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Research paper

Bifunctional viscous nanovesicles co-loaded with resveratrol and gallic acid for skin protection against microbial and oxidative injuries

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ABSTRACT

Resveratrol and gallic acid were co-loaded in phospholipid vesicles aiming at protecting the skin from external injuries, such as oxidative stress and microbial infections. Liposomes were prepared using biocompatible phospholipids dispersed in water. To improve vesicle stability and applicability, the phospholipids and the phenols were dispersed in water/propylene glycol or water/glycerol, thus obtaining PEVs and glycerosomes, respectively. The vesicles were characterized by size, morphology, physical stability, and their therapeutic efficacy was investigated *in vitro*. The vesicles were spherical, unilamellar and small in size: liposomes and glycerosomes were around 70 nm in diameter, while PEVs were larger (~170 nm). The presence of propylene glycol or glycerol increased the viscosity of the vesicle systems, positively affecting their stability. The ability of the vesicles to promote the accumulation of the phenols (especially gallic acid) in the skin was demonstrated, as well as their low toxicity and great ability to protect keratinocytes and fibroblasts from oxidative damage. Additionally, an improvement of the antimicrobial activity of the phenols was shown against different skin pathogens.

The co-loading of resveratrol and gallic acid in modified phospholipid vesicles represents an innovative, bifunctional tool for preventing and treating skin affections.

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1. Introduction

Phenols are ubiquitously distributed in plants and plant-derived products, including vegetables, fruits, spices, tea and wine. They are produced as a defence mechanism to ward off microbial threats or external stressors. For instance, *trans*-resveratrol is produced in grapevines after fungal infection or exposure to ultraviolet light [1,2]. The main classes of phenols found in plants and foods are phenolic acids, flavonoids, lignans and stilbenes, all possessing a base-common phenolic group with one or more hydroxyl groups, but ranging from simple phenolic molecules to complex polycyclic structures. All these molecules have antioxidant activity, mainly due to their redox properties, which plays an important role in adsorbing and scavenging free-radicals, quenching oxygen and decomposing peroxides. As a result, phenols have beneficial effects on human health, including protection against cancer, cardiovascular diseases, and inflammatory-based pathologies. In addition, some phenols, such as resveratrol and gallic acid, have shown antimicrobial and antifungal activities [3]. Resveratrol (3,5,4′-trihydroxystilbene) is a water insoluble stilbene with two phenolic rings at the extremities of an ethylene chain, and gallic acid (3,4,5 trihydroxybenzoic acid) is a water-soluble phenolic acid with three hydroxyl groups. Despite their chemical differences, they both possess powerful antioxidant and antibacterial activities [4–7]. Resveratrol was found to inhibit a large variety of filamentous

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fungi, yeasts, Gram positive and Gram negative bacteria, and other human pathogens [6,8]. The exact mechanism behind this antibacterial activity has not been clarified yet. In recent experiments performed *in vitro* on Gram negative bacteria *Escherichia coli*, Hwang et al. [9] reported that resveratrol caused DNA fragmentation, which interferes with the cellular division by suppressing the FtsZ protein involved in Z-ring formation. Gallic acid has also shown antimicrobial activity against different pathogenic bacteria [10–12]. Bacteria cell membranes appear to be the main target of gallic acid, leading to irreversible changes in permeability profile, rupture, pore formation, etc. [11].

Hence, the association of resveratrol and gallic acid, which have different physico-chemical properties but share antibacterial and antioxidant activities, in one formulation can provide an effective multi-target tool for the treatment of skin pathologies [13]. However, their biological efficacy is limited by low bioavailability, especially when applied on the skin. As previously reported, the loading of phenolic molecules in appropriate liposomal formulations can improve their local deposition in the skin and bioactivity [14,15]. Therefore, in the present work, resveratrol and gallic acid were co-loaded in liposomes, which were further modified by adding a co-solvent (propylene glycol or glycerol) to produce Penetration Enhancer-containing Vesicles (PEVs) and glycerosomes. The physico-chemical properties of the vesicles and their ability to improve the delivery of both phenols to the skin were probed. Additionally, the biocompatibility of the formulations and their ability to protect keratinocytes and fibroblasts from oxidative stress were evaluated, as well as their antimicrobial activity.

The novelty of this work is in simultaneously formulating resveratrol and gallic acid in innovative vesicle systems to exploit and maximize the properties of the phenols for the protection and treatment of skin pathologies associated with oxidative stress and microbial infections. A special focus was given to the ease of the preparation protocol, biocompatibility, stability, and activity of the formulations, which are key parameters to assess the feasibility of skin care products.

2. Materials and methods

2.1. Materials

Lipoid S75 (S75), a mixture of soybean phospholipids (70% phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine), triglycerides and fatty acids, was purchased from Lipoid GmbH (Ludwigshafen, Germany). Resveratrol (RSV) and gallic acid (GA) were from Sigma-Aldrich (Milan, Italy). Glycerol and propylene glycol (PG) were from Galeno (Potenza, Italy).

2.2. Vesicle preparation

Vesicles were prepared by dispersing S75 (120 mg/ml), resveratrol and gallic acid (5 mg/ml each) in water to produce liposomes, or in water/propylene glycol (1/1) to produce PG-PEVs, or in water/glycerol (1/1) to produce glycerosomes. The suspensions were sonicated for 30 cycles (5 s on / 5 s off) with an ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK). The samples (1 ml) were purified from the non-incorporated phenols by dialysis (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, Netherlands) against water (2 L) for 2 h, refreshing the water after 1 h.

Empty vesicles were also prepared, characterized and used as a reference.

2.3. Vesicle characterization

For cryogenic transmission electron microscopy (cryo-TEM), a thin aqueous film was formed by placing 5 μl of the vesicle dispersion on a glow-discharged holey carbon grid, and vitrified by plunging into ethane using a Vitrobot (FEI Company, Eindhoven, Netherlands). The vitreous film was transferred to a Tecnai F20 TEM (FEI Company), and the sample was observed in a low-dose mode, at 200 kV and −170/−175 °C.

The average diameter and polydispersity index were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). Samples were backscattered by a helium-neon laser (633 nm) at an angle of 173°, at 25 °C. Zeta potential was estimated by means of the Zetasizer nano-ZS, which measures the particle electrophoretic mobility.

The entrapment efficiency, expressed as the percentage of the phenol concentration after dialysis versus the amount initially used, was determined by high performance liquid chromatography (HPLC) after disruption of the vesicles with methanol (1:1000 v/v dilution), using an Agilent 1100 chromatograph (Madrid, Spain). The samples were diluted with an equal volume of water prior to the analysis, and 25 μl was injected. For resveratrol, the mobile phase was a mixture of acetonitrile and 1% acetic acid in water (25:75, v/v), delivered at flow rate of 1 ml/min through a Waters Nova-Pak C_{18} cartridge (4 μm, 3.9 × 150 mm), and detection was performed at 306 nm. For gallic acid, the mobile phase was a mixture of methanol and 1% acetic acid in water (15:85, v/v), delivered at 1 ml/min through a Waters Spherisorb ODS-2 column (5 μm, 4.6 × 250 mm), and detection was performed at 272 nm.

2.4. Evaluation of vesicle stability

The stability of the vesicles was evaluated by measuring the mean diameter, polydispersity index and zeta potential over a storage period of 90 days, at 25 °C, and by using the Turbiscan Lab Expert optical analyzer (Formulaction, l'Union, France). The instrument irradiates a cylindrical glass cell containing the sample: the light source is an electroluminescent diode in the near infrared (880 nm). Part of the incident light is backscattered by the sample or transmitted through it, and received by two sensors: the sensor that receives the transmitted light is located 180° from the incident radiation, while the sensor that receives the backscattered light is located 45° from the incident light.

Samples (without dilution) were placed in the test cells, and transmitted/backscattered light was monitored as a function of time (every 2 h) and cell height for 15 days at 25 °C using the Ageing Station (Formulaction, l'Union, France). The optical reading head scanned the sample, providing Transmission (TS) and Backscattering (BS) data every 40 μm in% relative to standards (suspension of monodisperse spheres and silicone oil), as a function of the sample height (in mm). The profiles build up a macroscopic fingerprint of the sample at a given time, providing useful information about changes in droplet size distribution, or appearance of a creaming layer, or a clarification front.

2.5. Rheological studies

Rheological measurements were carried out at 25 °C, using a Kinexus rotational rheometer (Malvern Instruments, Worcestershire, UK), equipped with data acquisition and elaboration software rSpace. A cone-plate geometry (CP1/60) was used [16]. Prior to the analysis, a thin layer of silicon oil (Dimethicone, RFE/Ph. Eur.) was laid on the free surface of the sample to prevent water evaporation. Steady shear experiments were performed in the stress range of 0.01–20 Pa. The viscosity of liposomes,

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