification (data not shown) no significant heterogeneity of particles was observed. The cream and gel had white and light cream colors, respectively, and both had a smooth or soft texture when applied to the skin behind the palm of the hand. Phase separation was not observed in any of the preparations. The creams and gels had an acceptable and consistent aroma. While it is not a part of this report, it was also noted that these aroma could be easily modified by the addition of small quantities (0.5%) of EO fragrances. Because fragrance components, also antimicrobials, might impact the microbial studies, they were not incorporated into the AC/AGs tested in this study for antimicrobial activity. The pH of the cream or gel base without the NLP-EOs and/or EOs was uniformly recorded as 5.0, whereas it was 5.5–6.0 in the presence of NLPs- EOs with free EOs or with free EOs only.

Stability of the Topical Creams and Gels. The cream and gel pH values were recorded every month over 6 months. Both the cream and the gel showed a slight increase in pH (0.1–0.2 pH) over the 6 month study (data not shown). These values are close to the optimal pH of human skin at 5.5.⁷⁸ The remaining properties listed in Table 4 were also

Table 4. Physical Properties and pH of the ACs and AGs Formulations

sample	color	texture	separation phase	consistency	pH	
cream	NLP-PERL	white	smooth	no	consistent	5.5
	NLP-PETC	white	smooth	no	consistent	6.0
	NLP-PETL	white	smooth	no	consistent	6
gel	NLP-PERL	light cream	smooth	no	consistent	5.5
	NLP-PETC	light cream	smooth	no	consistent	5.8
	NLP-PETL	light cream	smooth	no	consistent	6

tested in samples stored at 4, 25, and 45 °C. Overall, there were no significant pH changes in the creams or gels over 6 months (0.2 pH units) at these three temperatures. No other physical changes were observed over 6 months for the creams and gels stored at 4 °C. A slight change in color was noted after 6 months of storage at 25 and at 45 °C. Storage of the cream and gel at 45 °C also exhibited phase separation, loss of gel or cream consistency, and altered visual appearance of texture at the end of two months. These results clearly indicated that the creams and gels were not stable at 45 °C for more than one month. The values of viscosity of creams and gels with EOs and NLP-EOs is shown in Figure 4. The specific value was not the focus of this work, but it was reported nevertheless as an important property of skin care formulations.⁷⁹

Antimicrobial Activities of the Topical Creams and Gels with NLP-EOs. One of the major objectives of this study was to develop all-natural biodegradable biocompatible antimicrobial creams and gels containing EOs and NLP-EOs that were safe, environmentally friendly, potentially suitable for use in skin care products, and which exhibited useful

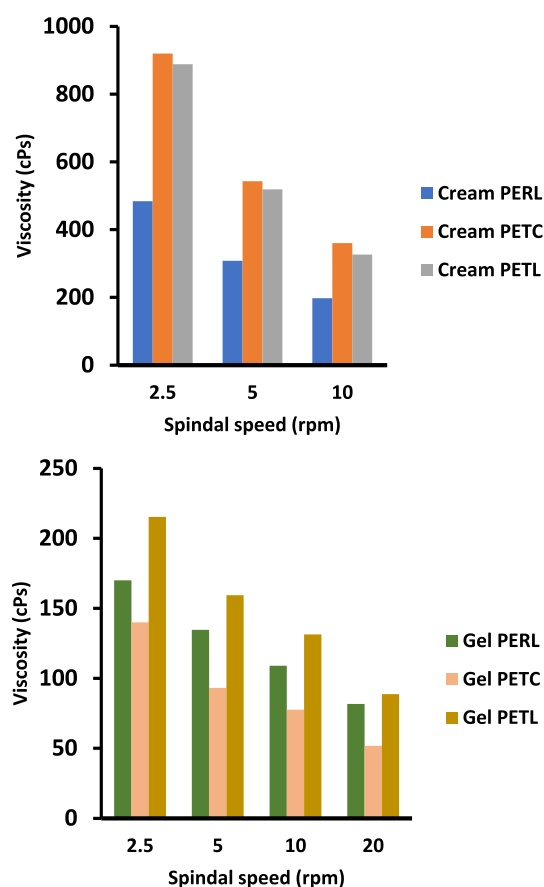


Figure 4. Viscosity data for creams and gels ($P \pm 1.75$).

antimicrobial activity. Creams and gels with EOs and NLP-EOs were tested for antimicrobial activities against Gram-positive and Gram-negative bacteria. The antimicrobial activity was measured using inhibition zones. The zone of inhibition (ZI) refers to the clear area around a well containing the antimicrobial agent (AC or AG), and the antimicrobial activity is inferred from the distance from the well at which visible cells do not show growth. The larger the ZI, the greater the antimicrobial potency of the agent placed in the well. Potent antimicrobials generally show large and easily measured ZI values,⁸⁰ typically in the range between 15 and 20 mm using this protocol. Typically, reproducible values could be recorded within 0.5 mm.⁸¹

The antibiotic kanamycin (30 μg) was used as positive control and its ZI was compared with those of the ACs and AGs. The results shown in Tables 5 and 6 indicated that the creams and gels with NLP-EOs and EOs all exhibited antibacterial activities regardless of the EO blends, the formulation (cream or gel) or the mode of delivery (NLPs or free EOs only). Controls consisting of the cream or gel matrix only, without EOs in any form, did not exhibit any measurable ZI. A similar product used as a lip balm that contained nonencapsulated EO ingredients (camphor and menthol) as well as phenol and olive oil was also included in the table for comparison.

In general, the creams (Table 6) showed more antimicrobial activity than the gels with the same active ingredients (Table 5). This was expected because, as noted above, the gel formulation limited the quantity of active ingredients that could be used compared to the cream by a factor of near 3.

Table 5. Inhibition Zones against *E. coli* and *B. subtilis* for AGs Containing Free EOs and NLP-EOs (PERL, PETC, and PETL) as Well as AGs with the Same Amount of Non-encapsulated EOs ($P \pm 0.2$ mm)

samples	zone of inhibition (mm)	
	<i>E. coli</i>	<i>B. subtilis</i>
G (gel matrix, blank)	0	0
olive oil	0	0
dilution solution (– control)	0	0
NLPs only	0	0
G + PERL and NLP-PERL	15	15.5
G + PECT and NLP-PETC	14	14.5
G + PETL and NLP-PETL	13	14
G + free PERL	13	14
G + free PETC	12	13
G + free PETL	11.5	12
lip balm (camphor and menthol)	14	15
kanamycin (+ control)	19	21

Table 6. Inhibition Zones against *E. coli* and *B. subtilis* for ACs Containing EOs and NLP-EOs (PERL, PETC, and PETL), as Well as ACs with the Same Amount of EOs ($P \pm 0.3$ mm)

samples	zone of inhibition (mm)	
	<i>E. coli</i>	<i>B. subtilis</i>
C (cream matrix, blank)	0	0
olive oil	0	0
dilution solution (– control)	0	0
NLPs only	0	0
C + PERL and NLP-PERL	21	23
C + PECT and NLP-PETC	20	21
C + PETL and NLP-PETL	18	22
C + free PERL	19	20
C + free PETC	18	19
C + free PETL	16	19
lip balm (camphor and menthol)	14	15
kanamycin (+ control)	19	21

Because the gels showed significantly less activity than the positive controls and creams, they were not considered in further studies. The ACs showed ZIs that were comparable with the positive controls. The creams with free EOs (PERL, PETC, PETL) showed ZIs of 19, 18, 16 mm against *E. coli*, whereas the positive control showed 19 mm. Likewise, the same free EOs gave ZIs of 20, 19, and 19 mm against *B. subtilis*, whereas the positive control gave 21 mm. In these experiments, the proprietary blend, PERL gave the best performance, the same as the positive control with *E. coli* and 1 mm less than positive control against *B. subtilis*. However, when part of the same quantity of EO was delivered as NLP-EOs, the antimicrobial cream performed better than the positive control in four of six experiments. The NLP-PERL cream showed higher inhibition than the positive control (+2 mm) for both bacteria. The NLP-PETC was better than the positive control for *E. coli* (+1 mm) but the same as the positive control for *B. subtilis*. Finally, NLP-PETL was better than the positive control for *B. subtilis* (+1 mm) but less active against *E. coli* (–1 mm). While the creams made with NLP- PETC and NLP-PETL showed performance measured against the positive control that was never more than + and –1 mm difference, the NLP- PERL showed 2 mm greater zones of inhibition against both

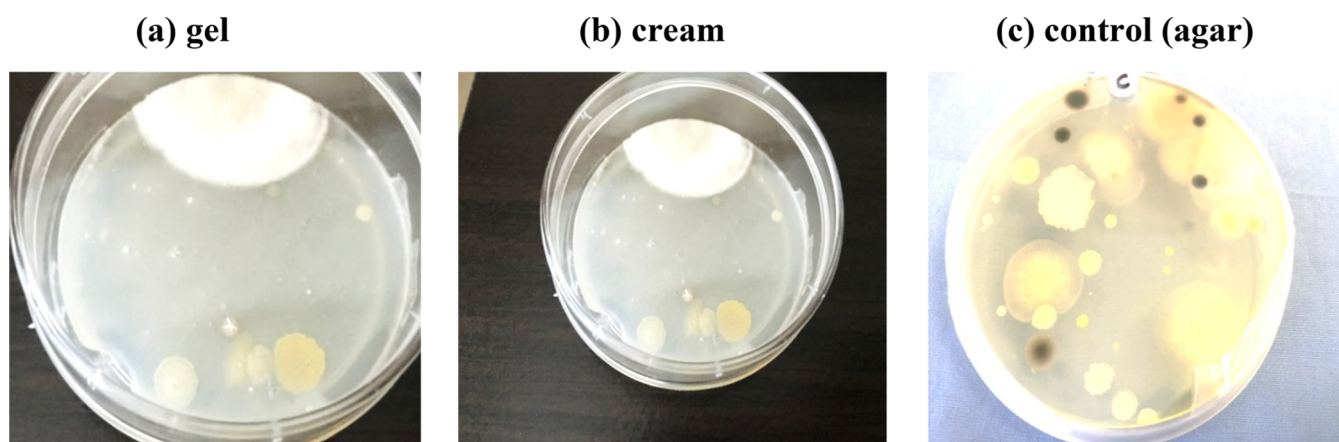


Figure 5. Biodegradability by environmental microbes after 10.5 h total exposure to natural environment over a period of 7 days: (a) gel, (b) cream, and (c) control (agar).

organisms. When the creams were retested after 90 days of storage at refrigeration temperatures, their antimicrobial activity did not exhibit degradation (data not shown).

The higher antibacterial activity of the commercial PERL blend was not unexpected. It was selected for comparison with the other blends because it had been used in commercial products for its antimicrobial activity. The headspace GC/MS results (Table 1) for the NLP-PERL showed a larger number of terpene components than either NLP-PETC or NLP-PETL. While the composition of PERL is proprietary, it has been known that this oil blend has more essential oil components than the others. It was likely that PERL included antimicrobial terpenes not found in the PETC or in the PETL blends. Because the formula was proprietary, two new laboratory-prepared blends (PETC and PETL) were tested against PERL in this study to demonstrate that antimicrobial creams could be developed using simple and readily available EOs, not just commercial proprietary blends. The major components in the two laboratory blends were, like the proprietary PERL, expected to include antimicrobial components, but they were also selected for other reasons, including cost, availability, and desirable properties such as minimal toxicity and pleasant odor. The tea tree oil was likely the most bioactive essential oil in PETC and PETL blends, but it was included at only 1 part in 30 in the blends to minimize potential adverse effects and unpleasant odor during EOs blending and NLPs-EOs preparation. Despite the fact that these EOs and their proportions were not selected for being among the most antimicrobial of EOs, most EOs do have some activity, and these EOs when delivered partially with NLPs, had activity similar to the kanamycin antibiotic positive control. This finding was consistent with previous reports of nanoenhanced bactericidal activity of EOs.⁸² Previous reports suggested, and this study confirmed, that while EOs have some antimicrobial activity, NLP encapsulation increased this activity. In this study, the increase appeared to be large enough to make them comparable to the synthetic antibiotic positive control. We expect that this result could be replicated using a variety of EOs from a large selection of regionally available or inexpensive materials. A study of pure EO extracts from 10 aromatic plants tested for antimicrobial activity using zone inhibition found activity in 90% of the oils tested.⁸³ Even at low concentrations, and against different microorganisms (e.g., *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and *Staphylococcus*

aureus) EO blends delivered in other nanoformulations have shown enhanced antimicrobial activities compared to the free essential oils. Other study found BD/BC nano formula treated Enterobacteriaceae infections at low concentrations of loaded antibacterial drug.⁸⁴ This was generally attributed to the ability of nanoscale materials to penetrate the bacterial membrane and the lysing effects after delivery of the EOs into the cell.^{85,86}

Our report confirmed these general findings and at a level of activity comparable to a conventional antibiotic, based on a topical formulation in a finished model-product rather than from testing of only the active components. This study also showed other advantages of our novel method with respect to the ingredients or their toxicity and simplicity of production.

Typically, the mechanism of the antimicrobial effect of EOs has been attributed to penetration of the cellular membrane and the subsequent disruption, causing cell lysis leading to leakage of cytoplasmic contents and the death of the microorganisms.^{9,87} While the nanoparticles themselves have been shown experimentally to penetrate cells, in our study, NLPs without the EOs demonstrated no ability to kill cells directly even at high concentrations (Tables 5 and 6). These results clearly demonstrated that the antimicrobial activity of the EOs was enhanced through nanoencapsulation but was not caused by an additional effect from the NLPs themselves. While there have been some reports that Gram-positive bacteria were more resistant to essential oils, other studies found the opposite.⁸⁸ In our study, the antimicrobial effect using all three EOs blends was greatest on Gram-positive bacteria. This observation had been attributed to the differences in the structure of the bacterial cell wall.⁸⁹

As a comparison, we also measured the antibacterial activity of a commercial product, CARMEX lip balm, marketed as a treatment for cold sores, which contained the antimicrobial aromatic synthetic compound phenol and two essential oil components, camphor and menthol. While it was not advertised as an antimicrobial, our results indicated that it had antimicrobial properties but was less active than the kanamycin positive control and significantly less active than our NLP-EOs creams.

Heavy Metal Contamination Evaluation of the Topical Creams and Gels. The creams and gels were evaluated for specific heavy metal contaminants, namely the top 4 of 20 hazardous substances reported by the U.S. Environmental Protection Agency and Agency for Toxic Substances and

Disease Registry (ATSDR)⁹⁰ as materials of concern, and those that could be present in cosmetics or the raw materials. The ICP MS results indicated that the levels of As, Cd, Pb, and Hg in the creams and gels were in the low ppb range, about 3 orders of magnitude below the maximum acceptable (ppm) levels established by the Food and Agriculture Organization/World Health Organization.^{91,92} This data suggested that the creams and gels did not contain significant levels of these four materials. The samples gave signals several orders of magnitude lower than the standards, corresponding to the low ppb range, and the samples were not quantified further at subppm levels. Their presence could preclude future use of these products as antimicrobials, and their absence in our formulations minimized our concerns about use and disposal of the materials in the laboratory setting.

Microbial Contamination Evaluation of the Topical Creams and Gels. Potential microbial contamination in the cream and gel ingredients was also evaluated. Because the creams and gels were prepared using natural ingredients without preservatives, contamination was possible and could have affected testing. However, no microbial growth on the samples tested in this study was observed over the observation conditions, except after deliberate exposure to either environmental microorganisms to test for biodegradation study or the model bacteria in the antimicrobial testing studies.

Biodegradability Evaluation of the Topical Creams and Gels. The products were not contaminated with microbes before use, but they were clearly biodegradable. Recent reports of consumer products accumulating in water, soil, and air have caused biodegradability to become an increasingly important concern.⁹³ Bacteria provide a major mechanism for degradation, but antimicrobials such as antibiotics have been accumulating in the food and water supplies, in part because they kill the bacteria that degrade them. For this reason, we designed experiments to demonstrate the biodegradability of the ACs and AGs containing EOs and/or NLP-EOs. Figure 5 showed that the ACs and AGs were degraded by environmental microorganisms within 7 days of exposure to outdoor air, whereas the nutrient agar, cream, and gel matrix (EO free materials) showed microbial growth after only 1 day of exposure. The antimicrobial activity of the EOs and NLP/EOs delayed natural environmental biodegradation but did not prevent eventual biodegradation after 7 days. Therefore, unlike conventional antibiotics which reportedly accumulated in water, land, and soil, the NLP-EOs and EOs in the creams and gels exhibited biological degradation when exposed to the natural environment. The environmental degradation of the creams and gels with NLP-EOs and EOs was not unexpected, as the plants and their contents, including the EO components, have been entering the ecosystem in significant quantities for as long as these plants have been present. Thus, while they could be degraded when exposed to environmental microbes of decay over time, they still have sufficient immediate potency to be used as antimicrobials or to be used to produce antimicrobial products, especially with optimization of delivery, which in this case was via biodegradable, biocompatible NLPs.

CONCLUSION

A novel, safe, inexpensive, and effective method for encapsulating blends of antimicrobial essential oils into biocompatible biodegradable NLPs, without the use of harmful organic solvents, expensive instrumentation, or high temperatures was developed. The method developed featured three

novel aspects: (1) use of liposomes to encapsulate EOs, (2) use of the EOs to replace synthetic organic solvents that are potentially toxic and/or leave harmful residues, and (3) an encapsulation process at temperatures below the boiling point of water. The LPs were made from soy lecithin, phytosterol, and α -tocopherol (vitamin E) that were synthesized using the EOs as the solvent. The liposomes were converted to nanoliposomes (NLPs) through a series of sonication, homogenization, and extrusion steps. Transmission electron microscopy indicated that the NLPs alone and nanoliposome encapsulated EOs were spherical in shape with sizes ranging between 50 and 115 nm diameter and with negative zeta potentials ranging from -34 to -43 mV. There was no significant heavy metal contamination [As, Pb, Cd, Hg] based on inductively coupled plasma mass spectrometry MS analyses. Nearly complete EO encapsulation (95% encapsulation efficiency) was confirmed by GC/MS. The NLP-EOs were used to prepare biocompatible biodegradable AC and AG topical formulations that exhibited antimicrobial activity against *E. coli* and *B. subtilis*. When EOs were present at least partially in NLPs, the activity of the creams was comparable to kanamycin, a synthetic antibiotic used as a positive control. Although beyond the scope of this study, the mechanism of antimicrobial activity observed in the EO blends in this study should be elucidated in the future.

The presence of the NLP-EOs and EOs delayed the onset of biodegradation by a factor of about seven compared to the other ingredients. The NLPs-EOs and the resulting ACs eventually degraded when exposed to the natural environment for 1.5 h a day after about 7 days (which was actually for a total of 10.5 h).

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Notes

The authors declare no competing financial interest.

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