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# Improvement of quercetin protective effect against oxidative stress skin damages by incorporation in nanovesicles

Maria Letizia Manca[a,*], Ines Castangia[a], Carla Caddeo[a], Daniel Pando[b], Elvira Escribano[c], Donatella Valenti[a], Sandrina Lampis[d], Marco Zaru[e], Anna Maria Fadda[a], Maria Manconi[a]

[a] Dept. Scienze della Vita e dell'Ambiente, CNBS, University of Cagliari, Cagliari, Italy
[b] Department Ingeniería Química y Tecnología del Medio Ambiente, University of Oviedo, Oviedo, Spain
[c] Biopharmaceutics and Pharmacokinetics Unit, Institute for Nanoscience and Nanotechnology, University of Barcelona, Barcelona, Spain
[d] Dept. Scienze Chimiche e Geologiche, CNBS and CSGI, University of Cagliari, Monserrato (CA), Italy
[e] Prigen srl, Sardegna Ricerche Ed. 3, Pula (Cagliari) 09010, Italy

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ABSTRACT

Quercetin was incorporated in glycerosomes, new phospholipid-glycerol vesicles, and their protective effect against oxidative stress skin damages was extensively evaluated. In particular, the concentration-dependent effect of glycerol (from 10 to 50%) on vesicle suitability as cutaneous carriers of quercetin was carefully assessed. All vesicles were unilamellar and small in size (~80–110 nm), as confirmed by cryo-TEM observation, with a drug incorporation efficiency ranging between 81 and 91%. SAXS studies, performed to investigate the bilayer arrangement, indicated a strong, dose-dependent interaction of glycerol with the polar portions of the phospholipid molecules, while quercetin did not significantly change the bilayer packing. *In vitro* studies on newborn pig skin underlined the concentration-dependent ability of glycerosomes to promote quercetin accumulation in the different layers, also confirmed by confocal microscopic observation of skin treated with fluorescent vesicles. Quercetin incorporated into liposomal and glycerosomal nanoformulations showed a strong ability to scavenge free radicals (DPPH test) and protect human keratinocytes *in vitro* against hydrogen peroxide damage. Moreover, quercetin-loaded vesicles were avidly taken up by keratinocytes *in vitro*.

Overall, results indicate 40 and 50% glycerosomes as promising nanosystems for the improvement of cutaneous quercetin delivery and keratinocyte protection against oxidative stress damage.



## 1. Introduction

Skin represents the largest human organ, with an important barrier function that allows the body to be protected against external agents. Skin is very often prone to oxidation, wounds and burns caused by environmental factors, pathological conditions or external trauma. Poor healing of such skin damages may result in serious complications and possible development of chronic skin lesions [1,2]. The healing of chronic wound is a complex process that involves more than one physiopathological factor, such as inflammatory response, over-production of destructive proteases and oxygen free radicals [3]. Recently, it has been found that topically applied antioxidants may play an important role in healing damaged skin, thanks to their ability to quench free radicals and stop the inflammatory process [4–7]. In addition, the topical administration can improve their local bioavailability and reduce their possible adverse systemic effects.

Polyphenols, such as flavonoids, are known as potent antioxidants and are commonly used for topical applications as a defence against natural skin damage (photo-ageing), skin cancer prevention, and skin care [8–10]. Among them, quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one), which may reduce inflammatory pain by inhibiting cytokine production, has shown superior antioxidant ability than many other antioxidant molecules [11,12]. Despite these promising properties, the skin permeability of quercetin is expected to be very low due to its poor solubility, making its topical delivery inefficient [13,14]. Many approaches have been developed aiming at overcoming this limit. Prodrugs, niosomes, conventional liposomes and novel vesicular systems have been used for the skin delivery of quercetin and other flavonoids [13,15–18]. Among others, innovative lamellar vesicles seem to be promising nanocarriers,

* Corresponding author. Tel.: +39 0706758582; fax: +39 0706758553.
*E-mail address:* mlmanca@unica.it (M.L. Manca).

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especially for improving their otherwise poor delivery to the skin [19–21].

In the present study, quercetin was entrapped in glycerosomes [22] to improve its antioxidant activity against skin damages. The nanocarriers were prepared with an unpurified mixture of phospholipids obtained from soy lecithin, and glycerol was used at different concentrations (10–50%, v/v). Liposomes were also prepared and all formulations were fully characterized for morphology, lamellar structure, particle size distribution, zeta potential, and stability during storage. The ability of these vesicles to act as cutaneous delivery systems of quercertin were investigated *in vitro* using Franz diffusion cells and pig skin, while confocal microscopy studies were performed in order to evaluate the carrier distribution in the skin. The antioxidant capacity of quercetin (free or loaded in the nanocarriers) was tested by assessing its ability to scavenge free 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH) and protect human keratinocytes *in vitro* against hydrogen peroxide damage. In addition, the *in vitro* uptake and distribution of quercetin-loaded vesicles in human keratinocytes were evaluated.

## 2. Material and methods

### 2.1. Materials

Phospholipon® 50 (P50) containing phosphatidylcholine (45%), phosphatidylethanolamine (10–18%), fatty acids (7%) and triglycerides (3%) was a gift from AVG S.r.l. (Milan, Italy); 1,2-dioleolyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) was purchased from Avanti Polar Lipids (Alabama, US). Quercetin, glycerol (Ph. Eur.) and all the other products were purchased from Sigma-Aldrich (Milan, Italy).

### 2.2. Vesicle preparation

Liposomes and glycerosomes, either empty or drug-loaded, were prepared by weighing P50 (60 mg/ml) and quercetin (5 mg/ml) in a glass test tube and adding water (to prepare liposomes) or glycerol/water mixture (10–50%, v/v) to obtain glycerosomes. Samples were left for one night at room temperature to facilitate swelling of the phospholipids. Dispersions were then sonicated 30 times (5 s ON and 2 s OFF) with a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, United Kingdom) at an amplitude of 15 microns [23,24].

Samples were purified from the non-incorporated drug by loading them into dialysis tubing (Spectra/Por® membranes, 3 nm pore size; Spectrum Laboratories Inc., Rancho Dominguez, United States) and dialyzing against water at 5 °C for 4 h (replacing the water every 30 min). Incorporation efficiency (*E*%) was expressed as the percentage of the quantity of drug found after dialysis versus that before dialysis. Quercetin content was determined by high performance liquid chromatography (HPLC) after disruption of the vesicles by dilution in methanol (1/100) and analysis of clear solutions at 255 and 367 nm using a chromatograph Alliance 2690 (Waters, Milan, Italy) equipped with a photodiode array detector and a computer integrating apparatus (Empower™ 3). The column was a SunFire C18 (3.5 μm, 4.6 × 150 mm), and the mobile phase was a mixture of acetonitrile, water and acetic acid (96:3.87:0.13, v/v/v), delivered at a flow rate of 0.7 ml/min.

Quantitative determination of phospholipids was carried out using the Stewart assay [25]. Vesicle dispersions (10 μl) were added to the reagent; the obtained solutions were maintained at 4 °C for 30 min and then analysed at 485 nm using a UV spectrophotometer (Lamba 25, Perkin Elmer). Aggregation efficiency (AE%) was calculated as the percentage of the amount of P50 aggregated in vesicular structures versus the amount initially used.

### 2.3. Vesicle characterization

Vesicle formation and morphology were evaluated by cryo-TEM analysis. A thin aqueous film was formed on a glow-discharged holey carbon grid. The resulting film was vitrified by plunging the grid (kept at 100% humidity and room temperature) into ethane, maintained at its melting point, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous film was transferred to a Tecnai F20 TEM (FEI Company), and the sample observed in a low dose mode. Images were acquired at 200 kV at a temperature between −170 and −175 °C, using low-dose imaging conditions with a CCD Eagle camera (FEI Company).

The average diameter and polydispersity index (P.I.) of each sample were determined by Photon Correlation Spectroscopy using a Zetasizer nano (Malvern Instrument, Worcestershire, United Kingdom). Zeta potential was estimated using the Zetasizer nano by means of the M3-PALS (Phase Analysis Light Scattering) technique [24].

### 2.4. Quercetin solubility

Quercetin solubility was determined by dispersing an excess amount of the drug in 50 ml of water or in aqueous mixture of glycerol (10, 20, 30, 40 and 50%, v/v). Dispersions were kept under constant stirring for 24 h at 25 °C in a thermostated bath. Then, after quercetin excess sedimentation, the clear solutions were centrifuged for 10 min at 10000 rpm and the supernatant analyzed by HPLC (Section 2.2) [22].

### 2.5. Stability studies

The stability of the vesicles was evaluated by measuring vesicle average size and P.I. over 90 days at room temperature (~25 °C). In addition, the Turbiscan Lab® Expert optical analyser equipped with an ageing station (Formulaction, Paris, France), was used to determine the accelerated stability of the vesicular dispersions, monitoring the samples under realistic conditions (without mechanical stress or dilution) [26]. The instrument scans the entire height of the sample (λ = 880 nm), acquiring transmission and backscattering data every 40 μm, and shows the backscattering variation (ΔBS) in the three sections (bottom, middle and top) of the cell. Sample variations at the bottom and top of the measuring cell are linked to migration phenomena (sedimentation and creaming, respectively). Variations in the middle are mainly related to changes in particles size. The analysis of each sample was performed at 25 °C every 3 h for 8 days. Backscattering and time are shown on the ordinate, while the height of the cell is indicated on the abscissa of the graph. The first profile is displayed in pink and the last one in red.

### 2.6. Small-Angle X-ray Scattering

Vesicle structure was studied by Small-Angle X-ray Scattering (SAXS). SAXS analysis was recorded at 25 °C using a S3-MICRO SAXS camera system (HECUS X-ray Systems, Graz, Austria). The working q-range was 0.003–0.6 $Å^{-1}$, where $q = (4\pi \sin\theta)/\lambda$ is the modulus of the scattering wave vector, $\theta$ is the scattering angle and $\lambda$ is the wavelength. All scattering curves were reproduced three times and a representative curve was selected, plotting the scattering intensity ($I$) as a function of the scattering vector ($q$). SAXS patterns were analysed using the GAP (Global Analysis Program) software developed by Pabst [27]. The GAP allows fitting the SAXS pattern of bilayer-based structures, i.e. vesicles and lamellar phases.