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A Poloxamer-407 modified liposome encapsulating epigallocatechin-3-gallate in the presence of magnesium: Characterization and protective effect against oxidative damage

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is a polyphenolic catechin from green tea, well known for being bioactive in age-associated pathologies where oxidative stress plays a preeminent role. The activity of this molecule is however contrasted by its high chemical and metabolic instability that determines a poor concentration of the antioxidant within the biological system after administration. In order to protect the molecule and increase its delivery efficiency, we have encapsulated EGCG inside anionic liposomes made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and cholesteryl hemisuccinate. To maximize EGCG internalization, magnesium salt was added in the preparation. However stable nanodispersions suitable for drug delivery were obtained only after treatment with Poloxamer-407, a polyethylene–propylene glycol copolymer. The structural and morphological properties of the produced dispersion were studied by X-ray diffraction, which showed a multilamellar structure even after EGCG addition and an ordering effect of Poloxamer-407; Dynamic Light Scattering demonstrated serum stability of the liposomes. The characterization was completed by evaluating both encapsulation efficiency (100%, in the final formulation) and *in vitro* EGCG release. Since oxidative stress is involved in numerous retinal degenerative diseases, such as age-related macular degeneration, the ability of these liposomes to contrast H_2O_2-induced cell death was assessed in human retinal cells. Morphological changes at the subcellular level were analyzed by Transmission Electron Microscopy, which showed that mitochondria were better preserved in cells treated with liposomes then those treated with free EGCG. In conclusion, the results demonstrated that the produced formulation enhances the efficacy of EGCG under stress conditions, thus representing a potential formulation for the intracellular delivery of EGCG in diseases caused by oxidative damage.

1. Introduction

Oxidative stress can be linked to several pathophysiological processes including neurodegenerative (Kim et al., 2015), cardiovascular diseases (Heitzer et al., 2001), cancer (Milkovic et al., 2014), aging (Gil-del Valle et al., 2015), obesity (Marseglia et al., 2014), chronic inflammatory disorders like rheumatoid arthritis (Tak et al., 2000) and with several retinal degenerative diseases, such as age-related macular degeneration (AMD) where an increase in the steady-state concentration of reactive oxygen species (ROS) is present (Hernández-Zimbrón et al., 2018). A possible strategy to contrast oxidative stress is to reduce the concentration of ROS, like superoxide anion, hydrogen peroxide, and hydroxyl radical, by enhancing the level of antioxidant molecules in the tissues.

Amongst the water-soluble antioxidants, catechins, the main polyphenolic compounds present in green tea, show considerable bioactivity in degenerative diseases associated with oxidative stress (Mandel et al., 2011; Frei and Higdon, 2003). Epigallocatechin-3-gallate (EGCG), the major constituent in green tea, is mainly responsible for the remarkable antioxidant activity due to the presence of the D ring in the galloyl group in addition to the other three rings present in its structure (A, B and C) (Fig. 1) which are sensitive to oxidation. (Severino et al., 2009). These structural characteristics account for EGCG's potent radical scavenging activity (Zhang et al., 2007; Nanjo et al., 1999)

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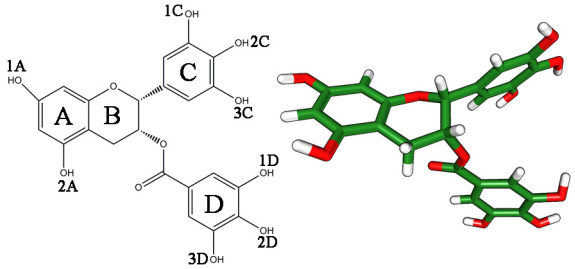

Fig. 1. Structure of epigallocatechin-3-gallate (EGCG).

towards both superoxide and hydroxyl radicals as well as peroxyl radicals, nitric oxide, carbon-centered free radicals and lipid free radicals (Salah et al., 1995).

In addition, EGCG can chelate metal ions like copper(II) and iron (III) which participate in the Fenton reaction and are responsible of the subsequent generation of ROS (Morel et al., 1993).

A possible setback in the employment of EGCG as antioxidant is its chemical and metabolic instability, which can determine a low concentration of the antioxidant within the biological system after administration (Lambert and Yang, 2003; Chen et al., 2001). Therefore, the bioavailability of EGCG may be modulated by using nano- or microparticles (Hu et al., 2013; Granja et al., 2017).

Among all drug delivery systems, liposomes offer several advantages because of their biocompatibility, their low toxicity and non-immunogenicity (Torchilin, 2005; Minnelli et al., 2018; Galeazzi et al., 2015; Mobbili et al., 2015; Crucianelli et al., 2014). These supramolecular aggregates can encapsulate enzymatic antioxidants but also hydrophilic and lipophilic chemical antioxidants, shielding and protecting them from inactivation or rapid clearance from cells. Moreover, the ability of polyphenols to interact with the lipid bilayer (Laudadio et al., 2018; Nakayama et al., 2000) promotes the encapsulation of these compounds inside lipidic nanoparticles and makes liposomes potential delivery systems for EGCG (Mignet et al., 2013).

Previously, we used an in silico approach combined with experimental methods to identify the best lipid matrix and salt composition for obtaining the highest percentage of encapsulated catechin inside liposomes (Laudadio et al., 2018, 2017). In particular, by using anionic multilamellar liposomes prepared from a ternary, lipidic system (POPC/ DOPE /CHEMS 1:1:1) in the presence of Mg^{2+} ions, we obtained complete EGCG encapsulation. However, the liposomes resulted very unstable, as the simultaneous presence of EGCG and magnesium salts was observed to induce the formation of large cluster aggregates.

In order to obtain nanoparticles useful for EGCG delivery applications in this study we describe the preparation and characterization of liposomes made from the same ternary lipidic system, containing EGCG and magnesium salts in addition to a stabilizing agent (the Poloxamer-407) which is able to prevent aggregation and to promote the formation of stable nanodispersions. The structural characteristics of the produced nanodispersions, as well as their *in serum* stability, encapsulation efficiency and *in vitro* EGCG release, were fully determined by different techniques such as X-ray diffraction, Dynamic Light Scattering (DLS), gel filtration and dialysis methods. Moreover, the *in vitro* ability of the nanodispersion to contrast the consequence of H_2O_2 exposure in Adult Retinal Pigmented Epithelium (ARPE-19) cells was evaluated. In particular, the morphological changes in the cytoplasm, mitochondria, endoplasmic reticulum and nucleus of cells induced by the oxidative stress were analyzed by Transmission Electron Microscopy (TEM) while cell viability was assessed by the MTT assay. Since ARPE-19 cells are considered a good *in vitro* model for studying age-related macular degeneration (AMD) (Zareba et al., 2006), the results are very promising in AMD prevention.

It is noteworthy that Poloxamer-407 is a non-ionic triblock copolymer composed by a central hydrophobic poly(propylene oxide) chain (PPO) capped by two hydrophilic chains of poly(ethylene oxide) (PEO), which functions as emulsifier and stabilizer (Wu et al., 2009; Müller et al., 1996). Poloxamer-407 is approved as an inactive ingredient by the FDA for various types of pharmaceutical formulations, and is accepted as GRAS (Generally Recognized as Safe) excipient (Dumortier et al., 2006), making it an excellent candidate for the preparation of drug delivery systems.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesteryl hemisuccinate (CHEMS), used for liposome preparation, were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Sephadex G-50, Poloxamer-407, $MgCl_2$ salt, 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), hydrogen peroxide and all solvents were obtained from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Epigallocatechin 3-Gallate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Adult human retinal pigment epithelial (ARPE-19) cells were a kind gifted from Dr. Dario Rusciano (Sooft Italia spa). All cell culture reagents were purchased from Euroclone (Euroclone, Italy). All other chemicals and buffer components were analytical grade preparations.

2.2. Preparation of bulk and nanodispersed liposomal phases.

Bulk liposomal phases were obtained by Reverse Phase Evaporation (REV) (Szoka and Papahadjopoulos, 1978). Appropriate amounts of chloroform solutions of DOPE, POPC, CHEMS and methanol solution of EGCG, when present, were mixed to obtain 1:1:1:0.6 mol/mol ratio and a final concentration of 3 mg mL^{-1} of lipids and 1 mM of EGCG. The solvent was removed under reduced pressure at room temperature to preserve the EGCG molecular structure (Price and Spitzer, 1994). After removal of residual solvent under nitrogen flow, lipids were redissolved in 3 mL of an ether/methanol mixture (2:1, v/v) and 1 mL of phosphate buffered saline (PBS, pH 7.4) was added with or without $MgCl_2$ salts ($MgCl_2$/EGCG, 5:1 mol/mol). With the aim to obtain an initial water-in-oil emulsion (W/O), the resulting two-phase system was briefly sonicated (2 min) with a vibra cell sonicator (Sonics Vibra Cell Mod. VCx130) equipped with a tapered micro tip. The organic solvent was removed under vacuum (Rotavapor, Büchi) to cause a phase inversion that gave an O/W emulsion. The obtained liposomes L, ML (magnesium containing liposomes), L-EGCG (EGCG loaded liposomes) and ML-EGCG (magnesium containing liposomes loaded with EGCG) were characterized fresh and/or after equilibration for 24 h. The liposomal suspensions containing Poloxamer-407, PxL (poloxamer liposomes), MPxL (magnesium-containing poloxamer liposomes), PxL-EGCG (poloxamer liposomes loaded with EGCG) and MPxL-EGCG (magnesium-containing poloxamer liposomes loaded with EGCG) were prepared in the same way, but Poloxamer-407 was added in PBS to obtain a polymer final concentration of 0.8 mg mL^{-1}. Note that the MLV suspensions were directly used for X-ray diffraction experiments, while samples for DLS characterization, turbidimetric analysis, encapsulation efficiency determination, *in vitro* release and cellular assays, were sonicated (sonic Vibracell) before being used for 30 min in pulse mode (30 s on; 2 s off, 50%) at 0 °C, until the liposome dispersion was completely clear.

2.3. X-ray diffraction

X-ray diffraction experiments were performed using a 3.5 kW Philips PW 1830 X-ray generator (Amsterdam, Netherlands) provided with a bent quartz crystal monochromator (λ = 1.54 Å) and a Guinier-type focusing camera (homemade design and construction, Ancona, Italy). Diffraction patterns were recorded on GNR Analytical