# Tocopherol-loaded transfersomes: *in vitro* antioxidant activity and efficacy in skin regeneration

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## ABSTRACT

Transfersomes were prepared by using different polysorbates (i.e., Tween 20, 40, 60 and 80) and loaded with tocopherol acetate, a naturally-occurring phenolic compound with antioxidant activity. The vesicles showed unilamellar morphology, small size (~85 nm), low polydispersity index (≤0.27), and high entrapment efficiency, which increased as a function of the length of the Tween fatty acid chain (from 72% to 90%). The long-term stability of the formulations was evaluated by means of the Turbiscan™ technology, which indicated their good stability, irrespective of the Tween used. The vesicles efficiently delivered tocopherol to the skin, and showed biocompatibility *in vitro* in keratinocytes and fibroblasts. Regardless of the Tween used, the transfersomes were able to protect skin cells from the oxidative damage induced by hydrogen peroxide. Additionally, transfersomes promoted cell proliferation and migration, which resulted in an acceleration of skin wound closure. These results demonstrated that tocopherol-loaded transfersomes bear potential as topical delivery system with antioxidant activity and wound healing properties.

**Keywords:** Tocopherol; transfersomes; Tween; skin delivery; antioxidant activity; skin wound.

## 1. Introduction

The use of naturally-occurring phenolic compounds to treat skin disorders has attracted considerable interest in the last years. Phenolic compounds exhibit low toxicity and a series of biological activities, including antioxidant, anti-inflammatory and antimicrobial activities with health-promoting effects. Evidence suggests that phenols can alleviate symptoms and inhibit the development of various skin disorders, such as skin aging, skin lesions, including wounds and burns, and even cancer development (Działo et al., 2016). The wound healing efficacy of phenols has been attributed to their ability to reduce the amount of reactive oxygen species (ROS) and inflammation developing in the wound, thus promoting the induction of the skin repair mechanisms and accelerating the healing process (Fitzmaurice et al., 2011).

Alpha-tocopherol represents the most abundant form of vitamin E, and is well-known for a strong antioxidant activity, which resides on its ability to stabilize reactive species (Pegoraro et al., 2017). Topically applied tocopherol has previously shown several beneficial and protective effects on the skin. It is considered a photoprotector, as it can reduce ultraviolet (UV)-induced oxidative damage (Lopez-Torres et al., 1998; McVean and Liebler, 1997). Tocopherol has also been shown to be able to accelerate wound closure (Bonferoni et al., 2018; Horikoshi et al., 2018). Its role in repairing injured skin is associated with its antioxidant activity, which counteracts the overproduction of ROS involved in the pathogenesis of wounds (Schäfer and Werner, 2008). In addition, it has been demonstrated that tocopherol accelerates wound repair by promoting cell polarization and migration in human keratinocytes via phosphatidylinositol kinase/protein kinase C signalling cascade (Horikoshi et al., 2018).

The administration of tocopherol is, however, limited by its poor water solubility and chemical instability. The former can be increased by using tocopherol salts, and both can be improved by incorporation in delivery systems (Pereira et al., 2015).

There are only a few scientific studies reporting on the effective incorporation of tocopherol in nanocarriers. Tocopherol has been loaded in lipid nanoparticles, which provided effective protection against UV-induced degradation, thus proving to be promising carriers for ensuring long-lasting antioxidant benefits to the skin (Chen et al., 2017). In another study, tocopherol was loaded in nanovesicles made of lecithin and hyaluronic acid, which were proposed as an innovative system for the treatment of burn wounds (Pereira et al., 2016). Alternatively, chitosan oleate nanoemulsions containing tocopherol were proposed as a wound healing system (Bonferoni et al., 2018). Tocopherol has been incorporated in phospholipid vesicles to improve its delivery to cells and tissues, while acting as membrane stabilizing agent (Gonnet et al., 2010; Kagan, 1989). Moreover, it was found that the incorporation of tocopherol in liposomes reduced lipid oxidation, which resulted in changes in bilayer permeability (Hunt and Tsang, 1981).

Given the poor literature on the topic, in the present study tocopherol in form of acetate salt was loaded in transfersomes, ultradeformable vesicles first introduced in the early 1990s (Cevc and Blume, 1992). Thanks to the elasticity generated by the incorporation of an edge activator in the lipid bilayer structure, transfersomes have been shown to permeate through the stratum corneum lipid lamellar regions as a result of their ability to squeeze through pores appreciably smaller than their own size, and of the hydration or osmotic force in the skin (Cevc et al., 1998; Gupta et al., 2012). Non-occlusive conditions are essential to generating a transepidermal osmotic gradient, which acts as the driving force for elastic transport into the skin. Over the past years, transfersomes have been used as carriers for a range of small molecules, peptides, proteins and vaccines, both *in vitro* and *in vivo* (Cevc and Blume, 2001; Gupta et al., 2012; Jain et al., 2003).

In this study, tocopherol acetate loaded transfersomes were prepared with phosphatidylcholine and different polysorbates (i.e., Tween 20, 40, 60 or 80), with the aim of investigating their feasibility for non-invasive delivery to the skin, along with their antioxidant and wound healing potential. The morphology, size, zeta potential and stability of the vesicles were evaluated, as well as their ability to

deliver tocopherol to the skin. The biocompatibility of the formulations was assessed *in vitro* in skin cells, epidermal keratinocytes and dermal fibroblasts. Moreover, the ability of the formulations to protect the cells from chemically-induced oxidative damage was studied, along with their ability to stimulate cell proliferation and migration in order to achieve wound closure.

## 2. Material and Methods

### 2.1. Materials

Soy phosphatidylcholine (Lipoid S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Alpha-tocopherol acetate, Tween 20 (polyoxyethylene monolaurate), Tween 40 (polyoxyethylene sorbitan monopalmitate), Tween 60 (polyoxyethylene sorbitan monostearate) and Tween 80 (polyoxyethylene sorbitan monooleate) were purchased from Galeno (Carmignano, Potenza, Italy).

### 2.2. Vesicle preparation

For the preparation of transfersomes, 180 mg of S75, 15 mg of Tween (20, 40, 60 or 80) and 15 mg of tocopherol acetate were weighed in a glass vial and dispersed in 3 ml of water. After 4 hours, the dispersions were sonicated with a high intensity ultrasonic disintegrator (20 cycles, 5 sec on and 2 sec off; 13 microns of probe amplitude). The vesicle dispersions (1 ml) were purified from the non-incorporated tocopherol by dialysis against water (2 l) using Spectra/Por® membranes (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, the Netherlands) at room temperature for 2 h by replacing the water every 30 min. The water used (8 l total) was able to theoretically remove the tocopherol contained in 1 ml of vesicle dispersion (5 mg).

### 2.3. Vesicle characterization

Vesicle formation and morphology were evaluated by cryo-TEM analysis. A thin film of each sample was formed on a holey carbon grid and vitrified by plunging (kept at 100% humidity and room temperature) into ethane maintained at its melting point, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 TEM (FEI Company) and the

samples were observed in a low dose mode. Images were acquired at 200 kV at a temperature of ~−173 °C, using low-dose imaging conditions with a CCD Eagle camera (FEI Company).

The average diameter and polydispersity index (PI; a measure of the size distribution width) of the vesicles were determined by dynamic light scattering using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility. For the mean diameter and zeta potential analyses, samples (n=6) were diluted with water (1:100) and analysed at 25 °C.

The entrapment efficiency (EE) of the transfersomes was calculated as the percentage of tocopherol recovered after dialysis *vs.* the amount initially used. The content of tocopherol was determined by high performance liquid chromatography (HPLC) after disruption of non-dialysed and dialysed vesicles with methanol (1:100 dilution), using a Perkin Elmer Flexar chromatograph (Madrid, Spain). The mobile phase was a mixture of methanol (97%) and ethanol (3%) delivered at a flow rate of 2 ml/min through a Brisa LC2 $C_{18}$ column (5 µm, 15 × 0.46 cm); detection was performed at 285 nm, and a calibration curve was built by using standard solutions ($R^2$ = 0.999).

### 2.4. Evaluation of vesicle stability

The stability of the vesicles was evaluated by static multiple light scattering using the Turbiscan Lab Expert (Formulaction, l'Union, France) (Vitonyte et al., 2017). Samples (without dilution) were placed in the test cell, and transmitted/backscattered light was monitored as a function of time (every 2 h) and cell height for 28 days at 30 °C using an Ageing Station (Formulaction). The optical reading head scanned the sample in the cell, providing Transmission (TS) and Backscattering (BS) data every 40 µm in % relative to standards (suspension of monodisperse spheres and silicone oil), as a function of sample height (in mm). The obtained profiles build up a macroscopic fingerprint of the sample at a given time, providing useful information about changes in droplet size distribution, or appearance of a creaming layer, or a clarification front with time.

## 2.5. *In vitro* skin permeation studies

Experiments were performed by using the dorsal skin of one day-old pigs (~1.5 kg) dead from natural causes, provided by a local slaughterhouse. The skin was stored at −20 °C after excision, defrosted and pre-equilibrated in saline (0.9% NaCl in water) 2 h before the experiments. The full-thickness skin specimens (n = 6 per formulation) were sandwiched between the donor and receptor compartments of Franz vertical cells with an effective diffusion area of 0.785 $cm^2$. The receptor compartment was filled with saline (~6.5 ml), thermostated at 37±1 °C, and continuously stirred. The transfersome dispersions were applied (100 µl) onto the skin surface, under non-occlusive conditions. Every 2 h and up to 8 h, the receiving solution was withdrawn, refreshed and analysed by HPLC for tocopherol content. After 8 h, the skin surface was gently washed with bidistilled water and blotted on filter paper. The stratum corneum was removed by stripping with adhesive tape (Tesa® AG, Hamburg, Germany). Epidermis was separated from dermis with a surgical scalpel. The tape strips and the skin strata were cut, placed each in a flask with ethanol, and sonicated for 2 min in an ice bath to extract the tocopherol. The tape and tissue suspensions were filtered out and assayed for tocopherol content by HPLC.

## 2.6. Cell viability assay

Human keratinocytes (HaCaT) and mouse embryonic fibroblasts (3T3) (ATCC collection, Manassas, VA, US) were grown as monolayers in 75-$cm^2$ flasks, incubated in 100% humidity and 5% $CO_2$ at 37 °C. Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, and 1% fungizone was used to culture both cell lines. The cells were seeded into 96-well plates at a density of $7.5\times10^3$ cells/well, and after 24 h of incubation were exposed for 48 h to tocopherol-loaded transfersomes properly diluted to yield tocopherol concentrations of 20, 10, 1, and 0.1 µg/ml. Cell viability was assessed by adding MTT [3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (100 µl, 0.5 mg/ml, final concentration) to each well. After 3 h, the formazan crystals were dissolved in dimethyl sulfoxide,

and the absorbance was read at 570 nm. The experiment was repeated at least three times, each time in triplicate. The results are presented as a percentage of untreated cells (100% viability).

### 2.7. Protection against cellular oxidative stress

HaCaT and 3T3 cells were seeded into 96-well plates and incubated at 37 °C for 24 h. The cells were exposed to hydrogen peroxide (1:40000 dilution) and incubated for 4 h with the tocopherol-loaded transfersomes at a final concentration of 1 μg tocopherol/ml. The cells were washed with phosphate-buffered saline (PBS), and the viability was assessed by the MTT test. The measurement of cell survival was used to quantify the protective effect of the formulations against hydrogen peroxide-induced oxidative stress. Untreated cells were used as a negative control (100% viability), and cells exposed to hydrogen peroxide-only were used as a positive control. The experiment was repeated at least three times, each time in triplicate.

### *2.8. In vitro* scratch assay

The ability of the tocopherol-loaded transfersomes to stimulate HaCaT and 3T3 proliferation and migration was evaluated by measuring the cell expansion on wound surface (scratch assay). The cells were seeded in 6-well plates until complete confluence was reached. Then, a linear scratch was generated using a sterile plastic pipette tip. The scattered fragments of cells were removed by gently washing with fresh medium. The cells were treated with the vesicular formulations (1 μg/ml tocopherol) and incubated for 24 and 48 h. Untreated cells were used as a control. The speed of cell migration and consequent wound closure was observed under a light microscope using a 10× objective, thus assessing the efficacy of the formulations in wound healing.

### 2.9. Statistical analysis of data

Results are expressed as the mean±standard deviation. Analysis of variance (ANOVA) was used to evaluate multiple comparison of means and Student's t-test was performed to substantiate differences between groups using XLStatistics for Windows. The differences were considered statistically significant for $p<0.05$.

## 3. RESULTS

### 3.1. Vesicle characterization

Cryo-TEM micrographs showed that all the transfersomes were spherical, unilamellar and small in size, below 100 nm (Figure 1), which points to the fact that the type of Tween used did not affect the vesicle assembly. To complement these results, the mean diameter of the vesicles was measured by dynamic light scattering, which provides a global estimation of the vesicle size calculated from the scattering intensity of each particle population. As shown in Table 1, empty transfersomes were around 85 nm in size, with good homogeneity (PI $\leq$ 0.27) and highly negative zeta potential (ZP ~−63 mV), due to the charge carried by S75. The encapsulation of tocopherol did not affect vesicle size, while the zeta potential values were more negative (~−80 mV; Table 1), due to the arrangement of the phenol within the vesicles. It is noteworthy that the type of Tween used had a negligible effect on the above parameters (i.e., size, PI, ZP), while it was a critical factor for the entrapment efficiency, which was found to increase progressively as a function of the length of the Tween fatty acid chain and the hydrophilic/lipophilic balance (HLB). The transfersomes containing Tween 20 (HLB 16.7) had the lowest entrapment efficiency (72%), while the transfersomes containing Tween 80 (HLB 15) had the highest entrapment efficiency (90%; Table 1).

The long-term stability of the transfersome formulations was evaluated by using the Turbiscan™ technology, which provides early information on possible destabilization processes occurring in a colloidal dispersion, as it discriminates between reversible particle migration (sedimentation, flocculation, or creaming), and irreversible particle-size change (coalescence). The Turbiscan™ measures the backscattering (BS) intensity versus sample height and aging time. This allows the monitoring of particle diameter evolution and concentration change, as the BS is directly dependent on the particle mean diameter and volume fraction. The main instability phenomena typically observed in colloidal systems are particle migration (i.e., local variation of the concentration of particles that causes a local variation of the BS level measured at the bottom and top of the sample)

and particle size increase (i.e., global variation of the particle size that causes a global variation of the BS level measured in the middle of the sample).

As can be seen in the profiles depicted in Figure 2, all the transfersomes possessed good stability, since BS variations ranged from 7% to 12%. Variations in size due to flocculation were observed in the samples, and a peak appeared approximately after 10 days in the right part of the cell for transfersomes containing Tween 20 and Tween 60, which indicates the occurrence of migration phenomena to the upper part of the cell (creaming) caused by density differences between the vesicles in dispersion and the continuous water phase.

These results point to the good stability of the prepared transfersomes, since even when instability phenomena occurred, their reversibility (especially in the case of flocculation) allowed the re-suspension of the vesicles by gentle shaking.

### *3.2. In vitro* skin permeation

The skin delivery capabilities of transfersomes were evaluated under non-occlusive conditions, which are crucial to generating a transepidermal osmotic gradient that drives the transport of the vesicles and their payload into the skin. The accumulation of tocopherol in the main layers of the skin (i.e., stratum corneum, epidermis and dermis) and in the receptor compartment of the Franz cells, which mimics the biological fluids, was quantified (Figure 3). The highest amounts were found in the stratum corneum: approximately 8.5% for all the transfersomes, with the exception of the transfersomes containing Tween 80, which provided a higher accumulation (>11%). The values of accumulation in the epidermis and dermis were 3.5% and 5.5%, with no statistically significant differences among the formulations. Overall, the accumulation of tocopherol in the whole skin was around 18% as an average of the four formulations, with transfersomes containing Tween 80 providing the highest value (>19%).

The amount of permeated tocopherol was approximately 6.5%, regardless of the formulation applied on the skin.

### *3.3. In vitro* biocompatibility and antioxidant activity

The biocompatibility of tocopherol loaded transfersomes was evaluated by using both epidermal keratinocytes (HaCaT) and dermal fibroblasts (3T3). No toxic effect was observed in HaCaT cells exposed to the formulations, as the viability was unchanged compared with control untreated cells, regardless of the tocopherol concentration tested (0.1-20 μg/ml; Figure 4), while a dose-dependent decrease in viability (from ~95% to 75%; Figure 4) was observed in 3T3 cells. Since no statistically significant differences were detected among the transfersomes ($p>0.05$), it can be concluded that the type of Tween used to prepare the transfersomes had no impact on the biocompatibility of the formulations.

The antioxidant activity of the formulations was evaluated as a function of their ability to protect the keratinocytes and fibroblasts from the damages induced by hydrogen peroxide as oxidative agent (Figure 5). Both cell lines showed a ~65% viability when stressed with hydrogen peroxide, due to the ROS produced in culture. When HaCaT cells were treated with the tocopherol-loaded transfersomes, viability reached ~100%, thus highlighting the protective effect of the formulations against ROS ($p<0.05$ versus the cells treated with hydrogen peroxide only). The results from 3T3 cells treated with the transfersomes disclosed an improved protection against the deleterious effects of hydrogen peroxide, and a proliferative response (~115% viability on average) that was stronger when the transfersomes containing Tween 40 were used (127% viability; $p<0.05$).

### *3.4. In vitro* wound closure

The ability of the formulations to promote both proliferation and migration of keratinocytes and fibroblasts was assessed by the scratch assay, which involves the evaluation of cells spreading on the wound area, consisting in a linear cut on the cell monolayer.

In HaCaT cells treated with transfersomes, a progressive decrease in the wound area was observed with time, due to newly formed cells (Figure 6). After 24 h, the regeneration of the cell layer was faster in the cells treated with transfersomes containing Tween 80, and the wound was almost

completely closed, as compared to untreated control cells. The cells treated with the other transfersomes showed an intermediate rate of wound closure. After 48 h, the wound was still open in untreated control cells, while it was closed in the cells treated with the transfersomes, with the exception of those containing Tween 60.

In 3T3 cells, the transfersomes promoted wound closure, as compared to untreated control cells, in a slower manner than in HaCaT cells (Figure 7). After 24 h, the scratch in untreated cells was basically unchanged, while the wound area decreased in the cells treated with transfersomes, with an almost complete closure when transfersomes containing Tween 80 were used. After 48 h of treatment with the vesicular formulations, cell proliferation was stimulated leading to a significant reduction in wound width when transfersomes containing Tween 20, 40 and 60 were used, and a complete closure when the transfersomes containing Tween 80 were used. These results point to a key contribution of the formulations in improving cell proliferation and migration at the wound site, and wound healing.

## 4. Discussion

Our objective in this study was to suppress the increased levels of ROS associated with wounds that damage the cells, by localized delivery of antioxidant tocopherol, and stimulate skin regeneration at the wound site. To achieve this objective, tocopherol-loaded transfersomes were developed to carry and deliver the phenol to the skin. The transfersome formulation was optimized using different polysorbates (i.e., Tween 20, 40, 60, and 80), which weakly affected the physico-chemical features of the vesicles (i.e., size, morphology, stability) and their technologically (entrapment efficiency and transdermal diffusion) and physiologically relevant properties required for wound healing (e.g., antioxidant and regenerative properties). The results showed that the transfersomes were small in size (<100 nm), with high entrapment efficiency (up to 90%), and stable on storage. The elastic vesicles provided a good distribution of tocopherol in the skin strata, as well as its permeation into the systemic circulation. Additionally, the biocompatibility of the formulations, their protective effect against oxidative damage, and their wound healing potential were demonstrated *in vitro* in keratinocytes and

fibroblasts. These skin cells have a relevant role in wound healing, and their proliferation is essential for the complete restoration of the epithelium damaged during lesions (Bainbridge, 2013; Mansbridge and Knapp, 1987). The tocopherol-loaded transfersomes applied at the wound site were able to inhibit the tissue damage caused by the free radicals generated during injury thanks to the antioxidant activity of tocopherol, stimulate the proliferation and migration of skin cells, and accelerate the wound healing process, as demonstrated by the assessment of the speed and degree of wound closure. Previous studies have demonstrated the beneficial effects in wound healing of raw tocopherol when given orally (Musalmah et al., 2005), or of a tocopherol cream applied topically in diabetic rats (Lin et al., 2012), or of tocopherol in chitosan oleate nanoemulsions tested *in vitro* in keratinocyte and fibroblast cell cultures and *ex vivo* in skin biopsies (Bonferoni et al., 2018). To the best of our knowledge, this is the first study reporting on the potential acceleration of wound healing by topical delivery of tocopherol via transfersomes, especially transfersomes containing Tween 80.

## 5. Conclusion

The study suggested the successful development of vesicular formulations that could be used to help control wound oxidative stress, and thereby accelerate wound healing. The type of polysorbate used to prepare the tocopherol loaded transfersomes weakly affected the properties and performances of the vesicles, but the transfersomes containing Tween 80 can be regarded as the most promising formulation, as the vesicles were small (76 nm) and capable of incorporating the highest amount of tocopherol (90%). In addition, they were able to improve, to a better extent, the deposition of tocopherol in the different skin strata, and to ensure the complete closure of the wound. Additional investigations, such as targeted *in vivo* tests, are needed to validate the *in vitro* antioxidant and wound healing activities of the prepared tocopherol loaded transfersomes.

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401 **Figure captions**

402 **Figure 1.** Cryo-TEM images of tocopherol transfersomes prepared with Tween 20, 40, 60 or 80: A,
403 T20Transfersomes; B, T40Transfersomes; C, T60Transfersomes; D, T80Transfersomes.

404 **Figure 2.** Stability study of the transfersomes performed by using the Turbiscan® technology.
405 Backscattering profiles of transfersomes containing Tween 20, 40, 60 or 80 over 28 days at 30 °C. A,
406 T20Transfersomes; B, T40Transfersomes; C, T60Transfersomes; D, T80Transfersomes.

407 **Figure 3.** *In vitro* skin permeation studies: amount of tocopherol accumulated in the skin layers (SC,
408 stratum corneum; Ep, epidermis; D, dermis) and receptor fluid (RF) after 8 h of non-occlusive
409 application of the transfersomes. Each value is the mean ± standard deviation of six experimental
410 determinations.

411 **Figure 4.** Viability of keratinocytes (HaCaT) and fibroblasts (3T3) incubated for 48 h with tocopherol
412 transfersomes. Data are reported as mean values ± standard deviations of cell viability expressed as
413 the percentage of control (untreated cells; 100% of viability).

414 **Figure 5.** Antioxidant protective effect of tocopherol transfersomes against hydrogen peroxide-
415 induced oxidative stress in keratinocytes (HaCaT) and fibroblasts (3T3). Data are reported as mean
416 values ± standard deviations of cell viability expressed as the percentage of control (untreated cells;
417 100% of viability).

418 **Figure 6.** Optical microscopy images of wound closure in keratinocytes (HaCaT) as a function of the
419 treatment with transfersomes for 0, 24 and 48 h, in comparison with untreated control cells. Bars
420 correspond to 500 μm.

421 **Figure 7.** Optical microscopy images of wound closure in fibroblasts (3T3) as a function of the
422 treatment with transfersomes for 0, 24 and 48 h, in comparison with untreated control cells. Bars
423 correspond to 500 μm.

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