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Decylglucoside-based microemulsions for cutaneous localization of lycopene and ascorbic acid

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ABSTRACT

Cutaneous delivery of combinations of antioxidants offers the possibility of enhanced protection against UV-radiation. In this study, we investigated the potential of sugar-based microemulsions containing monoglycerides to promote simultaneous cutaneous delivery of lycopene and ascorbic acid, and increase tissue antioxidant activity. Lycopene and ascorbic acid were incorporated (0.04% and 0.2% (w/w), respectively) in decylglucoside-based microemulsions containing isopropyl myristate mixed with monocaprylin (ME-MC), monolaurin (ME-ML) or monoolein (ME-MO) as oil phase. The microemulsions increased lycopene delivery into porcine ear skin by 3.3- to 8-fold compared to a drug solution. The effect of microemulsions on ascorbic acid cutaneous delivery was more modest (1.5–3-fold), and associated with an approximately 2-fold increase in transdermal delivery. According to their penetration-enhancing ability, the microemulsions were ranked ME-MC > ME-MO > ME-ML. This superiority of ME-MC coincided with a stronger effect in decreasing skin electrical resistance. After 18 h of treatment, the viability of bioengineered skin treated with ME-MC was 2.2-times higher compared to Triton-X100 (moderate irritant), demonstrating that ME-MC is less cytotoxic. Skin treatment with ME-MC containing both antioxidants increased the tissue antioxidant activity by 10.2-fold, but no synergism between the antioxidants was observed.

1. Introduction

Lycopene, a carotenoid found in red-colored fruits, has aroused great interest because of its strong *in vitro* antioxidant activity (Andreassi et al., 2004) and ability to inhibit UVB-induced ornithine decarboxylase and myeloperoxidase, reducing inflammation and skin thickness, and providing protection against photodamage (Cesarini et al., 2003; Fazekas et al., 2003; Mein et al., 2008). Because lycopene content in the skin seems to be inversely proportional to skin roughness, it may also be able to reduce furrows and wrinkles formation (Darvin et al., 2008). Based on all of these properties, cutaneous delivery of lycopene would be very attractive (Gonzalez et al., 2008).

In a previous study, we developed topical microemulsions in an attempt to address formulation and delivery challenges resulting from lycopene's strong lipophilicity, which makes it difficult to dissolve lycopene in aqueous systems as well as in oils used in food and cosmetics (Spernath et al., 2002), and prone to be retained within the stratum corneum (Lopes et al., 2010). Microemulsions containing mono and diglycerides of capric and caprylic acids as oil phase were most effective at increasing lycopene delivery into viable skin layers compared to those containing triglycerides of the same fatty acids (Lopes et al., 2010). In addition, incorporation of ascorbic acid in lycopene-containing microemulsions increased lycopene content in the skin by 25%, probably due to inhibition of its degradation (Biacs and Daood, 2000; Lopes et al., 2010).

Since ascorbic acid offered some protection against lycopene degradation, and a concomitant increase in lycopene and ascorbic acid plasma concentration improved protection from DNA oxidative damage (Lopes et al., 2010; Riso et al., 2004), development of a topical formulation that provides cutaneous localization of these two antioxidants could potentially maximize their benefits to the skin. However, lycopene and ascorbic acid have distinct structures, molecular weight (176 and 536.8 for ascorbic acid and lycopene respectively) and calculated log*P* values (approximately −1.64 and 17 for ascorbic acid and lycopene respectively), making their simultaneous delivery into viable skin layers challenging (Cotelle et al., 2003; Spernath et al., 2002; Vertzoni et al., 2006).

The ability of formulations to improve the release and transport of therapeutic agents into the skin may be influenced by the type of delivery system, its composition and concentration of components (Hosmer et al., 2011; Phelps et al., 2011; Savic et al., 2006).

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Thus, this study aimed at evaluating whether and how microemulsion composition affects skin penetration of lycopene and ascorbic acid. More specifically, we developed microemulsions containing sugar-based surfactants and various monoglycerides as oil phases to assess the influence of the acyl chain length of monoglycerides on skin permeability and cutaneous drug delivery. We used monoglycerides of caprylic acid (monocaprylin, 8 carbons, MC), lauric acid (monolaurin, 12 carbons, ML) and oleic acid (monoolein, 18 carbons, MO). While the penetration-enhancing ability of monocaprylin and monoolein has been described (Lopes et al., 2005, 2009), there is very little information on monolaurin, except that its antimicrobial properties can be advantageous in topical treatment (Fu et al., 2006). Our results demonstrate that the type of monoglyceride plays a major role on the ability of formulations to concomitantly maximize the cutaneous delivery of ascorbic acid and lycopene.

2. Material and methods

2.1. Material

Propylene glycol (PG), ascorbic acid, isopropyl myristate, neocuproine, ammonium acetate and copper chloride were obtained from Sigma (St. Louis, MO). Monoglycerides of caprylic acid (MC), lauric acid (ML) and oleic acid (MO) were kindly supplied by Abitec Corporation (Janesville, WI) or purchased from Nu-Check Prep (Elysian, MN). Decylglucoside was kindly supplied by Cognis (BASF, Cincinnati, OH). Lycopene standard was purchased from Wako Chemicals (Richmond, VA). Acetonitrile, methanol, ethanol and dichloromethane were purchased from Mallinckrodt Baker (Phillipsburg, NJ).

2.2. Methods

2.2.1. Lycopene extraction and purification

Lycopene was extracted from commercial tomato paste as previously described by ours and other groups (Lopes et al., 2010; Periago et al., 2004; Ronman, 1985). Briefly, tomato paste (100 g) was dehydrated with 95% ethanol, filtered and the residue was dissolved in dichloromethane and dried with anhydrous sodium sulfate. After solvent removal *in vacuo*, the extract was purified by flash column chromatography (silica gel, petroleum ether:dichloromethane 75:25, v/v) (Periago et al., 2004). The identity of the purified lycopene was confirmed by comparison to a commercially available standard. As previously described, purified and standard lycopene presented similar R_f values in thin layer chromatography, similar UV–vis spectra (characteristic peaks at 504, 472, and 446 nm), similar retention times when analyzed by HPLC (9.4 min, even though peak areas were generally 25% smaller for the extracted compound, suggesting the occurrence of some degradation during extraction), and presented a molecular ion of m/z 537 when analyzed by mass spectrometry (positive ionization mode, Waters TQD tandem quadrupole detector, Milford, MA) (Lopes et al., 2010). The purified lycopene was used for formulation development.

2.2.2. Phase diagram construction and sample preparation

Ternary phase diagrams were constructed using the water titration method at room temperature. Decylglucoside was used as surfactant and propylene glycol as co-surfactant. They were chosen due to their low irritation potential (Graf et al., 2008; Savic et al., 2006). Propylene glycol, as other short and medium chain length alcohols, is commonly added as co-surfactant to further reduce the interfacial tension and aid microemulsion formation (Fan et al., 2011; Lawrence and Rees, 2000). It is miscible with water and the oil used, and thus, able to partition into these phases (Lawrence and Rees, 2000). For consistency with previous studies, propylene glycol was considered a co-surfactant, and its weight fraction was included in the surfactant-co-surfactant blend (El Maghraby, 2008; Fan et al., 2011; Li et al., 2012; Peira et al., 2008). Surfactant and co-surfactant were mixed at 3:1, 1:1 and 1:3 (w/w). The oil phase consisted of mixtures at 1:9 (w/w) of isopropyl myristate and monoglycerides. We used monocaprylin (8 carbons, MC), monolaurin (12 carbons, ML) or monoolein (18 carbons, MO) as monoglycerides; isopropyl myristate was included to aid melting and mixing of monolaurin and monoolein with the surfactant blend. Mixtures of surfactant-co-surfactant and oil phase at 1:9 to 9:1 (w/w) were titrated with water under vortexing, and the systems were characterized by visual inspection. Phase diagrams were plotted to show the relationship between formulation composition and phase behavior. Formulations that were fluid, clear, and did not undergo phase separation were classified as microemulsions, and assigned to a monophasic region (black-shaded) in the phase diagrams (Hathout et al., 2010).

Based on the phase behavior of mixtures and dimensions of the monophasic region in phase diagrams (Fig. 1), surfactant–co-surfactant blends composed of decylglucoside:propylene glycol at 1:1 (w/w) were selected for the preparation of three microemulsions, all containing the same ratio of components (surfactant:oil:water at 50:20:30, w/w/w), but each with one type of monoglyceride: monocaprylin (ME-MC), monolaurin (ME-ML) or monoolein (ME-MO). By keeping the ratio between components constant, a comparison of the influence of monoglycerides can be made. The oil phase was set at 20% to increase cutaneous over transdermal delivery (Lopes et al., 2009). Lycopene was incorporated in the oil phases of these microemulsions at a final concentration of 0.04% (w/w), while ascorbic acid was incorporated in the aqueous phase at 0.2% (w/w).

2.2.3. Microemulsion characterization

Polarized light microscopy (Axiotop, Zeiss, Thornwood, NY) was used to verify the isotropicity of the selected microemulsions. The internal structure of the systems (water-in-oil, oil-in-water or bicontinuous) was assessed by electrical conductivity at $25 \pm 0.5^\circ$ C using a Jenway 4520 Conductivity/TDS meter (Techne Inc., Burlington, NJ). The ratio between surfactant:co-surfactant and oil was kept constant (2.5:1, w/w, surfactant blend:oil), while water was added at small increments along a dilution line (gray lines shown in the diagrams in Fig. 2A–C). Water was added until the microemulsions were transformed into turbid or gel-like systems (borderline of the monophasic region). Conductivity was measured after each water addition.

2.2.4. Effect of microemulsions containing various monoglycerides on cutaneous delivery of antioxidants

Skin penetration of lycopene and ascorbic acid was studied using Franz diffusion cells. Briefly, the skin from the outer surface of a freshly excised porcine ear was carefully dissected, stored at -20° C, and used within a month. On the day of the experiment, the skin was thawed and mounted in a Franz diffusion cell (diffusion area of 1 cm^2; Laboratory Glass Apparatus, Inc., Berkeley, CA). The receptor compartment was filled with 100 mM phosphate buffer (pH 7.4, containing 10% ethanol) and maintained at 37°C under constant stirring (Lopes et al., 2010). Microemulsions (100 mg) containing lycopene and ascorbic acid were placed in the donor compartment of diffusion cells for 6 or 12 h; solutions of 0.04% (w/w) lycopene and 0.2% ascorbic acid in isopropyl myristate/propylene glycol were used as control formulations.

After the abovementioned periods of time, skin samples were rinsed, blotted dry, and the stratum corneum (SC) was separated from the epidermis and dermis (ED) by tape stripping. Fifteen pieces of tape were used, and the pieces were placed

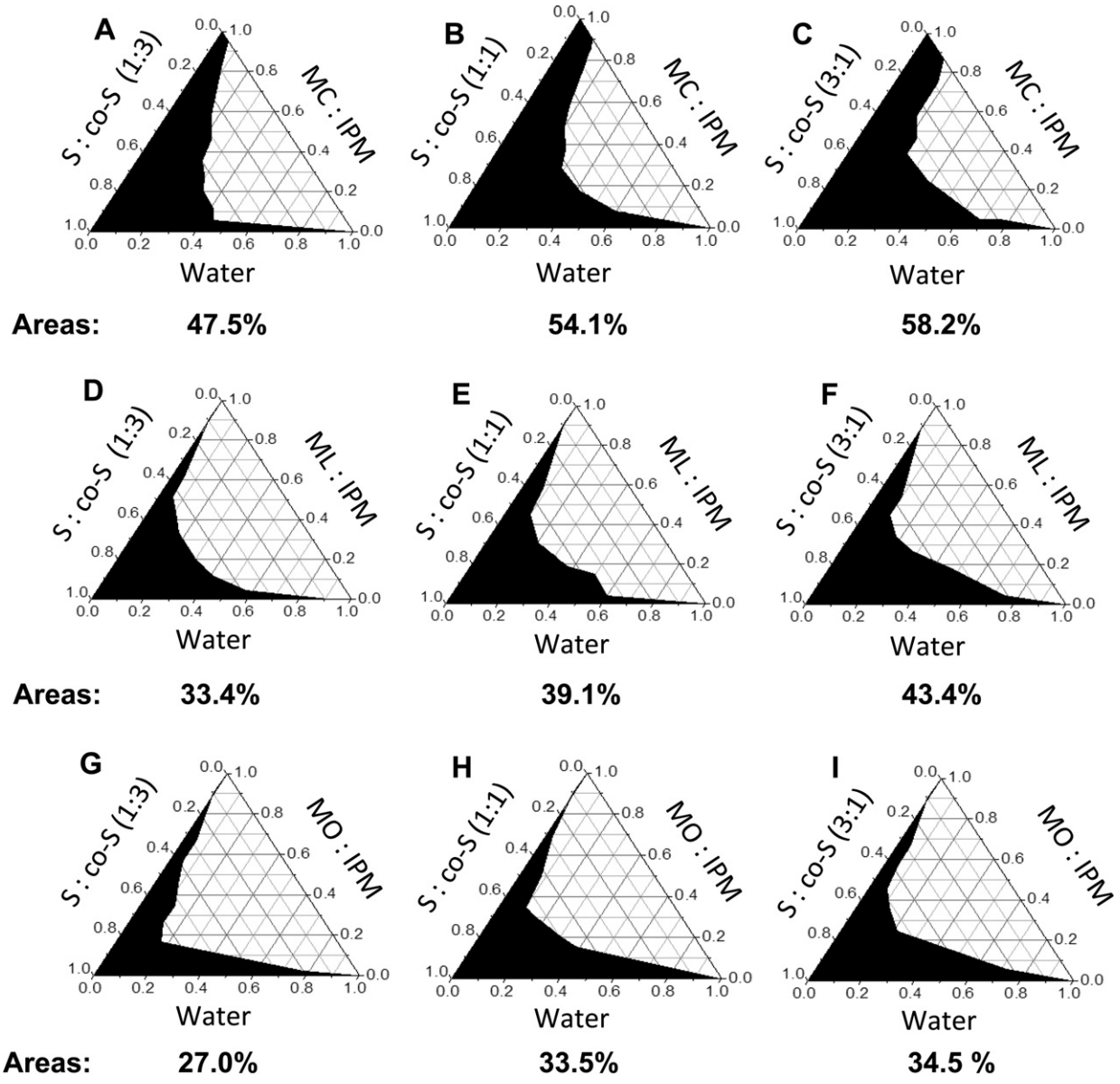

Fig. 1. Phase diagrams of mixtures composed of decylglucoside:propylene glycol (3:1, 1:1 or 1:3, w/w) as surfactant–co-surfactant blend, water and oil. (A–C) Phase diagrams of mixtures containing MC; (D–F) phase diagrams of mixtures containing ML; (G–I) phase diagram of mixtures containing MO. The black-shaded areas in the phase diagrams represent monophasic regions to which fluid and transparent formulations that did not undergo phase separation were assigned. The figure also shows the percentage area of the monophasic region.

in conical tubes containing 4 mL of acetonitrile:methanol (1:1, v/v, for lycopene) or methanol (for ascorbic acid). The remaining skin was cut and placed in 2 mL of either acetonitrile:methanol (1:1, v/v) solution, or methanol only, and homogenized using a hand-held tissue homogenizer (Biospec products, Bartlesville, OK). Samples were sonicated for 30 min, and the supernatant was filtered through 450 nm pore membranes. The amount of lycopene and ascorbic acid in the samples were quantified using HPLC. Aliquots of the receptor phase were assayed for lycopene and ascorbic acid. Because of its low solubility (below 2 ng/mL in plain water) (Vertzoni et al., 2006), lycopene from the receptor phase was concentrated 25-fold. Part of the receptor phase (1 mL) was subjected to drug extraction using 3 mL of chloroform (spiked with 0.1 mg/mL BHT for protection) (Vertzoni et al., 2005). After chloroform evaporation, the residue was suspended in 40 μL of acetonitrile:methanol, and the drug was assayed by HPLC. The concentrations of drugs in SC and ED are indices of topical delivery, whereas the concentration in the receptor phase is an index of transdermal delivery.

The stability of lycopene and ascorbic acid in the microemulsions was studied for the duration of the skin penetration assays (12 h). Plain microemulsions and those containing the antioxidants were diluted with methanol (1:10, 1:50 or 1:100, w/w), filtered (450 nm pore membranes), and assayed by HPLC. The content of lycopene and ascorbic acid ranged from 74 to 80% and 83 to 85%, respectively. Similar amounts of ascorbic acid were found in dodecylglucoside and cocoamide propylbetaine-based microemulsions after 24 h (Gallarate et al., 1999). Even though microemulsions and other aggregates increase lycopene and ascorbic acid stability compared to solutions, both compounds are easily degraded, and addition of another protective compound (as another antioxidant) could improve stability for longer periods of time (Boon et al., 2008; Gallarate et al., 1999; Vertzoni et al., 2006). However, we chose not to add it in this study as one of our goals was to evaluate whether

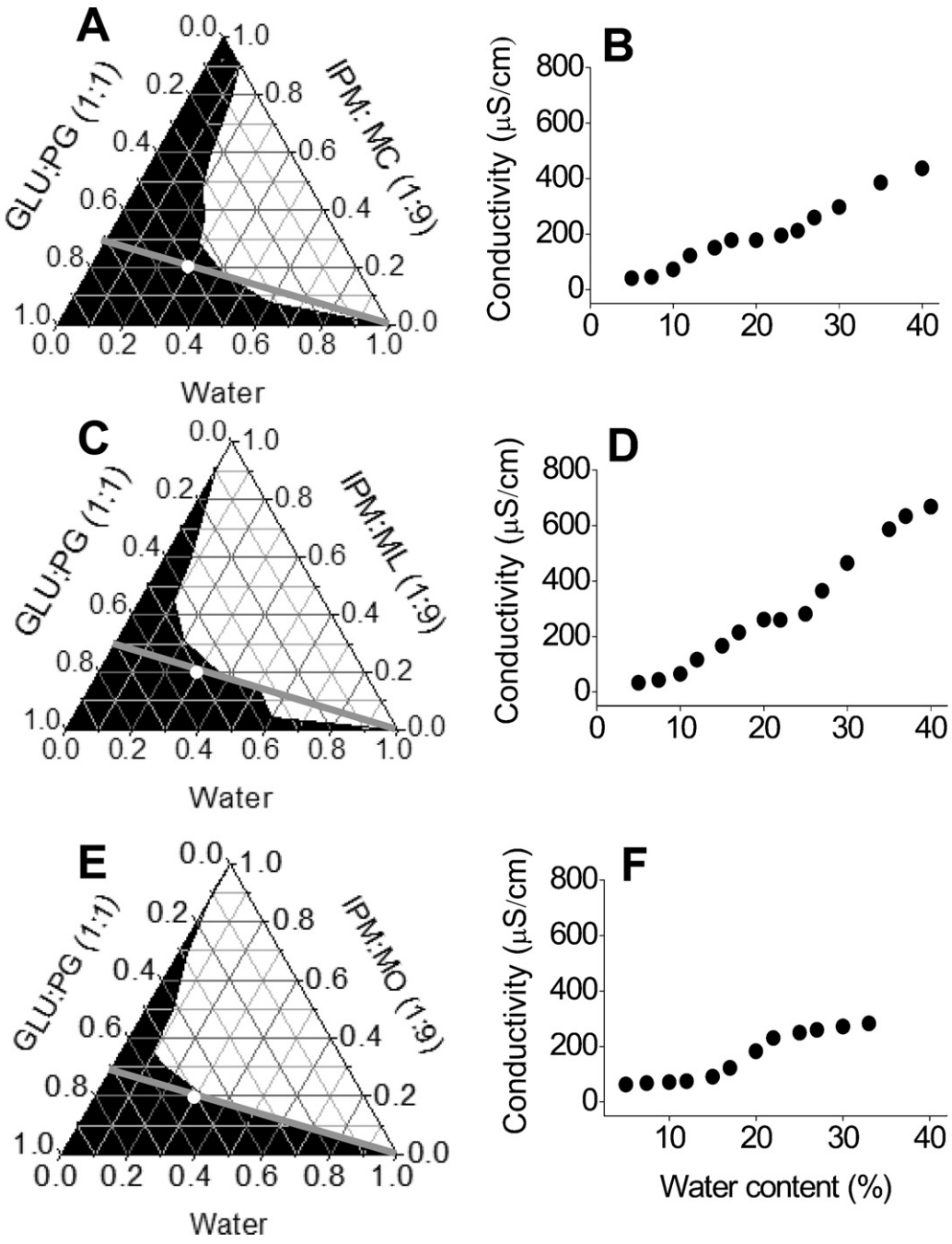

Fig. 2. Phase diagrams and conductivity as a function of aqueous content of mixtures containing MC (A and B); ML (C and D) and MO (E and F). The diagrams show the dilution lines investigated in the conductivity studies (gray lines in the diagrams), and the composition of microemulsions ME-MC, ME-ML and ME-MO.

lycopene and ascorbic acid displayed synergism, and addition of another antioxidant could interfere with their relationship.

2.2.5. Effect of microemulsions containing various monoglycerides on the electrical resistance of skin

The influence of microemulsions on the barrier function of the stratum corneum was studied by assessing changes in the electrical resistance of the skin tissues before and after application of the formulations using a LCR multimeter (Mod. 179, accuracy 0.8%, Fluke, Everett, WA). Skin samples were mounted in diffusion cells and PBS was added to both the donor and receptor compartments. After equilibration for 20 min, the baseline skin resistance was measured by inserting the electrodes in the donor and receptor compartments (Novotny et al., 2009; Rachakonda et al., 2008). Following, PBS in the donor compartment was replaced with 100 mg of water (control), decylglucoside–propylene glycol mixture (at 1:1, w/w) or the microemulsions for 12 h. By the end of the experimental period, skin samples were rinsed with water, carefully blotted dry, the donor compartment was refilled with PBS, and electrical resistance was measured.

2.2.6. Evaluation of the cutaneous irritation potential of a selected microemulsion

After determining that ME-MC was the formulation that delivered the highest amounts of lycopene into the skin, we evaluated its irritation potential in bioengineered skin equivalents in comparison to PBS and Triton-X100. These tissues are often used for primary screening of formulation safety, offering the advantage of

mimicking the complex structure of skin without exposing animals or human volunteers to potentially hazardous chemicals (Faller et al., 2002; Spiekstra et al., 2009). While PBS is considered safe (and used as negative control), Triton-X100 is considered a moderate irritant (Bagley et al., 1999; Verhulst et al., 1998), and was used as positive control as recommended by the manufacturer of EpiDerm tissues (MatTek corporation, Ashland, MA) for producing longer ET_{50} (time of exposure necessary to reduce viability to 50%) values compared to other surfactants, and allowing comparison among treatments for longer periods of time.

Treatments (75 mg) were placed in contact with the stratum corneum of the skin equivalents and incubated at 37 °C and 5% CO_2 for 2, 5 and 18 h (Kandárová et al., 2009). A colorimetric assay that measures the reduction of a yellow tetrazolium component into an insoluble purple formazan product by viable cells (MTT) was used to account for tissue viability. After treatment, tissues were rinsed with PBS, and incubated with 300 μL of MTT solution (1 mg/mL) for 3 h at 37 °C and 5% CO_2. MTT was then extracted by immersing the tissues in 2 mL of extracting solution (MatTek corporation, Ashland, MA) overnight. The optical density of the extracted samples was determined at 570 nm (background reading at 650 nm was subtracted from the readings), and percentage of tissue viability was plotted as a function of time of treatment. The time of exposure necessary to reduce cell viability to 50% (ET_{50}) was determined.

2.2.7. In vitro antioxidant potential of skin samples treated with antioxidant-loaded microemulsions

To evaluate whether lycopene and ascorbic acid that penetrated in the skin were active as antioxidants, the antioxidant capacity of skin samples treated for 12 h with ME-MC containing lycopene and ascorbic acid was compared to skin treated with unloaded ME-MC, and ME-MC containing only lycopene or ascorbic acid. The CUPRAC (cupric reducing antioxidant capacity) assay was used (Ozyurek et al., 2008). Skin homogenates were combined with copper chloride (0.5 mL, 10 mM), neocuproine (7.5 mM) and ammonium acetate (1 M, pH 7) (Lopes et al., 2010; Ozyurek et al., 2008), vortex-mixed for 1 min, incubated for 30 min at room temperature, and absorbance was determined at 450 nm. A standard curve of Trolox (0.01–0.5 mM) was used as reference to estimate the antioxidant capacity of skin samples.

2.2.8. HPLC analysis

Lycopene and ascorbic acid were assayed using a Shimadzu HPLC equipment (Prominence series, LC-20AB dual pump, SIL-20A autosampler, SPD-M20A photodiode array detector and Class-VP software). Separation was performed using a Prevail C_{18} column (150 mm × 4.6 mm, Alltech, Deerfield, IL), equipped with a C_{18} precolumn (7.5 mm × 4.6 mm, Alltech, Deerfield, IL), and isocratic mobile phase consisting of acetonitrile:methanol (52:48, v/v) at 1.5 mL/min for lycopene analysis and 0.05% phosphoric acid:methanol (90:10, v/v) at 0.8 mL/min for ascorbic acid analysis (Lopes and Reed, 2010).

2.2.9. Statistical analyses

The results are reported as means ± SD. Data were statistically analyzed using the ANOVA test followed by Tukey post hoc test. Values were considered significantly different when $p < 0.05$.

3. Results

3.1. Microemulsion characterization

Phase diagrams representing the phase behavior of mixtures containing different amounts of surfactant, oil and water are depicted in Fig. 1. Formulations that were fluid, clear, and did not undergo phase separation were classified as microemulsions, and assigned to a monophasic region (black-shaded region) in the diagrams (Hathout et al., 2010). Overall, the black-shaded region decreased as the length of the monoglyceride increased, regardless of the ratio between surfactant and co-surfactant. A decrease of approximately 20% in the area was observed when monocaprylin was replaced by monoolein, suggesting that the use of shorter monoglycerides allows incorporation of larger amounts of water.

The surfactant:co-surfactant ratio had a less marked effect on the area of the monophasic region. For the same oil phase, increases in the surfactant:co-surfactant ratio from 1:3 to 1:1 (w/w) increased the area of this region by 6–7%. A further increase in the surfactant:co-surfactant ratio to 3:1 increased the black-shaded area by 4% when monocaprylin and monolaurin were used, but had no effect in monoolein-containing samples. Based on the fact that a 1:1 ratio increased the monophasic area independently on the monoglyceride, this ratio was chosen for preparation of the microemulsions with each monoglyceride, all containing surfactant:oil:water at 50:20:30 (w/w/w).

The diagrams B, D and F of Fig. 2 show the influence of water on the electrical conductivity of systems along the dilution lines depicted in A, C and E. The differences in the conductivity values among the formulations might be a result of the different monoglycerides and their lipophilicity, as it was previously observed that the acyl chain length of surfactants and their solubility influenced the conductivity of microemulsions (Zielinska et al., 2008).

In the percolation model, used to distinguish the internal structure of microemulsions, it is generally accepted that a change in conductivity at a given aqueous volume fraction is consistent with phase transformation from reverse (w/o) to normal-type systems (o/w) through the emergence of bicontinuous structures (Hathout et al., 2010; Malheiro et al., 2007). In samples containing MC, very low values of conductivity were observed with less than 7.5% water, which is consistent with w/o systems. Addition of water at 7.5–17% increased conductivity (Fig. 2B), suggesting an increase in aqueous droplets' interlinking process and emergence of bicontinuous structures. Between 17 and 23% of water, the curve exhibited a trend towards more constant values of conductivity, and further addition of water increased conductivity until the system became turbid. Similar results were obtained for ME-ML (Fig. 2D). In MO-containing samples, the conductivity increased slightly up to 12% of water. We did not visualize the area where conductivity tends toward more constant values; instead, we observed a rise in conductivity as water content increased from 17 to 22%. This profile was observed by other authors and attributed to achievement of the percolation threshold (with formation of bicontinuous structures) (Araujo et al., 2010; Kogan et al., 2007). Above 25% of water, conductivity increased slightly until the system lost stability, which occurred at a lower aqueous content compared to the other formulations. A similar change in the conductivity profile among formulations (with microemulsion destabilization at lower aqueous contents) was previously observed with an increase in the formulation content of the lipophilic component (Malheiro et al., 2007), suggesting that the conductivity profile change in the monoolein-containing system compared to the others might relate to its higher lipophilicity.

Since all microemulsions contain 30% of water (being located at ascending portions of the conductivity plot), the w/o structure was excluded. Because the transformation between bicontinuous and o/w systems is less visible in conductivity curves (Naoui et al., 2011), the structure of the microemulsions could be consistent with bicontinuous or o/w systems. Techniques that can determine self-diffusion coefficients (such as NMR) could provide further differentiation (Hathout et al., 2010; Naoui et al., 2011). Since theoretically bicontinuous systems are not organized as droplets (even though they undergo continuous and spontaneous fluctuations with water- or oil-rich domains) (Hathout et al., 2010; Lawrence

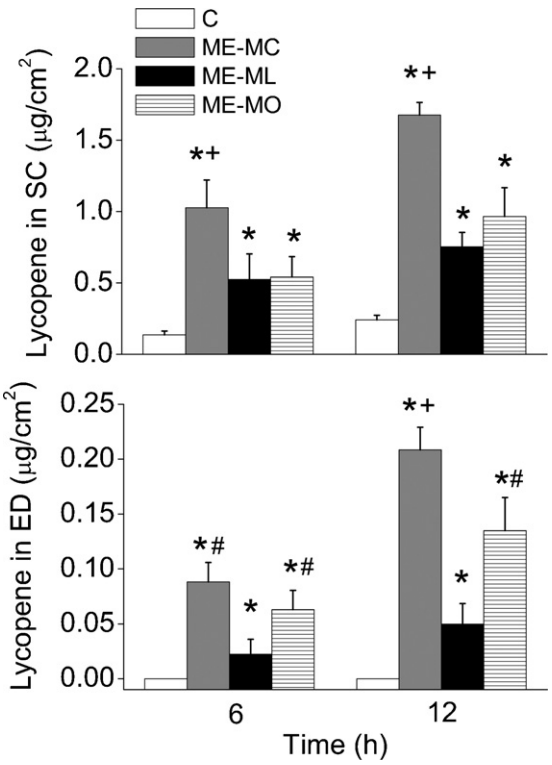

Fig. 3. Penetration of lycopene in the stratum corneum (SC) and viable skin layers (ED) after 6 or 12 h comparing the control solution (lycopene in isopropyl myristate:propylene glycol), ME-MC, ME-ML and ME-MO. $^{*}p < 0.05$ compared to the control solution, $^{+}p < 0.05$ compared to ME-ML and ME-MO, $^{\#}p < 0.05$ compared to ME-ML. Each point represents mean ± standard deviation of 5–9 replicates.

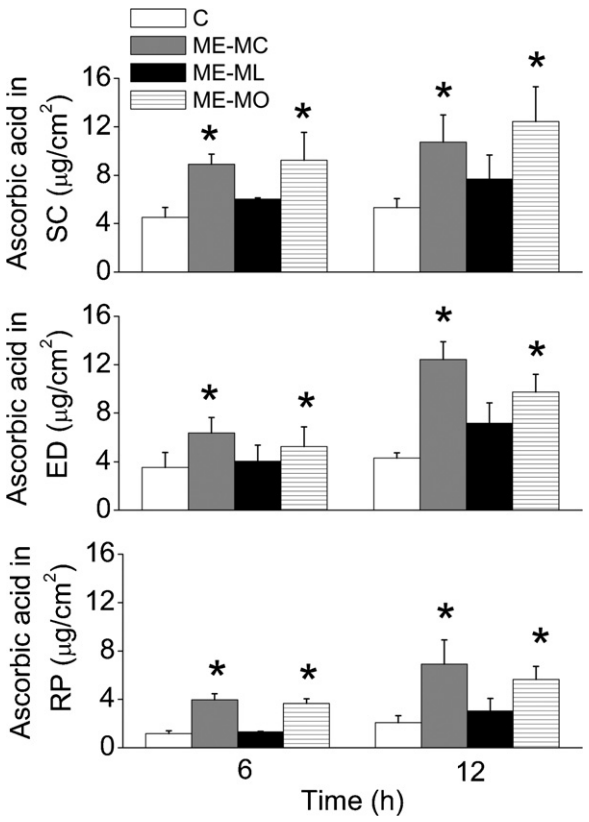

Fig. 4. Penetration of ascorbic acid in the stratum corneum (SC), viable skin layers (ED) and delivery into the receptor phase (RP) after 6 or 12 h comparing the control solution (ascorbic acid in isopropyl myristate:propylene glycol), ME-MC, ME-ML and ME-MO. $^{*}p < 0.05$ compared to the control formulation. Each point represents mean ± standard deviation of 4–6 replicates.

and Rees, 2000; Naoui et al., 2011), size determination was not included in this study.

3.2. Skin penetration assays

Skin penetration of lycopene from ME-MC, ME-ML and ME-MO is shown in Fig. 3. The microemulsions promoted a higher delivery of lycopene at all time-points studied compared to the control formulation. At 12 h post-application (the longest time-period studied), lycopene delivery into SC was 3–7-fold higher using the microemulsions. Lycopene failed to penetrate into viable skin layers when the control solution was used, whereas 0.20 ± 0.02, 0.05 ± 0.02 and 0.13 ± 0.03 μg/cm^2 of the drug was detected when ME-MC, ME-ML and ME-MO (respectively) were used. The effect of ME-MC was superior ($p < 0.05$ compared to other formulations) while ME-ML displayed the weakest penetration-enhancing ability. No lycopene was detected in the receptor phase even after extraction of the drug, which was concentrated 25-fold, allowing detection of 0.8 ng/mL. Thus, we could have detected lycopene after its extraction if it were reaching its solubility limit. The fact that lycopene was not detected suggests that its transdermal delivery may be extremely low.

The results for ascorbic acid skin penetration varied in several aspects compared to lycopene (Fig. 4). Larger amounts of ascorbic acid were delivered, but the effect of ME-MC was weaker and very similar to ME-MO. In addition, ME-ML failed to significantly increase ascorbic acid delivery into the stratum corneum and viable skin layers (Fig. 4). ME-MC and ME-MO significantly ($p < 0.05$) increased ascorbic acid transdermal delivery (2-fold) compared to the control formulation.

3.3. Microemulsion effect on skin electrical resistance

Delivery systems that contain penetration enhancers can increase the skin penetration of drugs by reversibly decreasing the skin barrier function, and consequently, its electrical resistance (Venuganti and Perumal, 2009). To test whether monoglycerides affect skin permeability differently, we evaluated changes in the skin electrical resistance. Treatment with water had a small effect on skin resistance (Fig. 5), which may be a result of tissue

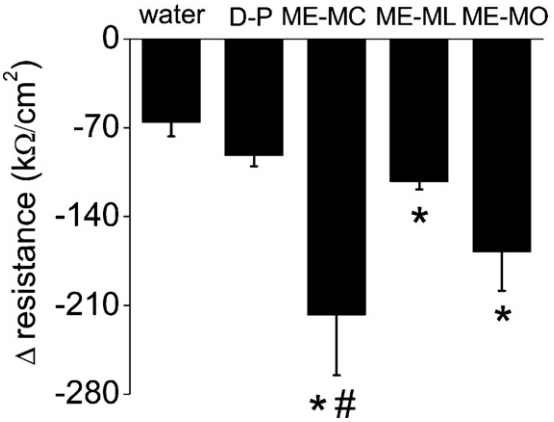

Fig. 5. Effect of treatment with water, decylglucoside–propylene glycol mixture (D–P), ME-MC, ME-ML and ME-MO on skin electrical resistance (as an index of permeability). The change in resistance was calculated based on the initial resistance of skin sections. Each point represents means ± standard deviation of 4 replicates $^{*}p < 0.05$ compared to water. $^{\#}p < 0.05$ compared to ME-ML.

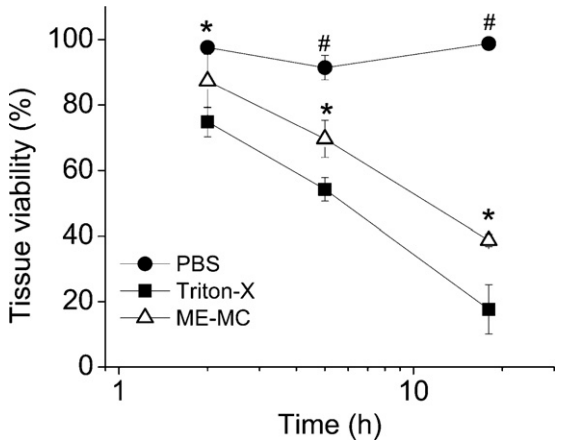

Fig. 6. Time-dependent effect of PBS, Triton-X100 and ME-MC on the viability of reconstructed skin equivalents. Each point represents means ± standard deviation of 3–5 replicates. $^{*}p < 0.05$ compared to Triton, $^{\#}p < 0.05$ compared to Triton and ME-MC.

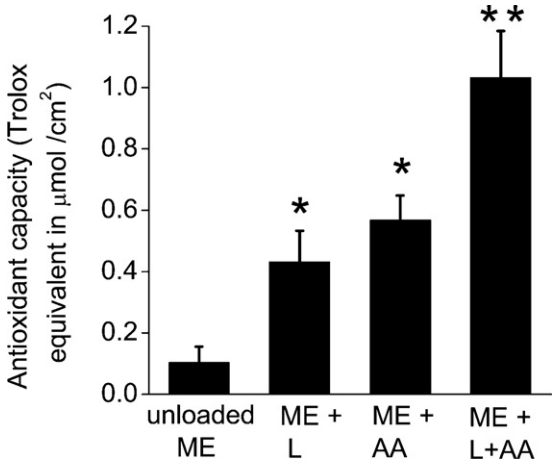

Fig. 7. Comparison of the antioxidant activity of skin sections treated with the unloaded ME-MC, and with ME-MC containing lycopene only, ascorbic acid only or both antioxidants. Each point represents means ± standard deviation of 4–5 replicates. $^{*}p < 0.05$ compared to unloaded ME, $^{**}p < 0.001$ compared to untreated.

manipulation. Compared to water, decylglucoside promoted a mild decrease in skin resistance. All microemulsions had a stronger effect, suggesting that their ability to disrupt the barrier does not depend only on the surfactant:co-surfactant effects, but on the combination of components. ME-MC had the strongest effect (3.4-fold decrease), followed by ME-MO (2.5-fold decrease), and ME-ML (1.9-fold decrease).

3.4. Cutaneous irritation potential of a selected microemulsion

Because ME-MC delivered the highest amounts of lycopene to the skin, this formulation was selected for further studies. The time of exposure to LP-MC necessary to reduce tissue viability to 50% (ET_{50}) was determined and compared to Triton-X100 and PBS (Fig. 6). PBS is considered safe, and as expected, did not reduce tissue viability at any of the time points studied. Compared to PBS, the viability of the tissues treated with Triton was significantly reduced to 78.1 ± 9.5% ($p < 0.05$) after 2 h. An effect of similar magnitude was observed approximately after 5 h when ME-MC was used, suggesting that ME-MC is better tolerated by the tissue. The estimated ET_{50} for triton and ME-MC was 4.5 and 12 h, respectively, demonstrating the lower irritation potential of ME-MC.

3.5. Antioxidant capacity of the microemulsion-treated skin

In this experiment, we evaluated whether lycopene and ascorbic acid were delivered in their active, antioxidant form, and whether their combination could further increase the antioxidant activity of the skin. Compared to skin treated with unloaded ME-MC, the antioxidant activity of the skin increased 4-, 5.6- and 10.2-times after treatment with ME-MC containing lycopene only, ascorbic acid only, and both antioxidants, respectively (Fig. 7). These results suggest that both antioxidants are active after penetrating the skin, and that their combination seems to result in an additive effect.

4. Discussion

The monoglycerides had a more pronounced effect on the monophasic, black-shaded region (to which isotropic, single-phase and fluid formulations were assigned) than propylene glycol content, with the smallest monoglyceride providing an area 20–24% larger. The water solubilization capacity of surfactants seems greater when lower molecular weight oils (with shorter chains or a smaller substitution degree of fatty acid chains) are used as oil phase in microemulsions (Djekic and Primorac, 2008). In line with our observations, it was previously described that the use of isopropyl myristate or ethyl oleate resulted in the formation of microemulsions over a wider range of aqueous content compared to decyl oleate (Djekic and Primorac, 2008). Similarly, use of medium chain mono/diglycerides led to a larger area of microemulsion formation than triglycerides (Lopes et al., 2010).

In addition to affecting the dimensions of the monophasic region in phase diagrams, the type of monoglyceride also affected the skin penetration of the antioxidants. Increasing the acyl chain length of the monoglyceride from 8 (ME-MC) to 12 (ME-ML) carbons significantly reduced the skin penetration of lycopene. However, the opposite effect was observed when the acyl chain was increased from 12 to 18 (ME-MO) carbons. The results in skin penetration coincide with changes in skin electrical resistance, and suggest that the superiority of ME-MC and inferiority of ME-ML result from differences on their ability to modulate the skin barrier. These results partially agree with those observed by Furuishi et al. (2007) describing the progressive reduction on the skin penetration of pentazocin as the monoglyceride acyl chain length increased from 6 to 18 carbons. The main difference between our and previous studies relate to the type of 18 carbon-acyl chain monoglyceride used: while monoolein is unsaturated, Furuish et al. used only saturated monoglycerides. Because the position and presence of double bonds affect the ability of fatty acids to disorganize the stratum corneum and modulate skin penetration (Taguchi et al., 1999), it is reasonable to suggest that not only the aliphatic chain length but also the presence of a double bond impact the penetration-enhancing ability of monoglycerides, making monoolein more effective than a saturated monoglyceride with 18 carbons (Taguchi et al., 1999; Takeuchi et al., 1998).

There were several differences between the delivery of ascorbic acid and lycopene. First, the amounts of ascorbic acid delivered into viable layers and across the skin were larger even if no penetration-enhancing technique was used, which is consistent with the fact that ascorbic acid is less lipophilic and smaller than lycopene (Garti et al., 2004; Lopes et al., 2010; Rozman et al., 2009). Similar observations were reported when delivery of ascorbic acid was compared to α-tocopherol (Rozman et al., 2009). Second, even though ME-ML had a weaker but significant effect on the skin electrical resistance and penetration of lycopene, it had no effect on ascorbic acid delivery. On the other hand, monocaprylin had the most pronounced effect on lycopene delivery, but displayed a similar effect as monoolein on ascorbic acid delivery. These results suggest that formulations able to decrease skin resistance by more than

2-fold increased ascorbic acid delivery independently of individual monoglyceride differences, whereas lycopene delivery was more sensitive to the effect of each monoglyceride.

The ET_{50} for ME-MC was approximately 3 times longer than that of Triton, which suggests that this formulation should be better tolerated by the skin. This is supported by previous observations that a polyglucoside-based emulsion capable of reducing skin equivalent viability to approximately 50% caused no significant changes in the erythema index in human subjects compared to those untreated (Savic et al., 2009). Even though there is no well-defined value of ET_{50} that ensures formulation safety, a previous study reported that several marketed topical formulations with good tolerability have ET_{50} values 3–9 times longer than Triton (Ayehunie et al., 2006).

Combining antioxidants can result in synergistic effects and a potential benefit to the skin. A previous study demonstrated that a regular intake of tomato products can increase cell protection from DNA damage induced by oxidant species, and since increases in plasma bioavailability of lycopene and vitamin C were observed, it was suggested that cell protection could derive from the synergism between these and/or other antioxidants (Riso et al., 2004). In addition, previous studies from our group suggested that ascorbic acid could provide some protection against lycopene degradation. Even though the skin treated with both compounds displayed a ten-fold increase in antioxidant activity (which is higher than the activity after treatment with each compound individually), our results support an additive, not synergistic, effect in the skin.

In conclusion, the type of monoglyceride used in the oil phase of decylglucoside-based microemulsions affected differently the permeability of the skin, and as a consequence, cutaneous drug delivery. The use of monocaprylin improved the formulation's ability to disrupt the barrier function of the skin and maximized the concomitant cutaneous delivery of lycopene and ascorbic acid, which led to an increased antioxidant activity in the tissue. These results reinforce the importance of the molecular structure of oil phases and formulation design when localization of multiple compounds into the skin is desired.

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