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Design of epigallocatechin gallate loaded PLGA/PF127 nanoparticles and their effect upon an oxidative stress model

Gina P. Hoyos-Ceballos[a], Vanesa Sánchez-Giraldo[b], Miguel Mendivil-Perez[b], Marlene Jimenez-Del-Rio[b], Ligia Sierra-Garcia[a], Carlos Vélez-Pardo[b,1], Betty L. López-Osorio[a,*,1]

[a] Grupo de Investigación Ciencia de los Materiales, Instituto de Química, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Calle 62 No. 52-59, Medellín, Colombia

[b] Grupo de Neurociencias de Antioquia, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Calle 62 No. 52-59, Medellín, Colombia

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ABSTRACT

The epigallocatechin gallate (EGCG) is the most abundant and powerful antioxidant found in green tea. This polyphenol has been proven to have great therapeutic potential in different diseases such as cancer, diabetes, and neurodegenerative diseases. However, the limitations imposed by their low stability and rapid systemic elimination lessen their bioavailability and therapeutic efficacy. To overcome the above problems, EGCG was incorporated into poly(lactic-*co*-glycolic acid) and Pluronic® F127 based nanoparticles (PLGA/PF127). Nanoparticles were prepared using the emulsion/solvent evaporation method resulting in particles with a mean size around 100 nm, low polydispersity index ≤0.1, high drug encapsulation efficiency (~86%) and *in vitro* sustained release. The antioxidant efficacy of the EGCG nanoparticulate formulation was evaluated upon an *in vitro* oxidative stress model. Remarkably, we found for the first time that EGCG encapsulated in PLGA/PF127 nanoparticles prevent the rotenone-induced ROS generation, loss of mitochondrial membrane potential and DNA fragmentation in nerve-like cells. Therefore, our findings suggest the use of these nanoparticles system might be a great opportunity to test EGCG delivery as a potential therapeutic strategy in *in vivo* models of neurodegenerative diseases.

1. Introduction

The epigallocatechin gallate (EGCG) is the major polyphenolic antioxidant present in green tea, representing about 10% of their extract dry weight [1]. This compound has been shown to have preventive effects for a broad range of disorders, which includes cancer [2], inflammatory processes [3,4], and neurodegenerative diseases [5,6]. This ability is mostly attributed to their radical scavenging and metal chelating properties [1]. However, the therapeutic application of EGCG is limited because of its poor absorption, rapid metabolism and systemic elimination that lessen their bioavailability and efficacy [7,8]. Over the last years, polymeric nanoparticles (NPs) have attracted considerable attention to overcome these challenges, since they can enhance drug's properties and prolong its circulation time in the body [9].

Poly-lactic-*co*-glycolic acid (PLGA), a FDA approved biomaterial, is the most commonly used synthetic polymer for development of drugs carriers. This biodegradable material can offer controlled and sustained drug release besides functionalization with vectors that provide a specific release site [10]. PLGA nanoparticles can be prepared through emulsion/solvent evaporation method where the selection of the stabilizer and its concentration are parameters that influence strongly the final physicochemical characteristics of the nanocarriers [11]. The Pluronic® F127 (PF127), a non-ionic triblock copolymer surfactant, has been used for coating and nanoparticles stabilization because of its low toxicity and ability to prevent protein adsorption and recognition by the reticuloendothelial system [12].

Recently, it has been demonstrated that rotenone (ROT), a neurotoxic compound widely used for *in vitro* and *in vivo* model of Parkinson's disease (PD) [13], induces apoptosis, a type of regulated cell death process in mesenchymal stromal cells derived nerve-like cells (NLCs) through an oxidative stress (OS) mediated intracellular signaling pathway [14]. The OS-induced mechanism involves nuclei/DNA fragmentation, loss of mitochondria membrane potential ($\Delta\Psi_m$), specific oxidation of H_2O_2-dependent DJ-1 interacting protein and activation of

* Corresponding author. Grupo de Investigación Ciencia de los Materiales, Instituto de Química, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Calle 62 No. 52-59, Medellín AA 1226, Colombia.
E-mail address: bettylope@gmail.com (B.L. López-Osorio).
[1] These authors share senior co-authorship.

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caspase-3, as end death marker.

The main aim of the present study was to design PLGA/PF127 NPs for incorporating the natural antioxidant (EGCG) and then to test them in an *in vitro* oxidative stress model.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA, 50:50 [MW] 24–38 kDa), Pluronic® F127 (PF127), epigallocathecin gallate (EGCG) and ethyl acetate were purchased from Sigma-Aldrich. Ultrapure water was obtained from Synergy® UV Water Purification System. All other chemicals used were of analytical grade purity.

2.2. PLGA/PF127 nanoparticles preparation

The polymeric NPs were prepared by the emulsion/solvent evaporation method according to ref. [15] with some modifications. Briefly, PLGA (4 mg) was dissolved in 2.0 ml of ethyl acetate to form the organic phase. This solution was then added drop by drop into an aqueous PF127 solution (0.01–1.00% w/v) at 600 rpm. The resulting emulsion was further sonicated using a probe-type sonifier (SONICS, VCX130 vibra-cell) at 130 W for 2 min to reduce the drop size. The organic to aqueous phase ratio (V_o/V_a) for the preparation of o/w emulsion was fixed to 1:2. Finally, the organic solvent was evaporated under magnetic stirring for 4 h at room temperature resulting in nanoprecipitation and formation of nanoparticles.

The best synthesis conditions determined for the PLGA/PF127 NPs, according to the particle size and polydispersity, were chosen for the incorporation *in situ* of EGCG during their preparation. An amount of EGCG (to obtain EGCG:PLGA weight ratios from 1:4 to 1:1) was dissolved in 0.5 ml of water and emulsified with 2 ml ethyl acetate containing 4 mg of PLGA. The obtained emulsion was dropped into the aqueous PF127 solution, as described above. The resultant particles were purified by ultrafiltration (Stirred Ultrafiltration Cell, Merck Millipore Ref. 5122) using regenerated cellulose membrane (MWCO, 1 kDa) in order to remove free EGCG. For some analyses, nanoparticles were freeze dried for 24 h to obtain powdered NPs.

2.3. Physicochemical characterization

2.3.1. Particle size and surface charge

The particle size and the polydispersity index (PDI) were determined by dynamic light scattering (DLS, HORIBA LB-550), taking the average of 5 measurements performed by using freshly prepared samples dispersed in MilliQ-water. The zeta potential (ξ) or the electrophoretic mobility was measured in Malvern, Zetasizer Nano Z equipment.

2.3.2. Morphological analyses

Morphological evaluation of the nanoparticles was done using Transmission Electron Microscopy (TEM) (FEI Tecnai G2-F20) following negative staining with uranyl acetate solution (2% w/v).

2.3.3. Drug encapsulation efficiency and loading capacity

The encapsulation efficiency (%EE) and loading capacity (%LC) of EGCG in PLGA/PF127 NPs were determined by UV–Vis spectroscopic analysis (Perkin Elmer Lambda 35 UV–Vis spectrophotometer with UV Winlab software) of the filtrate obtained from ultrafiltration of the prepared nanoparticle suspension. Briefly, nanoparticles were ultrafiltrated, and washed three times with MilliQ-water. Then, drug content in the filtrate was analyzed by measuring the UV absorbance at 273 nm (λ_{max}) and using a calibration curve: absorbance versus EGCG concentration to quantify the EGCG content. All the measurements were made by triplicate and reported as mean ± SD. The (%EE) and (%LC) were calculated according to the following expressions:

$$\%EE = \frac{\text{Amount of EGCG within NPs}}{\text{Total EGCG added}} x\ 100 \quad (1)$$

$$\%LC = \frac{\text{Amount of EGCG within NPs}}{\text{Total weight of NPs}} x\ 100 \quad (2)$$

2.3.4. Infrared and thermogravimetric analyses

Infrared spectra of PLGA/PF127 NPs, EGCG-PLGA/PF127 NPs, a physical mixture of drug and polymer and pure EGCG were obtained by using a FTIR spectrophotometer (Spectrum One equipment, Perkin-Elmer, MA, USA). The samples were dispersed in KBr and the spectra were recorded by 10 scans from 4000 to 500 cm^{-1}. Thermogravimetric analyses (TGA) of EGCG and EGCG-PLGA/PF127 NPs were performed in a thermogravimetric analyzer Q-500 TA instrument under air atmosphere. The scanning rate was 10 °C/min and the thermograms were recorded from 30 °C to 600 °C.

2.4. Colloidal stability study

Colloidal stability of PLGA/PF127 NPs and EGCG-PLGA/PF127 NPs was evaluated at 25 °C. The dispersed in water particles were stored at room temperature (~25 °C) and protected from light. Stability of the formulations was analyzed in terms of mean size and PDI for 30 days, by triplicate through DLS measurements at 37 °C.

2.5. In vitro drug release assay

The *in vitro* release profile of EGCG from the PLGA/PF127 NPs was investigated by a dialysis method using phosphate buffered saline solution (PBS; pH 7.4) as the release medium. Purified PLGA NPs were dispersed in 1.5 ml of MilliQ-water and then put in the dialysis bags (Sigma) with a molecular mass cut-off of 3500 Da. The bags were suspended in the release medium (PBS) at 37 °C under magnetic stirring at 100 rpm. 1 ml of medium was withdrawn at determined time intervals and replaced with the same volume of fresh medium. The collected samples were stored away from light and analyzed by UV detection (Perkin Elmer Lambda 35 UV–Vis spectrophotometer with UV Winlab software) at 273 nm. The kinetic analyses of the release data were performed using mathematics models, such as the zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Hixon-Crowell, and the best model was selected according to the highest correlation coefficient (R^2) [16]. These experiments were performed by triplicate.

2.6. Experiments with nerve-like cells (NLCs)

Nerve differentiated cells (Supplementary experimental material, Fig. 1s) were left untreated (vehicle) or pre-incubated with free EGCG (10, 25, 50 μM), PLGA/PF127 (40, 60, 80, 100 μg/mL) or EGCG (25 μM) encapsulated in PLGA/PF127 NPs (40 μg/mL NPs for 1 h at 37 °C). Then after, untreated (vehicle) or treated NLCs with EGCG, PLGA/PF127 or with EGCG-PLGA/PF127 NPs were left unexposed or exposed to rotenone (ROT, 50 μM) for 24 h at 37 °C. The evaluation of reactive oxygen species (ROS), assessment of the mitochondrial membrane potential ($\Delta\Psi_m$), and determination of DNA fragmentation were performed by fluorescent microscopy and/or by flow cytometry according to ref. [14]. The analysis of cell death markers was performed by immunofluorescent staining technique according to ref. [14].

2.7. Data analysis

Results (from 2.3 to 2.5 section) are presented as mean ± SD. Statistical analysis of the data was performed via one-way analysis of the variance (ANOVA) using Statgraphics Centurion software; a value of $P < 0.05$ was chosen to determine the statistical significance of