Alpha tocopherol loaded chitosan oleate nanoemulsions for wound healing. Evaluation on cell lines and ex vivo human biopsies, and stabilization in spray dried Trojan microparticles.

M.C. Bonferoni[1], F. Riva[2], A. Invernizzi[1], E. Dellera[1], G. Sandri[1], S. Rossi[1], G. Marrubini[1], G. Bruni[3], B. Vigani[1], C. Caramella[1], F. Ferrari[1]

[1] Department of Drug Sciences, University of Pavia, Vle Taramelli 12, 27100, Pavia, Italy

[2] Department of Public Health, Experimental and Forensic Medicine, University of Pavia, via Forlanini 2, 27100 Pavia, Italy

[3] Department of Chemistry, Physical Chemistry Section, University of Pavia, Viale Taramelli 16, 27100 Pavia, Italy

Corresponding author

Maria Cristina Bonferoni

Department of Drug Sciences, University of Pavia

Vle Taramelli 12, 27100 Pavia, Italy

Tel +39 0382 987375

E-mail: cbonferoni@unipv.it

Keywords: Chitosan, Oleic acid, Nanoemulsion, alpha Tocopherol, Wound healing

Abstract

An amphiphilic chitosan salt, chitosan oleate (CS-OA), was previously proposed for the physical stabilization of lemongrass antimicrobial nanoemulsions (NE) through a mild spontaneous emulsification process. As both chitosan and oleic acid are described in the literature for their positive effects in wound healing, in the present study CS-OA has been proposed to encapsulate alpha tocopherol (αTph) in NEs aimed to skin wounds. A NE formulation was developed showing about 220 nm dimensions, 36% drug loading, and αTph concentration up to 1 mg/ml.

Both CS-OA and αTph NE stimulated cell proliferation on keratinocytes and fibroblast cell cultures, and in ex vivo skin biopsies, suggesting the suitability of CS-OA and of the antioxidant agent for topical application in wound healing.

αTph stability, was further improved with respect of encapsulation, by spray drying the NE into a powder (up to about 90% αTph residual after 3 months). The spray drying process was optimized, to improve powder yield and αTph recovery, by a design of experiments approach. The powder obtained was easily re-suspended to deliver the NE and resulted able to completely release αTph.

1. Introduction

Immediately after wound occurrence, the inflammatory phase starts and immune cells, that invade the wound, secrete proteolytic enzymes and pro-inflammatory cytokines. Inflammatory cells produce and secrete large amounts of reactive oxygen species (ROS), whose levels are especially high in case of burns and diabetic lesions [1]. ROS have a defensive role, essential to protect the organism against invading bacteria and to reduce infections. At low concentrations, they have moreover a positive effect on wound healing, as they serve as signaling messengers in the cell, and facilitate angiogenesis [2, 3]. It is generally recognized, however, that excessive or too prolonged production of ROS causes oxidative stress, lipid peroxidation, and severe cell damage, contributing to the pathogenesis of chronic, non-healing wounds [4]. The positive role of endogenous antioxidants in wound healing seems confirmed by the evidence of their depletion in wounds, especially in aged, in immunosuppressed and in diabetic subjects. This correlation between low antioxidant levels and impaired wound healing process suggested the possible usefulness of antioxidant supplementation in case of chronic wounds [5]. Alpha tocopherol (αTph) represents the most abundant form of Vitamin E, and is well-known for its strong endogenous antioxidant activity, important to stabilize membranes and prevent lipid peroxidation. Supplementation with αTph was proposed in the literature to improve wound healing, although the presence of some conflicting findings suggests the necessity of further studies [6, 7]. Some evidence of the positive role of αTph even after topical delivery to wounds and burns is given. αTph in semisolid vehicles reduced lesions produced in rats by adriamycin

hydrochloride, accelerating skin regeneration [8], and enhanced healing of excision wounds in diabetic rats [9].

The administration of αTph presents, however, some challenges due to its poor stability and water solubility, due to marked hydrophobic properties. This aspect stimulated different studies aimed to encapsulate it in nanoparticles, emulsions and nanoemulsions, not only for oral but also for topical administration [10-13]**.** For αTph, like for the other liposoluble vitamins, encapsulation can improve stabilization and efficacy [14].

An amphiphilic chitosan salt, chitosan oleate (CS-OA), was previously proposed for the physical stabilization of an antimicrobial essential oil nanoemulsion (NE) prepared through a spontaneous emulsification process [15, 16]. In the present study, CS-OA has been proposed to encapsulate αTph in NEs aimed to wound healing. NEs represent a drug delivery system of recent interest as a vehicle of poorly soluble hydrophobic actives, although some questions are still open concerning the development of mild emulsification methods and the availability of effective well tolerated emulsifiers, aspects that require further studies [17]. Amphiphilic polymers are effective stabilizers of NEs due to the combination of surfactant activity and steric protection of dispersed phase [18]. Moreover, by assembling at the interface around the oil droplets, they can impart to the NEs some of the biological properties that characterize the polymer. In the case of the poorly soluble hydrophobic αTph the administration in NE formulations should improve the dispersion in the aqueous environment of the wound. Some advantages can be envisaged in wound and burn treatment for the combination of αTph and CS-OA, that, as amphiphilic polymer, has both stabilization action and wound healing activity. Both chitosan and oleic acid are in fact described in the literature for their positive effects on wound healing acceleration [19, 20]. To confirm this, in the present study the proliferative effect of unloaded CS-OA and αTph loaded NEs was evaluated on cell cultures of keratinocytes and fibroblasts and on ex vivo skin biopsies. This last model has the advantage of maintaining the intercellular signaling pathway not present in cell culture models, representing a complete substrate for the evaluation of the systems under study. The stabilization of αTph in NEs was checked and, to further improve it, a NE delivery system, based on Trojan microspheres as powder for cutaneous application, was developed by spray drying. The spray drying process was optimized by means of a design of experiments (DOE) approach based on a central composite design to improve powder yield and αTph loading. The NE dispersion and the αTph release rate from the so obtained powder were evaluated.

2. Materials and methods

2.1 Materials

The following materials were used: Chitosan (CS) was obtained as HCl salt from low molecular weight (LMW, 50-190 KDa) chitosan base, deacetylation degree 80% (Sigma Aldrich, Milan, I), by addition of HCl 0.5 N to chitosan until complete dissolution, dialysis in bidistilled water for 24 h and freeze-drying

(HetoDrywinner, Analitica de Mori, Milan, I). Oleic acid was from Fluka (Milan, I) α-tocopherol (αTph) was from Sigma-Aldrich, (Milan, I). Other reagents were all from Carlo Erba (Milan, I).

2.2 Methods

2.2.1 Preparation of nanoemulsions

Chitosan oleate (CsO) was obtained, as previously described [21, 22] in situ by self-assembling during the preparation of the NE samples. Acetone solutions of alpha tocopherol (αTph) (1 mg/ml) and oleic acid (10 mg/ml) were prepared. Different volumes of these solutions were mixed and, according to spontaneous emulsification [23, 16], added to 0.5 mg/ml aqueous solutions of chitosan HCl under stirring to obtain the intended chitosan (CS): oleic acid (OA) ratio (either 1:1 or 1:0.5). Acetone was removed in rotavapor (Laborota 400, Heidolph, Heidolph Instruments GmbH e Co., Germany) at room temperature. The samples were sonicated 15 minutes (Elmasonic S 80 H, Elma Hans Schmidbauer GmbH & Co, Singen, Germany) before characterization.

2.2.2 Dimensional and zeta potential characterization of dispersed phase

The particle size and the Polydispersity Index (PI) of the dispersed phase were measured by Photon Correlation Spectroscopy (PCS) (N5 Submicron Particle Size Analyser Beckman Coulter, IL, Milan, Italy). Samples were diluted in filtered bidistilled water and analyzed at 90° detection angle. PI indicates the width of the size distribution ranging between 0 (monodispersity) and 1. At least three replicates were performed. Zeta potential measurements were performed by means of a Zetasizer Nanoseries (Malvern Instrument) with a zeta DTS1060C cell. Three measurements were performed for each sample.

2.2.3 αTph HPLC analysis

αTph in NEs was evaluated by HPLC analysis. A Perkin Elmer Series 200 apparatus with UV-Vis detector was used, equipped with a C18 Waters column (3.9 mm diameter 150 mm length, particle size 10 µm). Mobile phase was CH_3CN:acetate buffer 95:5 (v/v) pH 4.0. Flow rate was 1 ml/min. Detection was performed at 292 nm wavelength (Perkin Elmer Instrument Lambda 25 UV/VIS Spectrometer, Monza, I) by comparison with a calibration curve. Both the standards and the samples were diluted in CH_3CN:acetate buffer (80:20 ratio) pH 4.0 before injection. This medium was previously found to be capable of dissolving all the nanoemulsion components. Three replicates were performed on each sample. The αTph Encapsulation Efficiency (EE%) and Loading capacity % were calculated by difference on the NE after centrifugation performed to separate the eventual not encapsulated oil, using the following ratios:

EE% = (amount of αTph quantified in NE)/(amount of αTph added to the system) x 100

Loading capacity % = (amount of αTph quantified in NE)/(amount of CS-OA + αTph added to the system) x 100

A preliminary stability evaluation was performed by analyzing residual αTph in the NE after definite times of storage until 1 month at 25 °C and 4-8°C. The αTph content was quantified in spray dried powder after 1 month and 3 months storage at room temperature. In all cases the analysis was performed by means of HPLC, as previously described.

2.2.4 Keratinocytes isolation and culture

For in vitro experiments, human primary cell cultures of epidermal keratinocytes were used, obtained from surgical biopsies according Häkkinen et al. [24, 25]. Biopsies was subjected to protease digestion to separate epidermis, that was in turn digested with trypsin (0.05% trypsin, 0.01% EDTA) under stirring for 10 minutes at room temperature to isolate keratinocytes. Cells were centrifuged 10 minutes 1200 rpm, and the pellet re-suspended in specific growth medium (EpiGRO^{TM}, Millipore, CA). Cells were directly seeded on round slides 12 mm diameter ($2*10^5$ cells/slide). Slides were introduced in 12 well plates and incubated at 37°C in 5% CO_2 atmosphere.

2.2.5 Keratinocyte proliferation test

Proliferation test was performed on keratinocytes grown to confluence on cover slides that were then introduced into a 12 well plate. Wells were added with amounts of αTph nanoemulsion corresponding to 67 µg αTph /well. Corresponding amounts of unloaded chitosan oleate were tested for comparison. Controls were represented by untreated cells. To each well 700 µl of growth medium EpiGRO^{TM} were added. The cells were maintained in culture for 24 h and 7 days. At the defined times and 1 h before cell fixation, DNA synthesis was analyzed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU). BrdU was added to the medium at 30 µM final concentration during the last hour in culture. The samples were then washed with PBS and fixed in 70% ethanol. The incorporated BrdU was detected by an immunostaining reaction with Amersham monoclonal anti-BrdU antibody (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England). Briefly, the dishes were washed with PBS and incubated with HCl 2 N for 30 min at room temperature. 0.1 M sodium tetraborate ($Na_2B_4O_7$ $10H_2O$, pH 8.5) was used to neutralize the solution for 15 min, and then the cells were washed twice for 5 min in PBS and incubated for 20 min in the blocking solution (1% w/v BSA and 0.02% w/v Tween 20 in PBS Tween Albumin (PTA)). Cells were then incubated for 1 h with mouse anti- BrdU antibody diluted 1:100 in PTA. The cells were washed three times (10 min each) in PTA and then incubated again for 30 min in PTA containing anti-mouse IgG FITC-antibody (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:100. The slides were extensively washed in PBS, counterstained for DNA with 0.5 µg/mL Hoechst 33258 (Sigma- Aldrich, Saint Louis, MO, USA), and mounted in Mowiol (Sigma- Aldrich, Saint Louis, MO, USA). The slides were observed by means of a

Confocal Laser Scanning Microscope (Leica, TCS SP5II, Leica Microsystems, Milano, Italy). Different images were recorded in different fields of each slide for a total amount of at least 500 cells, and replicating cells were counted with respect to the total number (blue nuclei).

2.2.6. Fibroblasts cell cultures and growth conditions

Normal Human Dermal Fibrobasts (NHDF, Promocell) were grown in Dulbecco's Modified Eagle's Medium (Lonza, Milan, Italy) supplemented with 10% (v/v) Foetal Bovine Serum (Euroclone, Milan, Italy), 1% v/v Penicillin–Streptomycin 100x containing 10,000 units/ml of Potassium Penicillin and 10,000 µg/ml of Streptomycin Sulfate and 25 µg/ml of Amphotericin B (VWR, Milan, I). Fibroblasts were maintained in a humidified (95 RH%) atmosphere with 5% CO_2 at 37 °C and routinely trypsinized every 7 days using trypsin–EDTA solution 0.25%. The cells used were between the 5th and the 13th passage.

2.2.7. Fibroblast cytotoxicity and proliferation test

Fibroblasts were seeded at a density of 20,000 cells/well into a 96-well plate (CELLSTAR Greiner®, Sigma Aldrich, MI, I) and were incubated with 200 µl of NE samples at different concentrations for 24 h in culture medium without serum (DMEM w/s). After that the Neutral Red test was performed: the medium was removed, fibroblasts were washed with PBS and treated with 200 µl of Neutral Red 0.5 mg/ml in HBSS. After 3h the medium was removed, the cells were washed with PBS. 200 µl of 50% v/v ethanol and 1% v/v glacial acetic acid were added to each well to disrupt cell membranes and solubilize NR present in vital cells. The plate was kept away from light for 5–10 min and the absorbance was read by a Microplate Absorbance Reader iMARK™ (Bio-Rad Laboratories S.r.l., Segrate, MI, Italy) at a wavelength of 490 nm, with a reference at 655 nm. The percentage of viability was calculated considering as 100% the viability of the control represented by the culture medium without serum (DMEM w/s). For BrdU test, 50.000 cells were seeded in growth medium without serum (DMEM w/s) on 2x2 cm^2 slides introduced in Petri dishes. After 30 minutes, 200 µl of samples were added and incubated with the cells for 24 h. 1 h before the end of the test, 20 µl of BrdU were added at 30 µM final concentration. Cells were washed with PBS, fixed with 70% ethanol and stored at -20°C. BrdU staining was performed as described for keratinocyte cell cultures.

2.2.8 Antioxidant activity evaluation on fibroblast cell culture

Sensitivity of fibroblasts to H_2O_2 was evaluated in cell culture in a citotoxicity test. 34.000 cells/well were grown in a 96 well plate in complete medium (DMEM 10%). After 24 h cells were incubated with increasing H_2O_2 concentrations in 0.5-2.5 mM final concentration range for 24 h. Cell viability was assessed by means of the Neutral Red test as previously described.

1.5 mM and 2.0 mM H_2O_2 concentrations were chosen to induce significant oxidative damage, and the test was repeated in presence of αTph NE. In this case, after 24 h of initial growth, 200 µl of NE diluted in

DMEM until αTph concentrations ranging from 0.1 to 100 μM were added to fibroblasts. Corresponding dilutions of unloaded CS-OA were also tested for comparison. After 4 h H_2O_2 at final 1.5 and 2.0 concentration was added and after 24 h NR test was performed. The percentage of cell viability was calculated referred to DMEM 10% control.

2.2.9 Ex vivo Punch tests

Punch tests were performed on human skin biopsies ex vivo obtained from breast reduction surgery from young healthy patients (kindly provided by the Plastic and Reconstructive Surgery, Department of Clinical Surgical Diagnostic and Pediatric Sciences, University of Pavia , Pavia, Italy − Plastic and Reconstructive Surgery Unit, Istituti Clinici Scientifici Maugeri, Pavia, Italy). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and received informed consent of all patients. The IRB approved the use of human tissue biopsies for experimental purposes (approval number 921 CEC) on 28/10/2013. The skin, cleaned of the hypodermic layer, was cut with a penknife into fragments of approximately 7x7 mm^2 and each of them was scratched with sterile punch (Ø = 3 mm). The fragments were placed in transwell inserts for 12-well plates (membrane pores: Ø = 0.40 μm, insert area 1,12 cm^2, Constar, Corning, New York, USA). The αTph NE (67 μg alpha tocopherol/punch hole) and corresponding amount of unloaded CS-OA were added to the biopsies. As negative control a non-treated skin fragment scratched with punch was used. In the basolateral compartment 700 μL of the culture medium for fibroblasts with FBS 10% were added to promote the vitality of the skin, ensuring hydration and adequate intake of nutrients through a homogeneous diffusion from the deep dermis. After incorporation for 1 hour with bromodeoxyuridine (BrdU), added to the growth medium at the concentration of 30 μM, at different time, histological analysis was carried out, as described in the literature [25].

Briefly, the skin fragments were fixed in a solution containing paraformaldehyde 4% and phosphate buffer PBS 0.1 M, pH=7.4 for 6 hours, dehydrated by an ascending alcohol scale (from 70% ethanol or absolute alcohol and xylene) and included in paraffin. Using a horizontal slide microtome Leitz (Leitz Wetzlar, Stuttgart, Germany), histological slices of 5-10 μm were obtained, then rehydrated, and stained with hematoxylin and eosin or processed for immune reaction with proliferation markers, either BrdU or proliferating cell nuclear antigen (PCNA), followed by washing in distilled water. Hematoxylin and eosin are dyes of choice for common histological sections, allowing the observation of the morphological appearance of the tissues. Hematoxylin is a basic dye which has affinity for all acid structures (and therefore basophilic) of cells and tissues; eosin stains pink/orange all the acidophilus basic structures. Mouse monoclonal primary antibodies were used for immuno-labeling reactions, anti-BrdU and anti-PCNA respectively, diluted 1:100 in PTA. Later, after two washes in PBS, the sections were incubated with secondary antibody supplied with the kit MACH1 Universal HRP-Polymer Detection Biotin-free in accordance with the instructions provided by the protocol. Finally, the sample sections were rehydrated by immersion in descending

gradation alcoholic solutions, attached with DPX and observed in transmitted light Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany). Proliferating cells positive for BrdU or PCNA were counted in each section. For comparison purpose, the number of cells was related to section areas (normalized cell number).

2.2.10 Nanoemulsions spray drying

NEs obtained as above described were further concentrated to obtain the αTph concentration of about 1 mg/ml, by means of a tangential flow ultrafiltration system (Vivaflow 50R 200, Sartorius Milan, I), with 14000 cut off dialysis membrane.

Spray drying of NEs was performed by means of a Mini Spray Dryer Buchi B-191 (Comaredo, Milan I). Inlet temperature was maintained constant and set at 150 °C, as suggested in the literature [26]. Nozzle pressure was maintained at 600 L/h. Aspiration and inlet flow rate were set according to a central composite design, as indicated in Table 1.

Table 1. Levels of the two spray drying parameters evaluated according to a central composite design.

levels	-1.414	-1	0	+1	+1.414
Inlet rate (ml/min)	7.2	8.0	10.0	12.0	12.8
Aspiration levels (m^3/h)	55.9	60.0	70.0	80.0	84.1

2.2.11 SEM Analysis

The SEM analysis of scanning electron microscopy measurements were carried out using a scanning electron microscope Zeiss, model EVOMA10 (CarlZeiss, Oberkochen, Germany). The samples were made conductive by the deposition of a gold layer by vacuum vapor phase.

2.2.12 DPPH antioxidant activity test

To 975 µl of 6.10^{-5}M of DPPH solution, 25 µl of sample were added and absorbance was read at 515 nm (Perkin Elmer Instrument Lambda 25 UV-VIS spectrometer, Monza, Italy) at definite times until plateau was reached.

Radical scavenging was calculated according to the following formula:

Radical scavenging % = $(1- Abs_0/Abs_{pl})*100$

Where Abs_0= absorbance at time zero; Abs_{pl} =absorbance at plateau

2.2.13 Release test

For release test of alpha tocopherol from spray dried powder, Franz cells were used. Cellulose acetate membrane (0.45µm, Sartorius Stedim Biotech GmbH, Gottingen, Germany) was used to separate the donor and acceptor chamber, that was filled with isopropyl myristate. An accurately weighed sample of powder was placed in the donor compartment and wetted with 1.5ml of physiological solution (NaCl 0.9% p/v). The temperature was set at 32°C. Known volumes of the receiving phase (600 µL) were taken at definite times (1, 2, 3, 4, 5 and 20 hours) and the volume was reconstituted with an equal amount of the receiving phase. The αTph concentration in the receiving phase was quantified by means of HPLC analysis.

2.2.14 Statistical analysis

Statistical evaluations were performed by means of Stat Graphics 5.0, Statistical Graphics Corporation, MD, USA. Differences were determined according to One-way ANOVA or by means of a multifactor ANOVA, as specified in the results. Differences were considered significant at $p < 0.05$. The same statistical package was used for the design and analysis of the Central Composite design used to optimize the spray drying process.

3. RESULTS AND DISCUSSION

3.1 Development and characterization of αTph nanoemulsions

In Figure 1 the mean diameters of four nanoemulsions prepared at two chitosan concentrations (CS 0.05% and CS 0.15%) and two chitosan:oleic acid ratios are reported. In all cases the dimensions are between 200 and 400 nm. This is in line with the results previously obtained with essential oil nanoemulsions [16]. In accordance with previous results, the increase of CS concentration had a significant positive effect on dimensions ($P<0.05$), while no significant effect of chitosan:oleic acid ratio on nanoemulsion dimensions could be evidenced (Multifactor ANOVA, $P<0.05$). The system based on CS 0.05% and CS:OA 1:0.5 ratio, corresponding to final concentrations of 0.5 mg/ml for chitosan, 0.35 mg/ml for oleic acid and 0.33 mg/ml for αTph, was therefore chosen to continue the study. For this NE, characterized by a particle size of 233 (±36) nm and polydispersity index of 0.24 (±0.02), an EE% of 93.64% (±7.97%) was obtained, corresponding to 36.47% (±2.17%) drug loading. The concentration of αTph was 311 (±26) µg/ml, confirming the potential usefulness of the NE to obtain relatively high concentrations of antioxidant in the colloidal dispersion. The chitosan coating was confirmed by positive zeta potential of 56.8 (±2,1) mV.

3.2 In vitro keratinocyte proliferation

Keratinocytes have a relevant role in wound healing and their proliferation is essential for the complete restoration of the epithelium damaged during lesions [27, 28]. The proliferation of keratinocytes in cell culture was here assessed after 24 h and 7 days of contact with the samples and in Figures 2 examples of CLSM microphotographs are given. As BrdU is a synthetic analog of thymidine, it is incorporated in DNA synthesis during cell replication, so that the green BrdU staining shows the cells that are in replication in S

phase. This is superimposed in the pictures to blue Hoechst staining that evidences nuclei independently on cell replication cycle. This approach was chosen to specifically put in evidence the effect of chitosan oleate CS-OA (b) and αTph nanoemulsion (c) on cell proliferation. Comparison was performed with controls represented by untreated cells at the same times (a). The quantification for the control and for the two samples at both contact times is illustrated in Figure 3, where the cells in proliferation were quantified by counting the BrdU positive cells with respect to the total number of cells evidenced in each image by Hoechst 33258 nuclear staining. In Figure 3 the ratio between the cells in proliferation, positive to BrdU staining, and the total cell population, evidenced by blue nuclei staining (Hoechst 33258), is illustrated for the control and for the cells treated with CS-OA and with the nanoemulsion (αTph NE). The αTph NE treatment corresponds to the highest and quickest proliferation of the cells, with a statistically significant increase with respect to controls both at 24h and at 7 days and with respect to CS-OA at 24h (One-way ANOVA, post hoc Fisher test, $P<0.05$). It is noteworthy to notice that even the unloaded CS-OA induced cell proliferation, although slower than in the case of NE, as significant difference between CS-OA and the controls was evidenced at 7 days, but not at 24 h. The literature reports evidence of the ability of chitosan to stimulate the proliferation of fibroblasts, especially in the case of low molecular weight and relatively high deacetylation degree polymer grades, while more controversial is the effect on keratinocytes, whose proliferation resulted inhibited by very low (13 kDa) molecular weight chitosan [28]. Other authors found that both low (50-190 kDa) and high (310-375 kDa) chitosan molecular weights increased proliferation of HaCaT cell line in vitro at 300 μg/ml, and inhibitory effect was observed only at concentrations as high as 1 mg/ml [29]. The positive proliferative effect of CS-OA observed in the present study is in line with these findings considering that a low molecular weight grade was used, and that the chitosan concentration in the final samples was lower than 500 μg/ml. Solubility limits of chitosan in cell culture media and viscosity effect of polymeric solution are moreover not relevant in the present case as CS-OA self-assembled into nanoparticles. Little information can be found in the literature about the effect of unsaturated fatty acids such as oleic acid on fibroblast and keratinocyte proliferation, although it is reported that fatty acid deficiency can delay wound healing [30], whereas their application on mice wound exert a positive effect on wound healing due to neutrophil migration [31].

3.3 Evaluation of nanoemulsions on fibroblasts

3.3.1 Fibroblast cytotoxicity and proliferation

Figure 4 illustrates the cell vitality of fibroblasts after contact with increasing concentrations of αTph NE or with unloaded CS-OA at the same concentrations as in NEs. In the considered range of αTph concentrations (0.1 – 100 μM) a statistically higher growth with respect to the DMEM w/s control was found between 0.25 to 10 μM (Student t test, $P<0.05$). In these cases proliferation increase was about 150-195% of the control, suggesting a positive stimulation of cell proliferation that can be useful in regenerative phases of skin

tissues. The effect of loaded alpha tocopherol can be highlighted by the difference between the two vitality curves given in the figure. Until 50 µM, αTph in NE induced higher proliferation than CS-OA alone. Above 10 µM, cell vitality decreases, but anyway a protective effect seems evident for the αTph NEs, for which vitality was anyway still close to 100% in the interval 20-40 µM. A BrdU test was performed also on fibroblasts, like previously described on keratinocytes, to assess the ability of the samples to stimulate proliferation. The results are illustrated in Figure 5a, where cells in proliferation evidenced by BrdU (green) are compared with the total number of cells stained with blue Hoechst. In Figure 5b the ratio between proliferating and total cells is quantified for the unloaded and the aTph loaded sample and for the controls. Both unloaded CS-OA and aTph NE determined a positive effect on the cell proliferation. It must be remembered that also in this case both CS-OA and αTph were tested on cells grown in DMEM w/s without serum, a poor medium that for which it is possible to note, as expected, a low proliferation. This choice was made to better put in evidence the role of the samples on cell growth and proliferation, that is confirmed higher for the antioxidant αTphNE than for unloaded CS-OA.

3.3.2 Antioxidant activity evaluation on fibroblasts

In Figure 6 (a and b) the results of the study performed to assess the protective effect of the antioxidant αTph NE on fibroblast cell cultures are illustrated. Figure 6a shows the preliminary evaluation of the fibroblasts sensitivity to oxidative damage induced by H_2O_2. On the basis of this curve the antioxidant effect of the αThp NE was assessed on fibroblasts exposed to 1.5 and to 2 mM H_2O_2, that induced strong damage. The results are illustrated in Figure 6b. For both the H_2O_2 concentrations, αThpNEs 5 and 10 µM corresponded to cell viability statistically higher with respect to the untreated control (One-way ANOVA, post hoc Fisher test, $P<0.05$), confirming the antioxidant activity and the protective effect of the encapsulated αThp.

3.4 Keratinocyte proliferation in ex-vivo human skin biopsies

Figure 7 shows histological images of human skin biopsies excised with 3 mm diameter punch, maintained in culture and treated for 24 and 48 h with unloded CS-OA and with αTph NE. In particular, a positive staining of keratinocytes for the nuclear PCNA and for the BrdU antigens can be seen especially after 48 h, indicating proliferating cells. It has been demonstrated in the literature that a reciprocal proliferative stimulation between keratinocytes and fibroblasts occurs in skin regeneration and favors both reconstruction of epidermis to cover wound surface and restore skin function, and collagen production to reconstruct dermis layers. This effect is sometimes indicated as double paracrine signaling [32, 33]. The model here used, based on ex vivo biopsies, is especially advantageous for maintaining the native capability of the skin layers to express mechanisms of communication between the cells involved in wound healing. A proliferation of keratinocytes can be observed in this model in the epidermal layers around the wounds, thus confirming, in spite of the biological variability, the effect previously observed on cell cultures. Figure

8 reports the number of keratinocytes that were positive to immunostaining with PCNA and BrdU. Although care was taken to normalize the number of counted cells to the tissue area, these results can be affected by biological variability and represent a semi-quantitative assay. They anyway contribute to confirm what observed in vitro in cell cultures. Also in this model keratinocyte proliferation appears sensitive to CS-OA and to αTph stimulation, thanks to the fact that the encapsulation of the antioxidant in CS-OA nanoemulsion efficiently brings it in contact with cell lines and wounded tissues. The stimulating effect on fibroblast proliferation could not be directly detected in this part of the study, but hematoxylin-eosin (HE) images (Figure 9) show that reconstitution of the layers underlying the punch wound is occurring in both samples, thus suggesting that fibroblasts are active in producing and reorganizing collagen network. The dermal structure underlining the punch in samples treated with αTph appears especially well organized showing light pink regular collagen bundles.

3.5 Stability of αTph nanoemulsion

In a previous work, NE samples encapsulating lemongrass essential oil showed good physical stability with dimensions stable for at least 3 months storage [16]. In this study similar results were obtained for NE physical stability (data not shown). αTph concentration was analyzed in αTph NE formulation stored at room (25 °C) and refrigerator (4 °C) temperature. As illustrated in Figure 10, after one week αTph content resulted of about 88% and 93% respectively, with respect of the initial amount. At longer times, however, a more relevant decrease, especially at room temperature, was obtained. About 66% and 75%of the initial content remained in fact after 4 weeks. This finding is in quite good accordance with results obtained with αTph encapsulated in different colloidal stabilizers, such as whey protein isolate [34], and indicates a quite good stabilization in the NE samples, considering the high sensitivity to oxygen and light of the molecule described in literature [35]. It seemed however worthwhile to further improve αTph NE stability. To attain this objective, a powder was developed by nanoemulsion spray drying. A similar process was proposed in literature for aTph emulsions [36] and nanoemulsions [26], with different stabilizing agents such as acacia gum. In the present work the spray drying process was developed by a design of experiment (DOE) approach according to a central composite design to assess the conditions of inlet and aspiration rate that allowed to obtain the highest powder yield and αTph content in the powder. In Table 2 the experiments performed are given with the powder yield (%) and the percentage of αTph remaining in the powder, as response variables. The NE dispersions described in Table 1 were concentrated up to final αTph content of about 1 mg/ml and were spray dried with 10% mannitol, added as bulk agent, calculated on the basis of total solid weight. Mannitol is largely used as an excipient in NP spray drying, also based on chitosan, for its quick dissolution and neutral nature. [37]

Table 2. Levels of the factors according to a central composite design and relative results of yield (%) and of percentage of αTph remaining in the powder with respect to the theoretical one.

inlet	aspiration	yield%	αTph%
-1	-1	26.0	67.6
1	-1	28.2	74.5
-1	1	28.9	85.6
1	1	35.7	93.0
0	0	30.6	95.7
0	0	30.1	90.1
0	-1,41421	25.0	74.8
1,41421	0	25.7	87.8
0	1,41421	36.7	94.9
-1,41421	0	23.3	75.3

Figure 11 illustrates the response surface and the contour plots for the αTph percentage of recovery response variables considered in this study. The response followed the equation here reported, while in Table 3 the statistical evaluation of the model is given, indicating good fitness of the model and significant effect for both inlet and aspiration factors:

αTph% = 92,9 + 3,99721*inlet + 8,11572*aspiration - 6,43128*inlet^2 + 0,125*inlet*aspiration - 4,78127*aspiration^2

The percentage of αTph recovered with respect to the initially loaded amount ranged in the different batches between 67 and 95%, confirming the overall good protective effect of the encapsulation of αTph during the spray drying process, that could be further improved by optimizing the choice of process parameters. In particular especially the increase of aspiration rate seems useful for the stabilization, probably for the positive effect of the quick drying and recovery of the powder.

Table 3. ANOVA table for αTph% response in the central composite design study of spray drying process

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:inlet	127,821	1	127,821	11,74	0,0266
B:aspiration	526,918	1	526,918	48,39	0,0022
AA	189,079	1	189,079	17,36	0,0141
AB	0,0625	1	0,0625	0,01	0,9432
BB	104,505	1	104,505	9,60	0,0363
Total error	43,5558	4	10,889		
Total (corr.)	910,401	9			

R-squared = 95,2158 percent
R-squared (adjusted for d.f.) = 89,2354 percent

The response Yield (%) followed the equation hereafter given, and in Table 4 the statistical evaluation of the model is reported. Also in this case the fitness of the model is acceptable but the only factor that

significantly influences the response appears to be aspiration, with a positive effect, indicating that yields increase for highest aspirations:

Yield% = 30,35 + 1,54927*inlet + 3,37183*aspiration - 2,4175*inlet^2 + 1,15*inlet*aspiration + 0,752512*aspiration^2

Table 4. ANOVA table for Yield% response in the central composite design study of spray drying process

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:inlet	19,2018	1	19,2018	4,50	0,1011
B:aspiration	90,9535	1	90,9535	21,32	0,0099
AA	26,7167	1	26,7167	6,26	0,0666
AB	5,29	1	5,29	1,24	0,3278
BB	2,58867	1	2,58867	0,61	0,4795
Total error	17,0608	4	4,26521		
Total (corr.)	177,137	9			

R-squared = 90,3686 percent
R-squared (adjusted for d.f.) = 78,3293 percent

The aspiration was therefore maintained at the maximum level (70%), with inlet rate at 13% to prepare the optimized formulation. The microparticle yield was in this case 39.7% and the analysis of αTph in the microparticles resulted in a recovery of 93.3 %, in quite good accordance with the model (about 94% theoretical value), and corresponding to 20.4 % αTph loading in the microparticles.

In Figure 12 the SEM images of this formulation are given. It is possible to appreciate, inside the spherical microparticles originated by the spray drying process, the spherical nanostructures of the nanoemulsion stabilized by chitosan oleate and embedded in the small amount (10%) of mannitol added. The microparticles obtained have the morphological characteristics of a so-called Trojan system, useful therefore to easily handle the nanoemulsion in a dry powder.

To confirm the ability of this powder to quickly restore original nanoemulsion, particle size after resuspension in distilled water was found 862 (±132) nm at 90°. An increase in dimensions with respect to the nanoemulsion before spray drying is clearly observed, probably due to aggregation, although the sample is still inside the nanoparticle range anyway suitable for topical application. Radical scavenging ability of αTph was maintained after encapsulation in CS-OA and following spray drying process, as demonstrated by 86,1% (±0,014) radical scavenging in DPPH test (performed on αTph concentration of 17,36 µg/ml obtained by microparticle resuspension). The recovery of αTph after storage of the powder at room temperature was 97.9 % (±0.3%) after 1 month and 92,0% (±1,1%) after 3 months. This result confirmed therefore the improvement of spray drying process to stabilize αTph nanoemulsion with respect to aqueous dispersion. The αTph release from the spray dried microparticles, evaluated in Franz cells, is illustrated in Figure 13. Isopropyl myristate was chosen as receptor medium, among the media considered in the literature for cutaneous release tests [38], to maintain αTph sink conditions. It is possible to see that

more than 80% of the antioxidant is released by the powder in the first 3 hours, indicating a release rate compatible with the intended use in wounds.

Conclusions

Chitosan oleate (CS-OA) appears suitable to stabilize NEs loaded with αTph, that are easily dispersible in aqueous media and therefore in wound fluids. The bioactive properties of both chitosan and oleic acid make the amphiphilic polymer especially useful for the encapsulation of αTph aimed to wound healing. The evaluation of the unloaded CS-OA and of the αTphNE systems, performed on cell cultures of fibroblasts and keratinocytes and on ex vivo human skin biopsies, confirm a proliferative effect on both cell lines and on biopsies especially for αTph loaded nanoemulsions, but also for unloaded CS-OA.

In the perspective of the design of a drug delivery system suitable for the NE administration to wounds and burns, a spray drying process was developed by means of a response surface DOE approach to optimize yield and αTph loading in a cutaneous powder. The stability of αTph, improved after encapsulation, was further increased by spray drying the nanoemulsion. This pointed out the possibility to formulate the nanoemulsion in Trojan microparticles, for which good antioxidant ability was assessed. The release of αTph from this powder, complete in a few hours, resulted compatible with a possible daily application in wound or burn treatment.

Aknowledgements

The authors wish to thank A. Farina (of the Histology and Embryology Unit, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Italy) for her much appreciated technical support, and Professors G. Nicoletti and A. Faga (of Plastic and Reconstructive Surgery Unit, Department of Clinical Surgical Diagnostic and Pediatric Sciences, University of Pavia, Salvatore Maugeri Research and Care Institute, Pavia, Italy) for kindly providing human biopsies.

References

[1] B. Latha, M. Babu, The involvement of free radicals in burn injury: a review, Burns 27 (2001) 309–317.

[2] N. Ojha, S. Roy, G. He, S. Biswas, M. Velayutham, S. Khanna, P. Kuppusamy, JL Zweier, CK Sen, Assessment of wound-site redox environment and the significance of Rac2 in cutaneous healing, Free Radic Biol Med. 44 (2008) 682–691.

[3] N. Bryan, H. Ahswin, N. Smart, Y. Bayon, S. Wohlert, J. A. Hunt, Reactive oxygen species (ROS) – A family of fate deciding molecules pivotal in constructive inflammation and wound healing, Eur Cell Mater. 24 (2012) 249-265.

[4] M. Schäfer, S. Werner, Oxidative stress in normal and impaired wound repair, Pharmacol Res. 58 (2008) 165–171.

[5] S.C. Blass, H. Goost, R.H. Tolba, B. Stoffel-Wagner, K. Kabir, C. Burger, P. Stehle, S. Ellinger, Time to wound closure in trauma patients with disorders in wound healing is shortened by supplements containing antioxidant micronutrients and glutamine: A PRCT, Clin Nutr. 31 (2012) 469-475.

[6] D. Altavilla, A. Saitta, D. Cucinotta, M. Galeano, B. Deodato, M. Colonna, V. Torre, G. Russo, A. Sardella, G. Urna, G.M. Campo, V. Cavallari, G. Squadrito, F. Squadrito, Inhibition of Lipid Peroxidation Restores Impaired Vascular Endothelial Growth Factor Expression and Stimulates Wound Healing and Angiogenesis in the Genetically Diabetic Mouse, Diabetes 50 (2001) 667-74.

[7] R. Hobson, Vitamin E and wound healing: an evidence-based review, Int Wound J. 13 (2016) 331–335.

[8] M.J. Lucero, J. Vigo, M.J. Leon, F. Martin, J.A. Sanchez, Therapeutic efficacy of hydrophilic gels of 7-tocopherol and tretinoin in skin ulcers induced by adriamycin hydrochloride. Int J Pharm. 127 (1996) 73- 83.

[9] T.S. Lin, A.A. Latiff, N.A.A. Hamid, W. Zurinah, W. Ngah, M. Mazlan, Evaluation of Topical Tocopherol Cream on CutaneousWound Healing in Streptozotocin-Induced Diabetic Rats, Evidence-Based Complementary and Alternative Medicine (2012) Article ID 491027, 6 pages doi:10.1155/2012/491027

[10] S. Trombino, R. Cassano, R. Muzzalupo, A. Pingitore, E. Cione, N. Picci, Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of β-carotene and α-tocopherol. Colloids Surf B Biointerfaces 72 (2009) 181–187.

[11] S. Matos de Carvalho, C. Montanheiro Noronha, C. Louise Floriani, R. Calegari Lino, G. Rocha, I. Casagrande Bellettini, P. J. Ogliari, P. L. Manique Barreto, Optimization of α-tocopherol loaded solid lipid nanoparticles by central composite design, Ind Crops Prod. 49 (2013) 278-285.

[12] J.M. Morais Diane, J. Burgess, Vitamin E nanoemulsions characterization and analysis, Int J Pharm. 465 (2014) 455–463.

[13] G. Garrastazu Pereira, C. Britto Detoni, G.A. Balducci, V. Rondelli, P. Colombo, S. Stanisçuaski Guterres, F. Sonvico, Hyaluronate nanoparticles included in polymer films for the prolonged release of vitamin E for the management of skin wounds, Eur J Pharm Sci. 83 (2016) 203–211

[14] M. Gonnet, L. Lethuaut, F. Boury, New trends in encapsulation of liposoluble vitamins, J Control Release 146 (2010) 276–290.

[15] M.C. Bonferoni, G. Sandri, S. Rossi, F. Ferrari, C. Caramella, Oil in water nanoemulsions, WO2016/063119 A1.

[16] M.C. Bonferoni, G. Sandri, S. Rossi D. Usai, I. Liakos, A. Garzoni, M. Fiamma, S. Zanetti, A. Athanassiou, C. Caramella, F. Ferrari, A novel ionic amphiphilic chitosan derivative as a stabilizer of nanoemulsions: Improvement of antimicrobial activity of Cymbopogon citratus essential oil, Colloids Surf B Biointerfaces 152 (2017) 385-392.

[17] Y. Singh, J. Gopal Meher, K. Raval, A.K. Farooq, M. Chaurasia, K.J. Nitin, K.C. Manish, Nanoemulsion: Concepts, development and applications in drug delivery, J Control Release 252 (2017) 28–49.

[18] T. Tadros, Polymeric surfactants in disperse systems, Adv Colloid Interface Sci. 147-148 (2009) 281–299.

[19] I. Bano, M. Arshad, T. Yasin, M. Afzal Ghauri, M. Younus, Chitosan: A potential biopolymer for wound management. Int J Biol Macromol. 102 (2017) 380–383.

[20] C.R. Cardoso, S. Favoreto, L.L. Oliveira, J.O. Vancim, G.B. Barban, D.B. Ferraz, J.S. Silva, Oleic acid modulation of the immune response in wound healing: a new approach for skin repair, Immunobiology 216 (2011) 409-15.

[21] E. Dellera, M.C. Bonferoni, G. Sandri, S. Rossi, F. Ferrari, C. Del Fante, C. Perotti, P. Grisoli, C. Caramella, Development of chitosan oleate ionic micelles loaded with silver sulfadiazine to be associated with platelet lysate for application in wound healing, Eur J Pharm Biopharm. 88 (2014) 643-50.

[22] M.C. Bonferoni, G. Sandri,E. Dellera, S. Rossi, F. Ferrari, M. Mori, C. Caramella, Ionic polymeric micelles based on chitosan and fatty acids and intended for wound healing. Comparison of linoleic and oleic acid, Eur J Pharm Biopharm. 87 (2014) 101-6.

[23] K. Bouchemal, S. Briancon, E. Perrier, H. Fessi, Nanoemulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization, Int. J. Pharm. 280 (2004) 241-251.

[24] L. Häkkinen, L. Koivisto, H. Larjava, An improved method for culture of epidermal keratinocytes from newborn mouse skin, Methods Cell Sci. 23 (2001) 189-96.

[25] F. Riva, A. Casasco, E. Nespoli, A. Cornaglia, M. Casasco, A. Faga, S. Scevola, G. Mazzini, A. Calligaro, Generation of human epidermal constructs on a collagen layer alone, Tissue Eng. 13 (2007) 2769-2779

[26] X. Li, N. Anton, T.M. Ta, M. Zhao, N. Messaddeq, T.F. Vandamme, Microencapsulation of nanoemulsions: novel Trojan particles for bioactive lipid molecule delivery, Int J Nanomed. 6 (2011) 1313-25.

[27] J.N. Mansbridge, M. Knapp, Changes in Keratinocyte Maturation During Wound Healing, J Invest Dermatol, 89 (1987) 253-263.

[28] G.I. Howling, P.W. Dettmar, P.A. Goddard, F. C. Hampson, M. Dornish, E. J. Wood, The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro, Biomaterials 22 (2001) 2959-2966

[29] Y.S. Wimardani, D.F. Suniarti, H.J. Freisleben, S.I. Wanandi, N.C. Siregar, M.A. Ikeda, Cytotoxic effects of chitosan against oral cancer cell lines is molecular weight-dependent and cell-type-specific, Int J Oral Res. 3 (2012) 1–10.

[30] Y. Kusakari, E. Ogawa, Y. Owada Y, N. Kitanaka, H. Watanabe, M. Kimura, H. Tagami, H. Kondo, S. Aiba, R. Okuyama, Decreased keratinocyte motility in skin wound on mice lacking the epidermal fatty acid binding protein gene, Mol Cell Biochem. 284 (2006) 183–188.

[31] L.M. Pereira, E. Hatanaka, E.F. Martins, F. Oliveira, E.A. Liberti, S.H. Farsky, R. Curi, T.C. Pithon-Curi, Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats, Cell Biochem Funct. 26 (2008) 197-204.

[32] S. Gomathysankar, A.S. Halim, N. Soriani Yaacob, Proliferation of Keratinocytes Induced by Adipose-Derived Stem Cells on a Chitosan Scaffold and Its Role in Wound Healing, a Review. Arch Plast Surg. 41 (2014) 452-457

[33] S. Werner, T. Krieg, H. Smola, Keratinocyte–Fibroblast Interactions in Wound Healing. J Invest Dermatol. 127 (2007) 998–1008.

[34] L. Wang, Y. Gao, J. Li, M. Subirade, Y. Song, L. Liang, Effect of resveratrol or ascorbic acid on the stability of a-tocopherol in O/W emulsions stabilized by whey protein isolate: Simultaneous encapsulation of the vitamin and the protective antioxidant, Food Chem. 196 (2016) 466–474.

[35] L. Liang, V. Tremblay-Hébert, M. Subirade, Characterisation of the β-lactoglobulin/α-tocopherol complex and its impact on α-tocopherol stability. Food Chem. 126 (2011) 821–826.

[36] C. Turchiuli, M.T. Jimenez Munguia, M. Hernandez Sanchez, Use of different supports for oil encapsulation in powder by spray drying, Powder Technol. 255 (2014) 103–108.

[37] A. Grenha, C. Remunan-Lopez, E. L.S. Carvalho, B. Seijo, Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins, Eur J Pharm Biopharm. 69 (2008) 83–93.

[38] S. Proniuk, S.E. Dixon, J. Blanchard, Investigation of the utility of an in vitro release test for optimizing semisolid dosage forms, Pharm Dev Technol. 6 (2001) 469–476.