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Chitosan Nanoparticles for Topical Co-administration of the Antioxidants Glutathione and Idebenone: Characterization and *In vitro* Release

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LM and AT designed the study, wrote the protocol, the first draft of the manuscript and revised it for submission. Authors LC, GGP and SB carried out the cellular studies and with authors PF, DM and NC managed the analyses and literature studies. Authors LM, GP and AL supervised the entire research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to explore the potential of novel nanoparticles (NPs) intended for topical administration of the hydrophilic antioxidant Glutathione and the lipophilic Idebenone. Glutathione was introduced into the NPs using two approaches: i) covalently bonded to Chitosan; ii) physically complexed with Idebenone and Sulfobutylether-β-cyclodextrin.

Methodology: NPs were formulated using the ionic gelation technique, by dissolving the polysaccharide-forming matrix (Chitosan, Glycol chitosan, Glutathionyl Chitosan) in water or in slightly acidic solution. Idebenone was physically entrapped whereas glutathione was either physically entrapped or covalently bonded to chitosan.

Physicochemical characterization of the resulting NPs included size, zeta potential measurements, antioxidant association efficiency, differential scanning calorimetry (DSC) and stability studies. Antioxidants *in vitro* release from the most stable NPs was assessed with Franz diffusion cells, and the *in vitro* antioxidant activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test. NP cytotoxicity was assessed on immortalized human keratinocytes (HaCaT) cell line.

Results: The NPs showed smaller particle size in acidic solution than in aqueous medium, whereas zeta potential values were always positive, irrespective of the medium. Stability studies led to the choice of the aqueous formulation where Glutathione was covalently bonded to Chitosan for this study. DSC highlighted amorphization of Idebenone in these NPs. *In vitro* release studies showed that only Idebenone was released from the NPs. The antioxidant activity test revealed a strong effect (close to 100%) of Idebenone loaded into NPs while its aqueous solution showed no activity. No cytotoxicity in human keratinocytes was observed for the investigated NPs.

Conclusion: The results of this study suggest that Idebenone can be loaded into a hydrophilic delivery system without organic solvents, often used for its solubilization, possessing high antioxidant activity. Therefore, these nanocarriers represent a promising strategy for the design of formulations for topical treatments with antioxidants.

Keywords: Polymeric nanoparticles; glutathione; idebenone; chitosan; cyclodextrins; topical delivery.

1. INTRODUCTION

The skin, outermost layer of our body, is constantly exposed to a variety of environmental insults, such as UV radiation, pollutants, xenobiotics, and cigarette smoke. As a response to these attacks, reactive oxygen species (ROS), including singlet oxygen, superoxide anions, the hydroxyl radical, hydrogen peroxide [1,2], and other free radicals are generated in the skin [3]. To counteract their deleterious effects, antioxidant agents, including glutathione (GSH, Fig. 1A), ascorbate, superoxide dismutase and glutathione peroxidase, are normally present in the cutaneous tissue [4]. An essential electron carrier in cellular respiration, coenzyme Q10, shows antioxidant properties, as well, by clearing ROS and protecting cells from oxidative stress [5,6]. When ROS production overwhelms the physiological cellular defenses, so-called oxidative stress occurs [7]. Cells respond to such oxidative stress with modified gene expression and DNA damage, resulting in abnormal cell morphology, cell apoptosis or necrosis, and oxidative alteration of lipids and proteins [8,9,10].

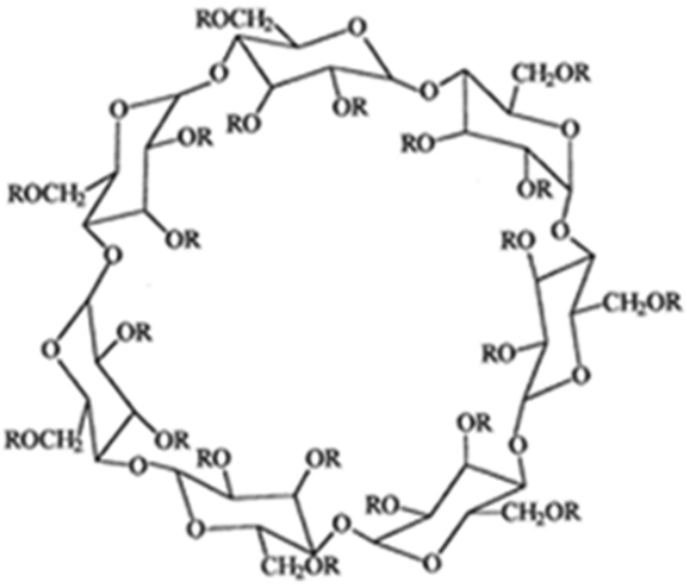
Several authors have suggested that topical use of antioxidants could be beneficial to reduce ROS-induced skin damage by improving skin antioxidant activity [3]. However, various problems have been reported when drugs are topically administered using conventional formulations such as poor skin penetration due to the barrier functions of the stratum corneum, and systemic absorption leading to unwanted systemic side effects [11,12]. Over past few decades, many colloidal carriers have been proposed to enhance drug absorption and delivery to the target site, such as liposomes, niosomes [12,13], solid lipid nanoparticles [14,15], nanostructured lipid carriers [16] or chitosan nanoparticles (CS NPs) [17]. The latter show several advantages since the polysaccharide CS is regarded as biologically safe, non-toxic, biocompatible and biodegradable [18]. Water solubility, mucoadhesion and absorption enhancing effects are additional CS properties making this polysaccharide suitable to form colloidal carriers. Furthermore, NPs based on CS can be obtained using simple and mild preparation methods [19].

Recently, the feasibility of using sulfobutylether-β-cyclodextrin (SBE-β-CD, Fig. 1A) for the formation of CS NPs has been reported [20]. Cyclodextrins (CDs) are commonly used in the pharmaceutical field as complexing agents to increase water solubility, bioavailability, and stability of poorly soluble drugs [21]. SBE-β-CD is a CD derivative, which shows improved solubility and complexation ability, without the significant toxicity associated with the parent or certain other alkylated CDs [22,23]. The inclusion of CDs in the NPs structures led to improved capacity of these carriers to load both hydrophilic and lipophilic drugs [24,25].

Therefore, we investigate here the feasibility of using CS/SBE-β-CD NPs as potential carrier systems for the topical delivery of two antioxidant agents, GSH and Idebenone (IDE) (Figs. 1B and 1C), with high and low water solubility, respectively.

As the skin consists of both lipid and aqueous compartments, the combination of the hydrophilic GSH with the hydrophobic IDE could be useful for topical antioxidant therapy. Recent studies showed that GSH treatment can reduce oxidative stress after topical application and is useful in wound repair [26]. IDE is a synthetic analogue of coenzyme Q10, whose potent antioxidant activity has been mainly attributed to inhibition of lipid peroxidation and protection of cell and mitochondrial membranes from oxidative damage [27]. Therefore, IDE antioxidant activity could be useful to prevent skin ageing and to protect the skin from oxidative damages due to its exposure to environmental oxidizing agents [28]. Previous studies confirmed the possibility of complexation of IDE with SBE-β-CD [29] and GSH with SBE-β-CD [20]. In this work, we study the introduction of both antioxidants into the same NPs via the use of SBE-β-CD, forming a complex called “SBE-β-CD/IDE and GSH”. Given that the chitosan derivative glycol chitosan (GlycolCS) is water soluble at physiological pH values [30], the formation of NPs for topical delivery starting from GlycolCS was also investigated. Furthermore, given the adhesive nature typical of thiomers [31], we also screened NPs obtained from a chitosan derivative formed through a covalent bond with GSH (CS-GSH) and, successively, cross-linked by the complex SBE-β-CD/IDE, to improve technological and antioxidant properties of the NPs.

The physicochemical characteristics of the resulting NPs vehicles were studied together with their ability to load and release IDE and GSH, their *in vitro* antioxidant activity and their potentially cytotoxic effects.



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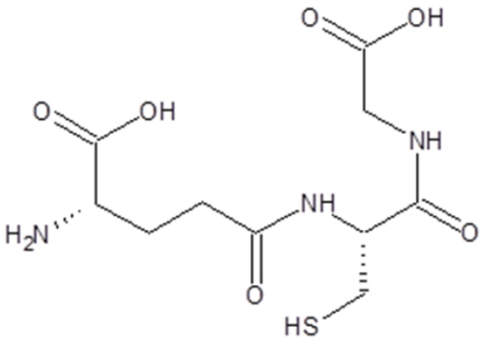

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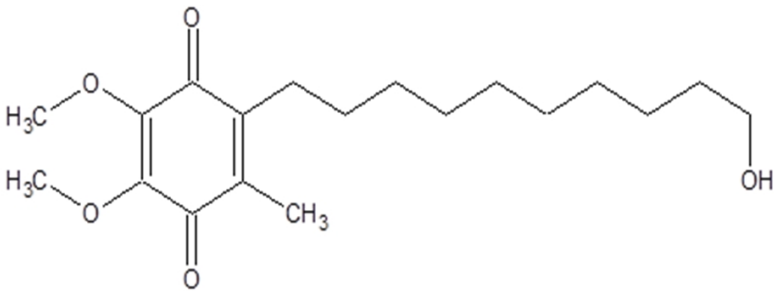

(C)

Fig. 1. Chemical structures of (A) Sulfobutylether-β-cyclodextrin sodium salt; (B) Glutathione; (C) Idebenone. For Sulfobutylether-β-cyclodextrin sodium salt, R = - (CH2)4-SO3$^-$

2. METHODOLOGY

2.1 Materials

The following commercial products were used as received: for NP formulation, Chitosan hydrochloride (CS) (Protasan, UP CL 113, *Mw* 110 kDa, deacetylation degree 86%) was purchased from Pronova Biopolymer (Norway); Idebenone (IDE) was a gift from Wyeth Lederle (Italy); Glycol chitosan (GlycolCS, Mw 400 kDa); Glutathione (GSH), glycerol (> 99.5% purity). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and all cell culture reagents (D-MEM; FCS; Glutamine, Penicillin/streptomycin) were purchased from Sigma-Aldrich, Italy. Sulfobutylether-β-cyclodextrin sodium salt (SBE-β-CD, Mw=2163 Da, average substitution degree=6.40, CyDex, Inc. USA) was kept in a desiccator until use. Double distilled water (Carlo Erba, Italy) was used throughout the study. All other chemicals were of reagent grade.Regenerated cellulose membranes (Spectra/Por CE; Mol. Wet. Cut off 3,000) were supplied by Spectrum (Los Angeles, CA, USA).

2.2 Nanoparticle Formulation

CS, GlycolCS and CS-GSH NPs, in the presence and in the absence of the antioxidants, were prepared according to a modified ionic gelation method [32,33]. Synthesis and characterization of conjugate CS-GSH, a chitosan derivative covalently bonded to GSH, were performed as described elsewhere [31].

As previously reported [20], the use of SBE-β-CD, through its anionic groups, allowed the cross-linking of CS with resulting NP formation. Two inclusion complexes were formed: SBE-β-CD/IDE and GSH in order to prepare CS and GlycolCS based NPs; SBE-β-CD/IDE in order to obtain CS-GSH based NPs. NPs preparation was carried out as follows:

a) IDE/GSH CS (or GlycolCS) based NPs: The SBE-β-CD/IDE and GSH complex was prepared at room temperature and under magnetic stirring for 24h. The reagents were poured into double distilled water in order to ensure an equimolar ratio of the antioxidants and SBE-β-CD. In particular, SBE-β-CD concentration was set to 0.45% w/v in order to promote nanoparticle formation, whereas IDE and GSH were set to the concentrations of 0.07% w/v and 0.06% w/v, respectively. When particles were formulated in acidic medium, 1.5 ml of a CS (or GlycolCS) solution (0.20% w/v) were dissolved in acetic acid (0.1% v/v) and maintained under magnetic stirring for several minutes prior to the addition of an aliquot of 0.5 ml of SBE-β-CD/IDE and GSH complex. The final CS (or GlycolCS)/SBE-β-CD mass ratio was 3/2.2 (w/w).

For particles formed in aqueous medium, 1.5 ml of a CS (or GlycolCS) solution (0.20% w/v) were first dissolved in double distilled water and maintained under magnetic stirring prior to the addition of 0.25 ml of SBE-β-CD/IDE and GSH complex. The final CS (or GlycolCS)/SBE-β-CD mass ratio was 3/1.1 (w/w).

b) IDE-loaded CS-GSH based NPs: The SBE-β-CD/IDE complex was formed as described in *a)* but without GSH.

Glutathionyl-Chitosan nanoparticles in acidic medium were obtained by dissolving 1.5 ml of CS-GSH (0.20% w/v) in acetic acid (0.1% v/v) and then adding 0.5 ml of the SBE-β-CD/IDE complex under magnetic stirring. The final CS-GSH/SBE-β-CD mass ratio was 3/2.2 (w/w).

Glutathionyl-Chitosan nanoparticles in aqueous medium were prepared by mixing 1.5 ml of CS-GSH aqueous solution (0.20% w/v) with 0.25 ml of the SBE-β-CD/IDE complex under magnetic stirring. The final CS-GSH/SBE-β-CD mass ratio was 3/1.1 (w/w).

The same NPs were prepared without IDE both in aqueous and acidic medium.

Finally, the resulting NPs were isolated by centrifugation on a glycerol bed (16,000 *g,* 45 min, Eppendorf 5415D, Eppendorf, Germany) and re-suspended in double-distilled water (water-formulated NPs) or in acetic acid (0.1% v/v) for NPs formulated in acidic medium, with manual shaking.

NP codes and the corresponding compositions are reported in Table 1.

Table 1. Code and composition of nanoparticles (NPs)

Code	Polysaccharide	Cross-linking agent
CS-GSH	Glutathionyl chitosan (CS- GSH)	Sulfobutylether β-cyclodextrin
CS-GSH IDE	Glutathionyl chitosan (CS- GSH)	Sulfobutylether β-cyclodextrin/ Idebenone (IDE) complex
GlycolCS IDE/GSH	Glycolchitosan (GlycolCS)	Sulfobutylether β-cyclodextrin/ Idebenone (IDE)/ Glutathione (GSH) complex
CS IDE/GSH	Chitosan (CS)	Sulfobutylether β-cyclodextrin/ Idebenone (IDE)/ Glutathione (GSH) complex

2.3 Physicochemical Characterization of Nanoparticles

Particle size and polydispersity index (PI) of all tested NPs were measured in double-distilled water by photon correlation spectroscopy (PCS) using a Zetasizer NanoZS (ZEN 3600, Malvern, UK). The ζ-potential was determined using laser Doppler anemometry (Zetasizer NanoZS, ZEN 3600, Malvern, UK). Particles were centrifuged on a glycerol bed and the resulting pellet, both for NP prepared in aqueous and acidic media, was diluted with aqueous KCl 1mM (pH 7), following a previously reported procedure [20].

2.4 Trasmission Electron Microscopy (TEM)

For transmission electron microscopy (TEM) characterization, an aqueous formulation of CS-GSH/ (SBE-β-CD/IDE) NPs was stained with 2% (w/v) of phosphotungstic acid. The resulting samples were deposited by solution casting on 300 mesh formvar-coated copper grids and observed at 120 keV, with a FEI Tecnai T12 TEM apparatus.

2.5 Antioxidant Association Efficiency (A.E.)

The Association Efficiency (A.E.) of GSH to the NPs was calculated indirectly. NPs were isolated from unloaded GSH by centrifugation (16,000 g, 45 min, Eppendorf 5415D, Eppendorf, Germany), and free GSH in the supernatant was quantified by HPLC as described below. Experiments were performed in triplicate and the association efficiency was calculated as follows (Eq. (1)):

$$\% \text{ A.E.} = 100 \times (\text{Total GSH} - \text{Free GSH}) / \text{Total GSH} \quad (1)$$

2.6 Analytical Determinations of IDE and GSH

High-performance liquid chromatography (HPLC) analysis of IDE was performed with a Waters (Waters Corp., MA) Model 600 pump equipped with a Waters 2996 photodiode array detector, a 20 µl loop injection autosampler (Waters 717 plus), and processed by Empower™ Software. For analysis, a C18 Varian OmniSpher column (15 cm× 4.6 mm; 5 µm particles) equipped with a precolumn C18 insert was eluted with 80:20 (v:v) methanol: water in isocratic mode, with a flow rate of 1.0 ml/min. Standard calibration curves for IDE determination were analyzed at 280 nm wavelength and calibration curve linearity (r^2>0.999) was maintained over the range of concentrations tested (10 µg/ml–0.1 µg/ml). The limit of quantification (LOQ) of the HPLC method was 0.08 µg/ml. The retention time of IDE was 6 min.

For the quantification of GSH in the conjugate CS-GSH, Ellmann assay [34] was used to determine the amount of free thiol groups. According to this method, CS-GSH contained 0.852 mmol –SH/g.

For quantification of free GSH deriving from the NPs, HPLC analysis was carried out using a reversed phase Synergy Hydro-RP (25 cm x 4.6 mm; 4 µm particles; Phenomenex, Torrance, CA) column in conjunction with a precolumn C18 insert eluted with 1:99 (v:v) acetonitrile:0.025 M phosphate buffer (pH 2.7) in isocratic mode, with a flow rate of 0.7 ml/min. Standard calibration curves were analyzed at 220 nm wavelength using 0.025 M phosphate buffer (pH 2.7) as solvent. Calibration curve linearity (r^2 > 0.999) was maintained over the range of concentrations tested (1000 µg/ml–10 µg/ml). The retention times of GSH and its disulfide degradation product (Glutathione disulfide, GSSG) were 7.2 and 18 min, respectively. Under these conditions, the LOQ limit was 2 µg/ml for both GSH and GSSG.

2.7 Size Stability Studies of NPs

NPs prepared both in aqueous and acidic media were checked for their ability to maintain their size over time at 20°C and 37°C. Freshly prepared NPs were incubated at these temperatures sheltered from the light and samples collected after 0, 1, 2, 3 weeks. Size of each aliquot was referred to NPs diameter measured in water using PCS. Each assay was performed in triplicate and stopped once agglomeration was detected.

2.8 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) analyses were carried out with a DSC Q200 (TA Instruments, DE, USA) with TA Universal Analysis software and calibrated with indium. To determine NPs water holding capacity, all NP samples were lyophilized (Lio Pascal 5P, Italy) whereas IDE, GSH and SBE-β-CD were used as received. Equimolecular mixtures of IDE and SBE-β-CD were prepared by kneading and physical mixing. 1-2 mg of each sample was placed into a standard aluminium pan, crimped and heated from 5°C to 210°C at a constant rate of 10°C/min under a nitrogen flow of 50 ml/min. An empty aluminium pan was used as reference. All samples were run in duplicate.

2.9 DPPH Test for Antioxidant Activity

The *in vitro* antioxidant activity of NPs formulated in aqueous medium was evaluated using a DPPH test with slight modifications of the protocol reported by Fir et al. [35]. Briefly, the

DPPHl (DPPH·) was dissolved in ethanol (0.001% w/v) to obtain a stock solution and, then, diluted to 8 x 10-4% (w/v). 0.5 ml of each sample was reacted with 2.5 ml of the diluted DPPH· solution for 60 minutes at room temperature, sheltered from the light. The absorbance changes were measured at a wavelength of 514 nm.

Blanks were run by filling the cuvettes with 3 ml of the 8 x 10^{-4}% (w/v)(DPPH·) solution. A saturated aqueous solutions of IDE (3 µg/ml) [36] and an aqueous solution of GSH (1 mg/ml) were also analyzed in order to distinguish their behavior from those in the nanostructured vehicles. The saturated IDE solution was prepared by adding excess IDE to water, stirring the resulting suspension for 24 h at room temperature and then filtering the suspension to obtain a clear solution. Antioxidant activity (AA) was calculated from Equation (2) and expressed in percentages:

$$AA\ (\%) = (1 - A_s/A_b) \times 100 \quad (2)$$

A_s is the sample absorbance, and A_b the absorbance of the radical.

2.10 *In vitro* Release Studies

IDE and GSH release rates from the aqueous CS-GSH IDE NPs were determined through regenerated cellulose membranes using Franz-type diffusion cells (LGA, CA). As reported in the literature [*37*], this technique provides a suitable method for evaluating drug release from topical formulations.

Prior to carrying out release experiments, the cellulose membranes were moistened by immersion in water for 1 h at room temperature. Diffusion surface area and receiving chamber volume of Franz-type diffusion cells were 0.75 cm^2 and 4.5 ml, respectively. The receptor was filled with water/ethanol (50/50 v/v) for ensuring pseudo-sink conditions by increasing IDE solubility in the receiving phase [38,39]. In previous *in vitro* release studies of IDE from solid lipid nanoparticles, with the same receiving phase no change in nanoparticle integrity was observed [40]. The receiving phase was constantly stirred (700 rpm) and thermostated at 35°C to maintain the membrane surface at 32°C. As reported in the literature, the receiving chamber and the membrane should be maintained at a constant temperature close to normal skin temperature of 32±1°C, since drug release and skin permeation are affected by temperature [41].

500 µl of each formulation (50 µg of IDE) were applied to the membrane surface in the donor chamber and the experiments run for 24 h. Due to IDE photo-instability, all the release experiments were performed without light exposure. At intervals (0, 2, 4, 6, 24 h), 200 µL of the receptor phase were withdrawn and replaced with an equal volume of receiving solution, pre-equilibrated at 35°C. The receptor phase samples were analyzed by HPLC as described above to determine IDE and GSH content. At the end of the experiments, no change in NPs size and P.I. was observed by PCS analysis of samples withdrawn from the NPs formulation applied to the membrane surface. Each experiment was performed in triplicate.

2.11 Cellular Viability

The human keratinocyte cell line HaCaT was grown in D-MEM culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2.0 mM glutamine and 1%

antibiotics. Cultures were maintained in exponential growth at 37°C in a humidified atmosphere of 95% air-5% CO_2.

Toxicity in HaCaT cell line was tested at various points in time using Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega, WI, USA), a colorimetric method for the determination of the number of viable cells in proliferation as previously described [42]. Briefly, 4×10^4 cells/ml were seeded in a 96-well plate (100 µl/well) in the presence of NPs suspensions at concentrations ranging from 0 to 2.0 mg/ml of CS-GSH NPs and CS-GSH IDE NPs. The pure polymer CS-GSH was also screened at the same concentrations. Experiments were performed using the same vehicle dilutions but, due to the different concentrations of the starting material (pure polymer CS-GSH: 17.4 µg/µl; CS-GSH NPs: 13.8 µg/µl; CS-GSH IDE NPs 16.2 µg/µl), different final concentrations were obtained. A control containing only cell medium nanoparticles in the same range was included to evaluate potential colorimetric interference. Cell viability was tested after 24 h and 48 h by adding 20 µl of Cell Titer 96® Aqueous One Solution Reagent. The absorbance was measured at 490 nm using a Benchmark microplate reader (Bio-Rad, CA, USA). Each experimental point was obtained in sixtuplicate and each experiment was repeated three times.

2.12 Data Analysis

Plotting the cumulative amount of active compound released against time, the lag time was determined from the x-intercept value of the regression line. Active compound release rates were determined by dividing the slope of the steady-state portion of these graphs by the area through which diffusion took place.

Data from physico-chemical characterization and *in vitro* release studies were compared by a one-way ANOVA and differences were considered significant at 95 % level of confidence ($p<0.05$) using the GraphPad Prism v. 5.00 computer program (GraphPad Software, Inc. CA, USA). Bonferroni tests were used for post-hoc contrast.

3. RESULTS AND DISCUSSION

3.1 Nanoparticles Characterization

Tables 2A and 2B show the main physicochemical properties of the investigated NPs containing the lipophilic antioxidant IDE and/or the hydrophilic GSH. To design particles loading both the agents, two critical parameters were*:* i) the crosslinking agent enabling ionic gelation and ii*)* the medium for polycation dissolution.

Concerning the crosslinking agent, preliminary studies were carried out to load IDE in the CS network via the use of sodium tripolyphosphate (TPP), but, unfortunately, less than 10% of Association Efficiency (A.E.) was found (data not shown). Recent studies on the complexation of IDE with the anionic SBE-β-CD [29] revealed high IDE affinity for SBE-β-CD and the formation of 1:1 molar ratio stoichiometry. In a previous study, we observed that the use of TPP, as cross-linking agent, provided a lower association of GSH compared to SBE-β-CD [20,43]. Therefore, in the present work we tried to obtain novel biocompatible NPs via the use of sugar based polycations and oligoanion to load both IDE and GSH.

To achieve stable NPs, two media were used for particle formulation, namely a slightly acidic solution or distilled water. In acidic medium, the high charge density of the polycation led to the formation of nanoparticles with smaller size (Table 2A) [44] compared to those formed in distilled water (Table 2B). As expected, the solvent used for polysaccharide dissolution also influenced the final pH values of the nanosuspensions, NPs arising from an acidic medium showed a pH range 2.8-4.4 whereas NPs formed in distilled water a pH range 5.2-7.1. In particular, in the case of particles formed under aqueous conditions, in agreement with previous data [31], GlycolCS is water soluble also under physiological conditions and, therefore, the corresponding particles gave pH 7.1. In the case of CS-GSH based particles, the conjugation affected the pH of the medium, thus leading to nanocarriers at pH 5.2. Overall, the effect of pH could also be invoked to explain the differences in terms of zeta potential values. For all investigated particles, zeta potential values were always found to be positive, given the positive charges arising from chitosan (and glycol chitosan) chainsand measured values were in the range +20 - +30 mV, as expected on the basis of the molecular weight of the polycations.

In acidic medium, the high charge density of the polycation was responsible for the formation of nanoparticles with smaller size (Table 2A) [44] compared to those arising from the use of distilled water as dissolution medium (Table 2B). As expected, the solvent used for polysaccharide dissolution also influenced the final pH values of the nanosuspensions and, therefore, NPsformed in acidic medium gave pH 2.8-4.4 whereas NPs formed in distilled water gave pH 5.2-7.1.

Table 2. Physicochemical properties of NPs in acidic medium (Table 2A) and in aqueous medium (Table 2B). Control nanoparticles were CS-GSH. PI: polydispersity index. % A.E.: GSH Association Efficiency percentage. Mean ± S.D. are reported, n = 6. *Significantly different from the control (*P* =.05)

Table 2A

NP code	pH	Size (nm)	PI	Zeta potential (mV)	%A.E
CS-GSH	2.8	511±19	0.40-0.42	+30.7±1.9	99.3±2.2
CS-GSH IDE	3.6	474±33	0.33-0.42	+28.4±0.9	98.9±3.0
CS IDE/GSH	3.4	418*±53	0.55-0.66	+23.7*±2.9	12.0±1.3
GlycolCS IDE/GSH	4.4	687*±79	0.40-0.44	+26.0*±1.3	24.0±3.1

Table 2B

NP code	pH	Size (nm)	PI	Zeta potential (mV)	% A.E.
S-GSH	5.7	875±76	0.28-0.40	+20.4 ±1.5	98.8±3.3
CS-GSH IDE	5.2	376*±78	0.33-0.48	+23.0*±0.9	99.4±2.7
CS IDE/GSH	6.9	≥ 1000	0.34-0.47	+26.2*±1.4	70.5±3.0
GlycolCS IDE/GSH	7.1	740*±76	0.65-0.76	+20.2±0.6	40.7±6.2

The pH of the medium for polysaccharide dissolution influenced the protonation of the $-NH_2$ groups of the polycation. For this reason, the zeta potential values were higher for acidic NPs (up to +30.4 mV) than for aqueous NPs, exhibiting the lowest value of +20.2 mV for of GlycolCS IDE NPs. Interestingly, only for acidic formulations, zeta potential values of IDE-loaded NPs were lower than control, suggesting that the IDE could be located on the

external shell of the particles, partly masking the inherent positive charges of the polycation. On the other hand, for IDE-loaded NPs formulated in aqueous medium, an increase in zeta potential values was found compared to control NPs. This finding is in good agreement with Tantra et al.'s work [45], suggesting that the external electrical charges of these nanocarriers were influenced not only by the net chemical positions but also by the mutual molecular organization of all the substances at the surface.

We did not determine IDE association efficiency experimentally because IDE is a poorly water-soluble drug and if it were not entrapped into the NPs it would precipitate, as the NPs were prepared in water. As these NPs formulations were clear, with no sign of precipitation, all the IDE added during NP preparation had to be entrapped within the NPs. Therefore, IDE association efficiency was taken as 100%. The association efficiency of other lipophilic drugs loaded into nanoparticle carriers has been determined with this method [46].

Concerning GSH quantification, interestingly, no trace of GSH (or its oxidized form, GSSG) was detected in the supernatants from CS-GSH based NPs, thus showing a quantitative presence due to the GSH covalent bonding. In the case of physical complexation via SBE-β-CD (i.e., for CS and GlycolCS based NPs), different % A.E. values were found (see Tables 2A and 2B). These data suggest that changing NPs preparation conditions led to changes in peptide association to the NPs. It seems that encapsulation of GSH is influenced by the net electrical charges shown by the polycation. For instance, when pH increases under the aqueous formulation conditions, the electrostatic repulsion between $–NH3^{+}$ charges of CS (or GlycolCS) and GSH diminishes, thus allowing higher % A.E. values of the peptide.

As antioxidants were loaded into the nanoparticles as complexes with SBE-β-CD, the loading capacity (LC) of these nanoparticles depended on the mass ratio CS/SBE-β-CD and the stoichiometry of the complex SBE-β-CD/drug. Therefore, to increase the amount of drug loaded into these nanoparticles, changes in the mass ratio CS/SBE-β-CD and/or the stoichiometry of the SBE-β-CD/antioxidants complex were needed.These changes could not be accomplished without altering NPs physico-chemical properties and morphology, as SBE-β-CD was used as cross-linking agent in NPs preparation. Therefore, antioxidant loading into these nanoparticles could not be modified and, hence, LC corresponded to antioxidant association efficiency.

NPs prepared in aqueous medium seemed more promising than those obtained in acidic medium in view of possible dermal application, as pH values close to 5.0 are required to avoid skin damage. Therefore, stability studies were performed only on NPs prepared in aqueous medium.

Under the temperature condition tested (20°C and 37°C) CS IDE NPs and GlycolCS IDE/GSH NPs produced aggregates in less than a week (data not shown) whereas, the CS-GSH IDE NPs were stable for up to three weeks (Fig. 2).

The greater stability of CS-GSH based NPs compared to those containing CS and GlycolCS could be likely due to the increased hydrophilicity of this modified polymer in comparison to the other two polysaccharides [47]. However, during NPs storage both at 20°C and 37°C, we observed a slight decrease in particle size, which could be due to the swelling dynamics, occurring in the aqueous medium. During the centrifugation process, as chitosan and cyclodextrin-based nanoparticles are subject to swelling mechanisms, a hydration shell could be formed, which could then have slowly receded during storage, leading to a decrease in particle size. For the stable aqueous formulation, namely CS-GSH IDE NPs,

TEM imaginggave microphotographs (Fig. 3) confirming the approximate particle size rangesestimated using PCS. Furthermore, the TEM images showed no significant particle aggregation, along with light-grey filamentous areas due to solution residues related to the phosphotungstic acid-processing of the samples.

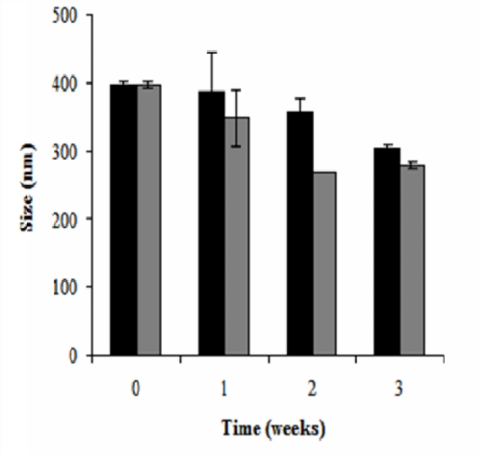

Fig. 2. Particle size of CS-GSH IDE NPs upon incubation at 20 °C (black bars) and 37°C (grey bars). Values represent means ± SD (n = 3)

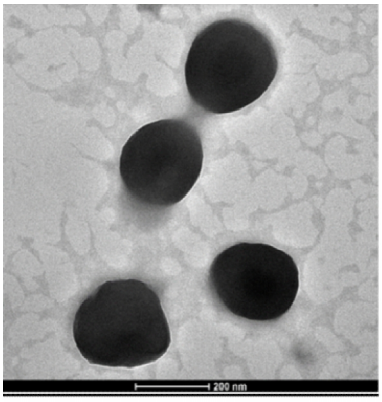

Fig. 3. Transmission Electron Microscopy image of nanoparticles composed of aqueous CS-GSH IDE (pH 5.2)

3.2 DSC Analysis

As shown in Fig. 4, pure IDE and GSH each show an endothermic peak at 55.8°C and 188.60°C, respectively. The IDE peak is sharp and rather symmetric whereas the GSH peak is asymmetric and shows, soon after the melting temperature, the beginning of other thermal processes, likely associated with GSH thermal degradation.

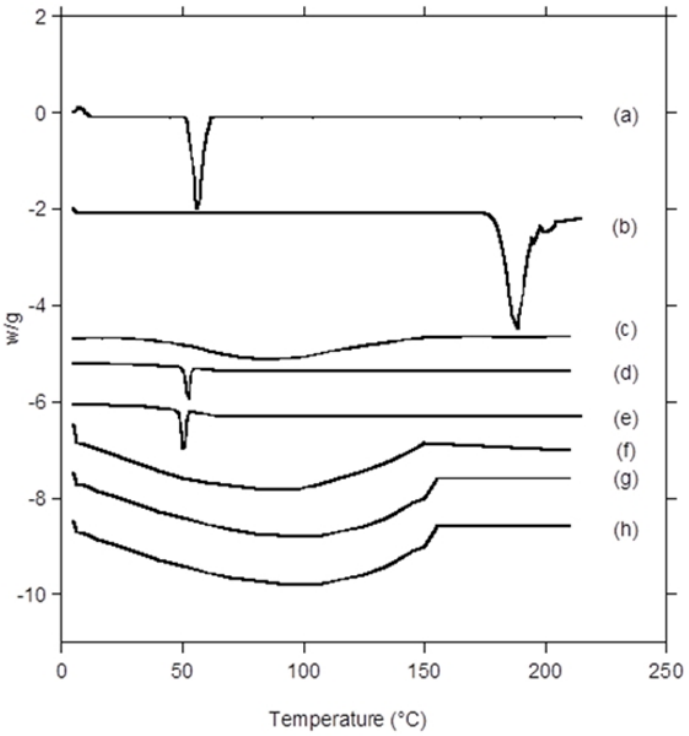

Fig. 4. DSC thermograms of: (a) IDE, (b) GSH, (c) SBE-β-CD, (d) IDE/SBE-β-CD physical mixture, (e) IDE/SBE-β-CD kneaded mixture, (f) CS IDE/GSH NPs, (g) GlycolCS IDE/GSH NPs, (h) CS-GSH IDE NPs

The SBE-β-CD thermogram had only one broad peak centered at 85.5°C, associated with the release of bound water from the sample. This broad peak was not present in SBE-β-CD/ IDE physical nor in kneaded mixture thermograms. This phenomenon along with the decrease in the IDE melting peak intensity and its shift to a temperature lower than that of the pure component seems to confirm a high affinity between the components of the binary systems. The interaction between IDE and SBE-β-CD was more evident in the kneaded than in the physical mixture, suggesting IDE amorphization and/or inclusion complex formation.

The complete disappearance of the IDE and GSH endothermal peak was observed in all NPs loaded with these drugs, indicating that, in these carrier systems, IDE and GSH were in an amorphous or disordered crystalline phase or a solid solution state [48].

Close examination of these latter thermograms revealed that there were some differences in the endothermic peak position around 100°C, associated with the loss of water, indicating that CS IDE/GSH NPs, GlycolCS IDE/GSH NPs, and CS-GSH IDE NPs differed in their water holding capacity and strength of water/NPs interaction.

It is well known that polysaccharides usually have a strong affinity for water, and in the solid state these macromolecules may have disordered structures which can be easily hydrated [49]. The insertion of hydrophilic groups, such as covalently bonded GSH on the chitosan -NH_2 moiety or ethylene glycol on the chitosan -CH_2OH moiety, increased the hydrophilic character of the NPs, which also depended on the supra-molecular structure of the systems.

The position of the endothermic peak of GlycolCS IDE/GSH NPs and CS-GSH IDE NPs at higher temperature than that observed for CS IDE/GSH NPs, indicated that these two systems had the best water holding capacity and the strongest water/NPs interaction. The highest hydrophilicity of CS-GSH IDE NPs could explain their greater stability in water observed in our stability studies.

Furthermore, none of the DSC curves of IDE loaded NPs formulations showed any degradation processes, so these systems, in the solid state, are thermally stable up to 210°C.

3.3 DPPH Test for Antioxidant Activity

The antioxidant activity of the nanoparticles prepared in this study was estimated using a slightly modified spectrophotometric method [35], based on the DPPH free radical. This test was designed to assess the ability of compounds to act as free radical scavengers or hydrogen donors, allowing an evaluation of the antioxidant activity of various antioxidant compounds [50,51].

As reported in Table 3, IDE in aqueous solution did not show anyradical-scavenging activity with the DPPH radical whereas aqueous solutions of GSH showed 100% activity. For IDE, the lack of antioxidant activity could be attributed to its poor water solubility, preventing interaction of sufficient amounts of antioxidant with the radical, whereas inclusion of IDE into the NPs showed an antioxidant activity (close to 100%), likely due to increased water solubility of IDE upon loading into these nanocarriers. When the NPs contained IDE and GSH, the percentages of antioxidant activity slightly decreased (91.6%) compared to GSH antioxidant activity.

Table 3. Antioxidant activity (% AA) of CS IDE NPs, CS-GSH IDE NPs, free IDE saturated aqueous solution (3 µg/ml) (IDE) and free GSH aqueous solution (1 mg/ml) (GSH). Data represent means ± S.D (n = 3)

Sample	% AA ±S.D.
CS IDE NPs	99.8±2.2
CS-GSH IDE NPs	91.6 ±3.5
IDE	0
GSH	98.9±3.1

3.4 *In vitro* Release Studies

An essential requisite of a pharmaceutical colloidal system is its ability to deliver the active ingredients, allowing their diffusion out of the vehicle following its application.

In this work, we assessed *in vitro* IDE and GSH release from CS-GSH IDE NPs, intended for topical administration, using the infinite dose technique, i.e. applying a large amount of formulation on the membrane surface. As previously reported [52], this technique avoids compound depletion from the donor compartment during the experiment, thus ensuring a constant driving force for the release process and steady-state conditions.

In vitro release profiles of IDE from CS-GSH IDE NPs is shown in Fig. 5. IDE was released from the vehicle following pseudo-first order kinetics due to the linear relationship of drug

concentration vs time and the excessive amount of drug in the donor chamber with respect to the receiving chamber (r^2=0.98, Flux = 0.33±0.04 μg/cm^2/h, Lag time = 1.33 h). On the contrary, no release of GSH was observed. This finding could be expected due to the covalent bond of GSH with CS. Under the conditions used, it was unlikely that this strong bonding between GSH and CS could break, providing free GSH and its diffusion out of the vehicle. However, *in vivo*, this linkage could be susceptible of hydrolysis by means of the enzymes normally present in the skin. Therefore, the lack of *in vitro* GSH release from these NPs cannot be regarded as a limit to their potential *in vivo* topical antioxidant activity.

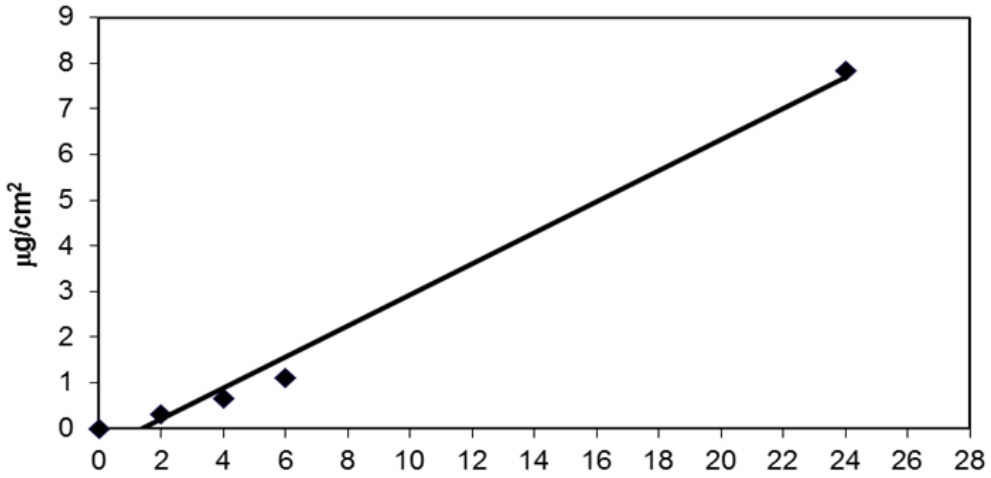

Fig. 5. IDE release from aqueous CS-GSH IDE NPs via Franz diffusion cells

3.5 Cellular Toxicity

Promising nanoparticle systems, intended for topical delivery of antioxidant compounds (GSH and IDE), must be capable of delivering sufficient levels of the active agent without compromising the target cells.

Therefore, dose and time-response experiments were conducted in a human keratinocyte HaCaT cell line (Fig. 6) using an MTS-based assay as described in the 'Methodology' section. A HaCaT cell line was chosen because this cell line is a largely used model for human skin keratinocytes.

The controls for colorimetric interference, containing only cell medium and the NP suspensions, showed no absorbance at 490 nm. The absorbance of this cell line was not significantly affected by the compound CS-GSH (Fig. 6, panel A), also present in both NPs formulations, indicating that, the compound CS-GSH did not compromise the proliferative functional activities of HaCaT cells.

The cellular toxicity of CS-GSH NPs (panel B) and CS-GSH IDE NPs (panel C) was investigated. Fig. 6 (panel B and C), shows that NPs had little (< 20%) at lower doses or no effect on cell proliferation, indicating the lack of cytotoxicity of these NPs on the HaCaT cell line, as indicated by cell viability (%).

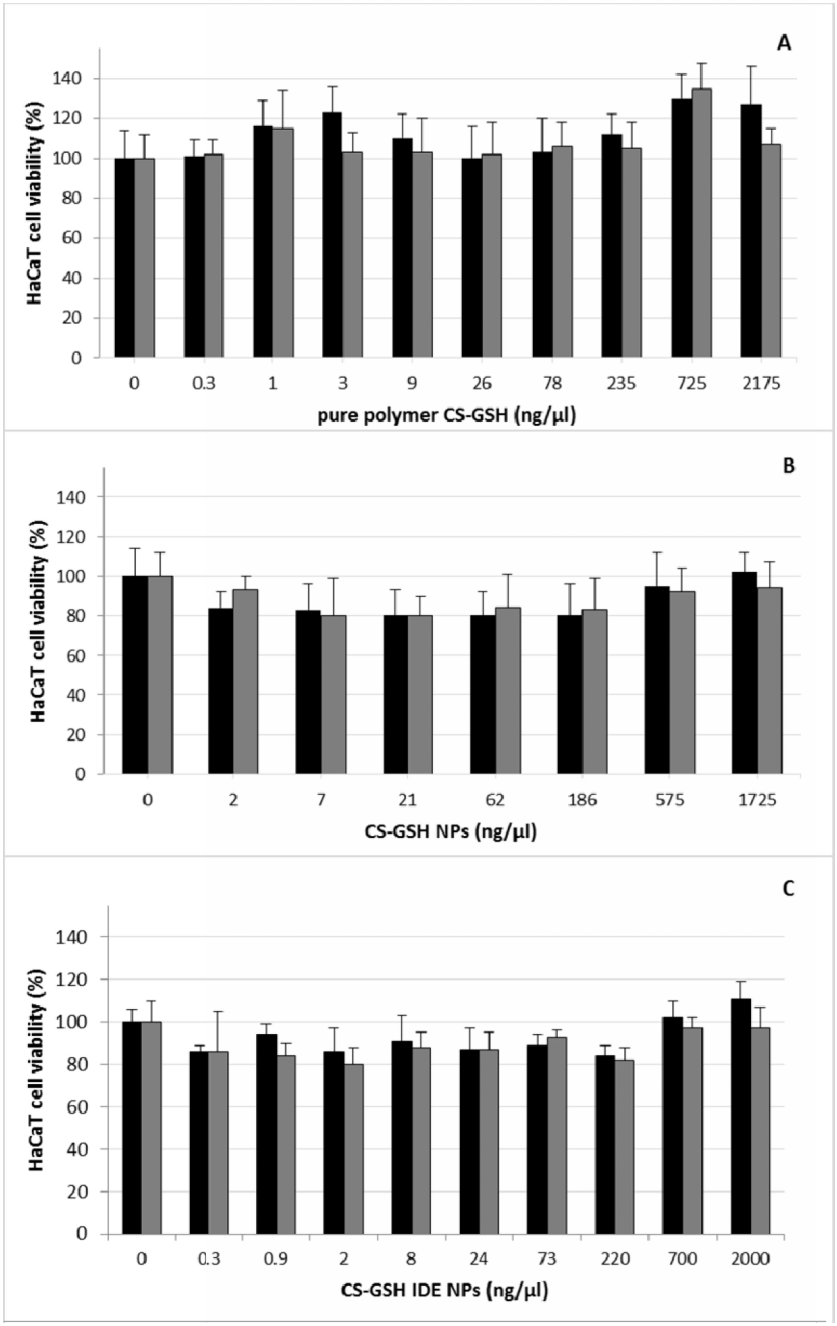

Fig. 6. Cytotoxicity studies in the HaCaT cell treated with pure polymer CS-GSH in the range 0-2.1 µg/µl (panel A); CS-GSH NPs in the range 0-1.7 µg/µl (panel B); CS-GSH IDE NPs in the range 0-2.0 µg/µl (panel C) for 24h (black bars) and 48h (grey bars). The viability was determined using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay as described in the Materials and Methods section. Each data point represents the mean value ± SD of sixtuplicate samples of three different experiments

4. CONCLUSION

In the present study, polysaccharide-based NPs containing IDE and GSH were successfully prepared under mild conditions for topical application of the two antioxidants agents. The different conditions selected for particle formulation allowed modulation of the main physiochemical properties of the resulting NPs in terms of size, zeta potential and GSH loading. The aqueous formulation CS-GSH IDE NPs were found to be the most promising. Stability studies showed that CS-GSH IDE NPs did not significantly change their particle size under storage at room temperature while *in vitro* release experiments showed a constant IDE release over 24 h. These NPs did not show any cytotoxic effect on HaCaT cell line, suggesting the possible applicability of these nanoparticles as multiple antioxidant delivery systems.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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