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EGCG intestinal absorption and oral bioavailability enhancement using folic acid-functionalized nanostructured lipid carriers

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ABSTRACT

This work aimed to develop folic acid-functionalized nanostructured lipid carriers (NLC) loading epigallocatechin-3-gallate (EGCG) to increase its oral bioavailability. An active targeting strategy was used and these nanoparticles (NPs) were fully characterized. The NP's effect on Caco-2 cell viability was evaluated and the apparent permeability (P_{app}) on a Caco-2 cell monolayer was determined. The results demonstrated that the developed NPs exhibited adequate physicochemical characteristics for oral administration and were found to be biocompatible with epithelial Caco-2 cells. Further, folic acid-functionalized EGCG-loaded NLC significantly increased EGCG transport across the intestinal barrier, promoting a 1.8- fold increase in its apparent permeability (P_{app}). Taken together, these results support that the developed NLC can be used as a promising carrier for safer and efficient management of several diseases since the pharmacokinetic (PK) properties of EGCG were improved with this nanomedicine-based strategy.

1. Introduction

Epigallocatechin-3-gallate (EGCG) is the major polyphenol found in green tea, being responsible for most of its associated health benefits, including in the prevention and treatment of inflammatory, infectious and cancer diseases [1, 2]. Despite all of these pleiotropic properties, most of EGCG's health-promoting effects are compromised following oral administration due to its poor intestinal permeability and stability [3]. In this context, nanotechnology-based strategies have been developed to increase EGCG oral bioavailability and thus potentiate its health benefits. Several types of nanoparticles (NPs) have been already used as EGCG delivery vehicles, including among others, lipid NPs [4, 5, 6]. Lipid NPs are a promising type of nanovehicles due to their many advantages over other types of NPs, such as high stability and biocompatibility, controlled release properties, low-cost production methods and easy scale-up [7, 8]. Different types of lipid NPs can be produced, including solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), the latter comprising a liquid lipid, which contributes to a more imperfect lipid matrix, leading to a higher drug loading and limited drug leakage during storage [7]. Due to the above-mentioned advantages, NLC were used in this study as a nanovehicle of EGCG. Moreover, to improve the specificity and efficacy, the NPs were functionalized with folic acid as a targeting ligand. Folic acid is a non-immunogenic water-soluble B vitamin critical for DNA synthesis, methylation and repair, being also a common cancer-cell targeting moiety [9, 10]. This vitamin binds to the folate receptors located within caveolae, being internalized in cancer cells through endocytic pathway [10]. In addition, folic acid has been explored to improve the intestinal permeability of NPs [11]. This approach is based on the expression of folate receptors at the gastrointestinal tract, leading to the absorption of folic acid through an acidic pH-dependent carrier-mediated process [11, 12, 13].

The present study aims to explore the effects of folic acid functionalization on the transport of NLC-encapsulated EGCG across an intestinal epithelial barrier and demonstrate that the NPs developed increase the oral bioavailability of EGCG. Thus, NLC were developed to be comprised in the optimal mean diameter range for NPs oral absorption (<500 nm) [14, 15, 16]. In addition, NLC were functionalized with folic acid to increase the bioavailability of the nanocarriers. The NLC developed were characterized according to their average diameter, polydispersity index (PDI), ζ potential and entrapment efficiency (EE). Caco-2 is a well established model to study the ability of different compounds, including NPs to cross the intestinal epithelium [17, 18]. Therefore, this cell line was chosen to evaluate the biocompatibility and intestinal permeability of the NPs developed in this study.

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2. Materials and methods

2.1. Materials

Precirol® ATO 5 was provided by Gatefossé (Nanterre, France). Miglyol 812 was purchased from Acofarma® (Madrid, Spain). Tween® 60, Triton™ X-100, Thiazolyl Blue Tetrazolium (MTT), Trypan Blue powder, dimethyl sulfoxide ≥99.9% and (-)- epigallocatechin gallate (EGCG) ≥80% (HPLC) from green tea were obtained from Sigma-Aldrich® (St Louis, MO, USA). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was obtained from Takara Bio Inc. (Shiga, Japan). Hanks' Balanced Salt Solution [-] CaCl2, [-] MgCl2 (HBSS), Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAXTM-I, 0.25% Trypsin-EDTA (1X), Penicillin-Streptomycin (Pen Strep), Fungizone (amphotericin B, 250 μg mL−1) and Heat Inactivated Fetal Bovine Serum (FBS) (origin: South America) were purchased from Gibco® by Life Technologies™ (Paisley, UK). Caco-2 cell line (passages 30 to 45) was purchased from the American Type Culture Collection (ATCC, Wesel, Germany). Double-deionized water used was obtained from a Millipore system with conductivity less than 0.1 μS cm-1.

2.2. Methods

2.2.1. Development of the NPs

NLC were produced by the high shear homogenization and ultrasonication technique, as reported previously [4]. Briefly, the lipid phase containing solid and liquid lipids and surfactant was melted at 70 °C in a water bath (Medingen E5 Bath- Thermostat). After complete melting, pre-heated ultrapure water was added to the lipid phase followed by stirring in an ultra-turrax (Ystral X10/20 E3; Ballrechten-Dottingen, Germany) at 822×*g* for 30 s, and ultrasonication using a probe sonicator (VCX130, Sonics and Material Vibra-Cell™ with a CV-18 probe; 115 Newtown CT, USA) at 70% for 5 min. The produced nanoemulsion was cooled at room temperature and stored at 4 °C until further use. For the production of EGCG-loaded NLC, EGCG was dissolved in the aqueous phase and added to the lipid phase followed by mixing in ultra-turrax and ultrasonication. For the development of DSPE-PEG-FA functionalized NLC, DSPE-PEG-FA conjugate was synthesized as reported previously [19]. Briefly, 1 g of FA was dissolved into a mixture of 40 mL of dimethyl sulfoxide (DMSO) and 0.5 mL of trimethylamine (TEA) under anhydrous conditions overnight. The resulting solution was then mixed with 0.5 g of dicyclohexylcarbodiimide (DCC) and 0.52 g of *N*-hydroxysuccinimide (NHS) for 18h in the dark, filtered with a 0.45 μM filter and placed under vacuum to evaporate the solvents. The resulting solution (2mL) was then added to 50 mg of DSPE-PEG_{2000}- NH_2 dissolved in 1 ml of DMSO followed by overnight stirring in the dark. Evaporation of DMSO was obtained by placing the solution under vacuum. After that, 6 mL of water were added followed by dialyzsis in 500 mL of ultrapure water for 48h. The resulting solution was lyophilized and the powder obtained was stored at -20 °C until further use. For the development of DSPE-PEG-FA NLC, the conjugate powder was added to the lipid phase in a ratio of 1% w/w of the total formulation mass.

2.2.2. Particle size and ζ potential measurements

The mean size and PDI of the produced NPs were determined using a Particle Size Analyzer (Brookhaven Instruments Corporation; Software: Particle Sizing v.5 Brookhaven Instruments; Holtsville, NY, USA) operating at a scattering angle of 90°, at room temperature, with dust cut-off set to 30 and refractive index of 1.33. ζ potential was measured using an electrode and zeta potential analyzer (ZetaPALS, Brookhaven Instruments Corporation, Software: PALS Zeta Potential Analyser v.5, Brookhaven Instruments; Holtsville, NY, USA).

2.2.3. Entrapment efficiency (EE) and loading capacity (LC) evaluation

EE and LC were determined by UV-vis spectrophotometry using an indirect method. EE corresponds to the percentage of EGCG that is entrapped in the lipid NPs in relation to the initial amount used, while LC is amount of EGCG per unit weight of the total NPs mass.

Nanoformulations were diluted in ultrapure water and filtered using centrifugal filter units (Amicon® Ultra Centrifugal Filters Ultracell-50 kDa, MERK Milipore, Ltd; Cork, Ireland) at 2851×g for 20 min. The amount of non-encapsulated EGCG present in the supernatant was quantified by UV-vis spectroscopy (Jasco V-660 Spectrophotometer, Software: Spectra Manager v.2, Jasco Corporation, USA) at 273 nm. EE was determined by calculating the difference between the initial amount of EGCG added for the preparation of NLC and the amount of EGCG present in the supernatant, as shown by the Eq. (1), while LC was calculated by dividing the amount of entrapped EGCG by the total weight of the lipid NPs as defined in Eq. (2):

$$EE = \frac{Total\ amount\ of\ EGCG\ -\ amount\ of\ EGCG\ in\ the\ supernatant}{Total\ amount\ of\ EGCG} \quad (1)$$

LC was calculated as indicated in Eq. (2):

$$LC = \frac{EE\ \times\ total\ amount\ of\ EGCG}{Total\ amount\ of\ lipids + surfactant + EGCG} \quad (2)$$

2.2.4. Caco-2 cell culture

Caco-2 cells (ATCC® HTB-37™) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum, 1% (v/v) Penicillin-Streptomycin and 1% (v/v) Fungizone at 37 °C in a 5% CO_2 atmosphere. For every 2–3 days cells were supplied with fresh supplemented DMEM. Cells were subcultured at 80–90% confluence by chemical detachment with trypsin- EDTA. Cell counting was performed in a Neubauer chamber after the addition of 25% (v/v) Trypan Blue solution (0.4% (w/v) in HBSS) to exclude non-viable cells. Cells were seeded at a density of 7.5×10^5 cells per 75 cm^2 flasks in 10 mL of supplemented DMEM

2.2.5. MTT Caco-2 cell viability assay

The effect of the NPs in Caco-2 cell viability was assessed by the MTT assay. Cells were cultured in 96-well plates (6.65×10^3 cells per well) for 20 h at 37 °C in an atmosphere of 5% CO_2. Cells were then incubated with different concentrations (corresponding to 5–100 μM of EGCG) of each NPs formulations and free EGCG for 24 h at 37 °C, 5% CO_2. A positive control containing fresh DMEM and a negative control (Triton™ X-100 1% (v/v) in sterile water) were also included. After 24 h, the medium was transferred to another culture plate and reserved for LDH assay, while the cells were treated with 0.5 mg/mL MTT for 3 h at 37 °C, 5% CO_2. Finally, 200 μL of dimethyl sulfoxide (DMSO) were added to each well followed by incubation as 37 °C for 15 min and protected from light. The absorbance was measured using a Synergy™ HT Multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at 550 nm and 690 nm for background subtraction.

2.2.6. LDH Caco-2 cell toxicity assay

The cytotoxic effect of the NPs in Caco-2 cells was assessed by the LDH assay. The medium collected after incubation of the NPs with cells was centrifuged at 250 g for 10 minutes at room temperature (Centrifuge 5810R, Eppendorf, Germany). The resulting supernatant was transferred to another 96-well plate and the LDH was quantified using the LDH cytotoxicity detection kit according to the manufacturer's instructions. Absorbance values were measured at 490 and 690 nm in a microplate reader.

2.2.7. Caco-2 cell permeability assay

Caco-2 cells were seeded on Transwell devices (6 wells, 4.67 cm^2 polycarbonate membrane with pore diameter of 0.4 μm) at a density of 4.5×10^5 cells per insert and grown for 21 days, at 37 °C and 5% CO_2 in supplemented DMEM. These growing conditions were followed to obtain a polarised monolayer with fully developed tight junctions [20]. Every 3–4 days cells were replenished with fresh DMEM and the integrity of the

cell monolayer was monitored by measuring the trans-epithelial electrical resistance (TEER) with an epithelial voltohmmeter (EVOM) from World Precision Instruments (Sarasota, FL, USA). After 21 days, only monolayers with TEER values higher than 200 Ω cm^2 were used, indicating the formation of a polarized monolayer. Cells were then washed twice with HBSS and incubated with free EGCG, EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC on the apical side, at a concentration of 25 uM of EGCG using HBSS (adjusted to pH 6.0) as a transport media, as previously reported [21] for 24 hours. At the end of the experiment, aliquots from the basolateral side were collected and treated with 60% acetonitrile to disrupt lipid NPs matrix and release the entrapped EGCG. TEER was measured at the end of the experiment to assure that the integrity of the monolayer was maintained. A blank HBSS solution was used as a negative control. Quantification of EGCG was performed by measuring the absorbance at 273 nm in a microplate reader. The apparent permeability coefficients (P_{app}) were calculated according to Eq. (3):

$$P_{app}\left(cm\ s^{-1}\right) = \frac{Q}{A \times C \times t} \quad (3)$$

where Q represented the total amount of permeated EGCG (μg), A is the surface area of the insert (cm^2), C is the initial EGCG concentration (μg/cm^3) and t is the experiment time (s).

2.2.8. Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics software (v.22.0.0.0; IBM, Armonk, NY, USA). The measurements were repeated at least 3 times and data expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) and differences between groups compared by Bonferroni, Tukey and Dunnet post-hoc tests with a P value of <0.05 considered statistically significant.

3. Results

3.1. Effects of EGCG lipid NPs on Caco-2 cell viability

Lipid NPs were produced using the high shear homogenization and ultra-sonication techniques as previously described [4]. Size, polydispersity index and ζ potential were measured using a particle size and ζ potential analyser. The size of the NPs was approximately 300 nm, which is suitable for an efficient intestinal uptake and therefore adequate for oral administration [14, 15, 16]. Polydispersity index (PI) was below 0.2 confirming that the NPs population is monodisperse and ζ potential was in the range of -30 mV, suggesting a good stability of the nanoformulation in suspension [22, 23]. EGCG EE was measured by UV-vis spectroscopy and values of approximately 90% (LC of 2.5%) were obtained.

To evaluate the effects of the NLC produced in the intestinal mucosa, viability and cytotoxicity assays were performed in Caco-2 epithelial cell line (Fig. 1). Caco-2 cells are a well-established *in vitro* model for the study of the intestinal absorption of different drugs and compounds, due to their ability to differentiate in culture and form a monolayer with similar characteristics to the enterocytes [24, 25, 26]. Cells were incubated for 24 hours with free EGCG, EGCG-loaded NLC, and folic acid-functionalized EGCG-loaded NLC. Concentrations of 5–100 μM of EGCG or the equivalent solid amounts of unloaded NLC were tested. Treatments with Triton X-100 and culture medium (DMEM) were used as controls.

The MTT assay (Fig. 2) demonstrated that free EGCG did not significantly affect Caco-2 cell metabolic activity in any of the concentrations tested, which is in agreement with a previous work showing that concentrations of EGCG up to 100 μM did not cause significant alterations on cell viability [27]. Concerning the effects of the NPs, a cell viability of approximately 80% was found after treatment with concentrations up to

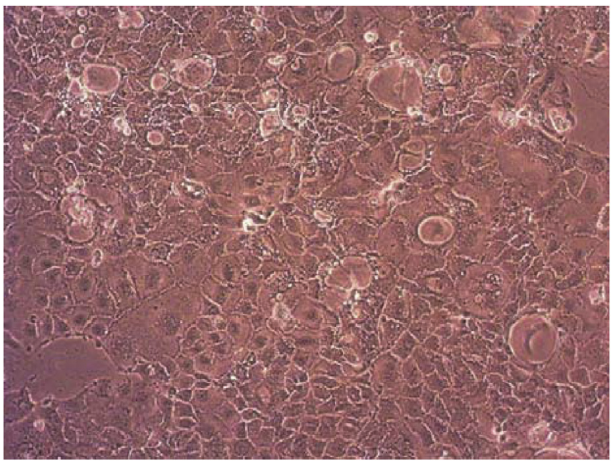

Fig. 1. Photograph of 100% confluent Caco-2 cells. Magnification 100x.

25 μM EGCG in both EGCG-loaded NPs and their unloaded counterparts. Additionally, the folic acid functionalization seems not to induce increased levels of cytotoxicity, comparing to their non-functionalized counterparts. This data suggests that nanoformulations up to concentrations of 25 μM of EGCG could be used for permeability studies, without compromising cell viability. In general, only treatments with 50 and 100 μM of EGCG lead to considerable effects on Caco-2 cell viability, although 50–60% of cells are still viable. These results suggest that the produced NLC have a satisfactory biocompatibility in the intestinal barrier and therefore are suitable for an oral delivery route.

LDH assay (Fig. 3) corroborate the results obtained in the MTT assay, showing that the highest concentrations (≥50 μM) of NPs tested induced some level of cytotoxicity in the Caco-2 cells, however concentrations up to 25 μM did not induce significant levels of cytotoxicity, being once more considered suitable for the permeability assays.

Both assays demonstrated a dose-dependent cytotoxic effect of the NPs, with absence of cytotoxic effects at the lowest concentrations tested (25 μM and lower). It is also noticeable that the folic acid functionalization did not induce additional cytotoxicity in the cells, when comparing the folic-acid functionalized NPs with their non-functionalized counterparts, demonstrating the biocompatibility of the synthesized conjugates.

In summary, the MTT and LDH results, point out the most suitable concentration of EGCG for permeability studies without compromising the cell viability of Caco-2 cells, therefore preserving the integrity of the cell monolayer. A concentration of 25 μM EGCG was then selected to proceed with the intestinal permeability assays.

3.2. Caco-2 permeability assay

Caco-2 cells were grown on Transwell polycarbonate inserts for 21 days to obtain a polarised cell monolayer with complete tight junction formation [20]. To ensure that the cell monolayer maintained its integrity during all the experiment, the transepithelial electrical resistance (TEER) was monitored. Caco-2 monolayers were then incubated with free EGCG, EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC. HBSS adjusted to pH 6.0 was used as transport media, as reported previously [21]. The intestinal permeability of free EGCG, EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC was determined after 24 hours and the results expressed as apparent permeability (P_{app}) values are presented in Fig. 4. The value of a P_{app} obtained for free EGCG was 1.35×10^{-6} cm s^{-1}, which is consistent with a previous report [28]. A similar value was obtained for EGCG-loaded NLC (1.28×10^{-6} cm s^{-1}). Folic acid-functionalized NLC, however, presented a P_{app} of 2.38×10^{-6} cm s^{-1}. This shows that folic acid functionalized EGCG-loaded NLC induced a significant 1.8-fold increase ($p < 0.05$) in this value after 24

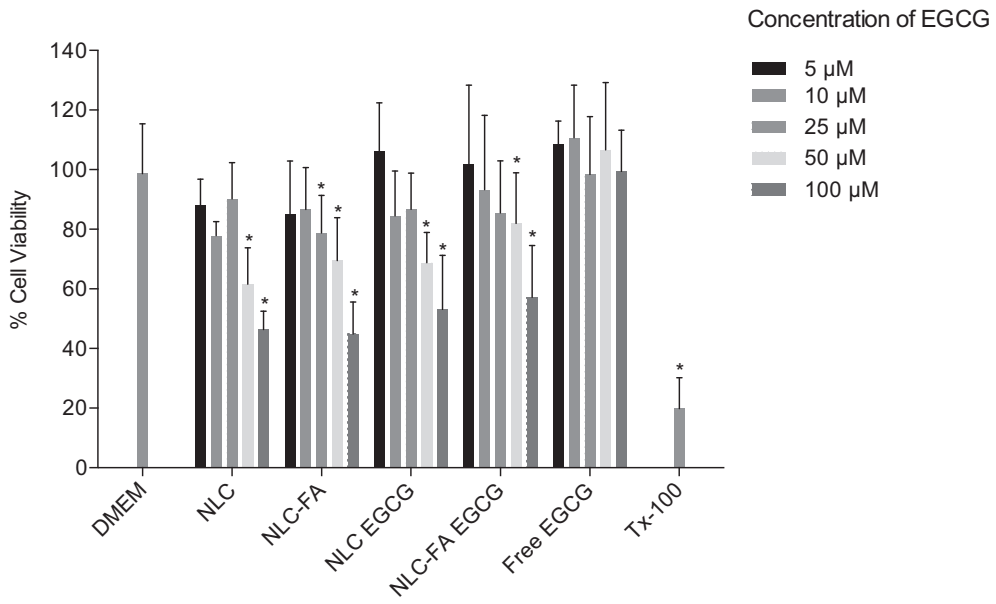

Fig. 2. Caco-2 cell viability assessed by MTT assay after 24 hours of exposure to the different NPs at increasing concentrations of EGCG (or the equivalent solid amount of unloaded NLC). Values represent the mean ± SD (n > 3). Results were compared with DMEM medium, which represents the maximum of cell viability. (*) denotes statistically significant differences ($P < 0.05$).

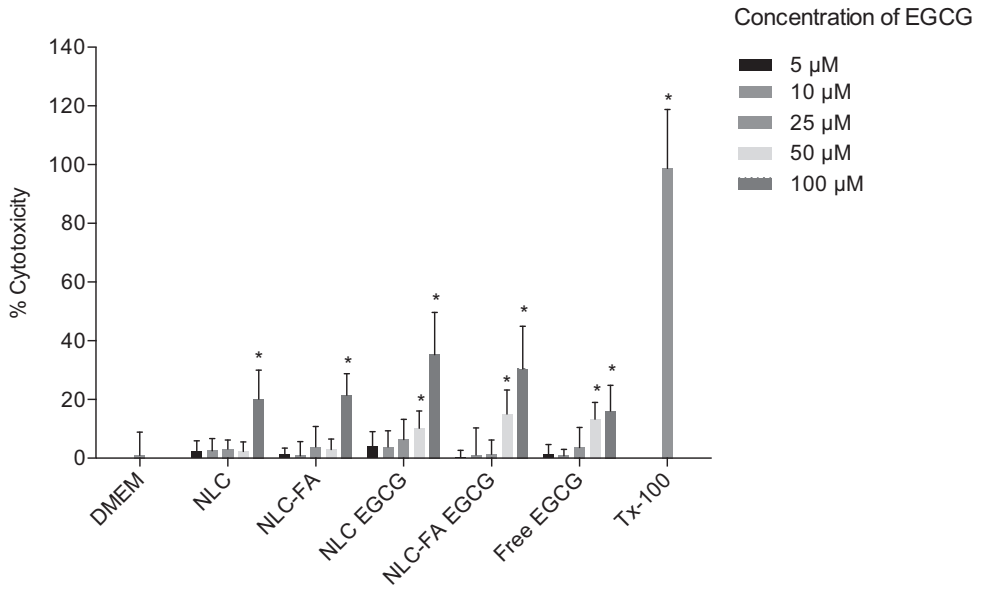

Fig. 3. Caco-2 cytotoxicity assessed by LDH assay after 24 hours of exposure to the different NPs at increasing concentrations of EGCG (or the equivalent solid amount of unloaded NLC). Values represent the mean ± SD (n > 3), Results were compared with DMEM, which represents the minimum of cytotoxicity. (*) denotes statistically significant differences ($P < 0.05$).

hours of transport. This supports the efficacy of the functionalization of lipid NPs, further suggesting that folate targeting associated with NLC is an efficient method to improve the intestinal permeability of EGCG.

To confirm that NPs are able to cross the Caco-2 monolayer, particle size, PDI and ζ potential measurements were performed on basolateral side as well as on the apical side of the transwell device after the permeability experiment (Table 1). NPs with sizes around 120–130 nm on the basolateral side of both EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC were obtained, suggesting that NPs could indeed cross the Caco-2 monolayer and therefore increase EGCG intestinal permeability. It is also possible to observe that NPs size decrease from apical to basolateral side, probably reflecting some rearrangement inside cells during crossing. Interestingly, particles with sizes of approximately 95 nm were found in the basolateral side, but not in the apical side of both control wells (HBSS) and free EGCG, where no NLC were present. This could be attributed to the secretion of cellular particles, such as extracellular membrane particles or exosomes from Caco- 2 cells to the basolateral side of the monolayer, as previously reported elsewhere [29, 30]. Nevertheless, the NPs found on the basolateral side of the transwells containing NPs presented higher sizes than the control and free EGCG. Moreover, the presence of two populations with sizes of approximately 70 nm (attributed to membrane particles and/or exosomes) and 350 nm (attributed to the presence of the NPs) only exist in the case of NLC and FA-NLC loading EGCG (data not-shown), confirming that this increase in particle size is due to the presence of the lipid NPs that have crossed the Caco-2 monolayer. It is also relevant to highlight

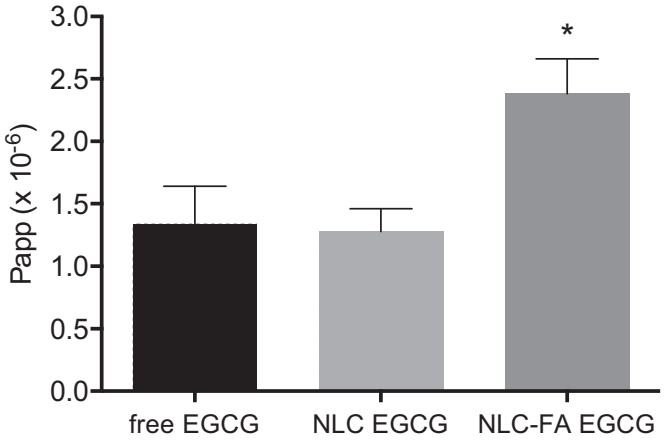

Fig. 4. Apparent permeability (P_{app}) of free EGCG, EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC for 24 h of transport across Caco-2 cell monolayer. Values represent the mean ± standard deviation (n = 3). (*) denotes statistically significant differences compared with the free EGCG ($p < 0.05$).

Table 1
NPs size, polydispersity index, and ζ potential from apical and basolateral sides of Caco-2 cells monolayer after 24 h incubation with EGCG lipid NPs. Control refers to only HBSS medium without any EGCG NPs sample. n.a. means that no data were detected.

	Size (nm)		Polydispersity Index		ζ Potential (mV)	
	Apical	Basolateral	Apical	Basolateral	Apical	Basolateral
Control	n.a.	94 ± 12	n.a.	0.26 ± 0.06	n.a.	-6±4
Free EGCG	n.a.	96 ± 15	n.a.	0.23 ± 0.04	n.a.	-11 ± 4
NLC EGCG	419 ± 41	127 ± 2	0.20 ± 0.04	0.34 ± 0.05	-16 ± 5	-13 ± 2
NLC-FA EGCG	510 ± 108	121 ± 11	0.28 ± 0.06	0.30 ± 0.01	-17 ± 3	-8±5

the values of PDI around 0.2 and 0.3, which demonstrates the presence of a population of lipid NPs with acceptable homogeneity [31] and also confirms the presence of the NPs on the basolateral side of the monolayer thus supporting the ability of the lipid NPs to cross the intestinal barrier. Regarding the values of ζ potential, an increase on the surface charge of the NPs was obtained comparing to the initial nanoformulations that were prepared in ultrapure water (values changing from around -30 mV to around -10 mV to -20 mV) on both apical and basolateral sides. It is well established that zeta potential is strongly influenced by the surrounding media of the NPs. Since the permeability assays were performed using HBSS, which is rich in electrolytes, lower absolute values of zeta potential are expected [32].

Taken together, these results support that the entrapment of EGCG into folic acid-functionalized NLC significantly increase its transport across Caco-2 monolayer, suggesting that folic acid functionalization of EGCG-loaded lipid NPs is an efficient method to improve its transport across the intestinal barrier.

4. Conclusion

In this study, folic acid-functionalized NLC for EGCG loading were efficiently produced. Cell viability and cytotoxicity studies in Caco-2 cells demonstrated that the NLC produced are non-toxic to Caco-2 epithelial cells during 24 h at concentrations up to 25 μM of EGCG. This concentration was then chosen to perform intestinal permeability studies. Cells were incubated with free EGCG, EGCG-loaded NLC and folic acid-functionalized EGCG-loaded NLC. A 1.8-fold increase in the intestinal permeability of the functionalized EGCG-entrapped lipid NPs comparing to free EGCG was found, suggesting that folic acid functionalization of EGCG-loaded lipid NPs can successfully increase its transport across the intestinal barrier and therefore enable it to reach the systemic circulation in higher amounts. Our previous results have demonstrated that folate-targeted NLC can efficiently protect EGCG in simulated gastrointestinal conditions with a controlled release profile during more than 24 hours [4]. This data together with the results shown here further support the potential of the NLC developed as effective EGCG delivery system for oral administration. Additionally, the NPs developed have the potential to enhance the oral absorption of drugs with poor bioavailability. Our results are comparable to a previous report where similar values for EGCG and catechin respectively were obtained (0.88 and 1.68 × 10^{-6} and cm s^{-1}) and increased to 1.42 and 2.39 1.68 × 10^{-6} cm s^{-1}, respectively after encapsulation into niosomes [33]. Other types of NPs such as chitosan-coated bovine serum albumin [34] and ovalbumin-dextran NPs [35] have been used and similar results regarding the increase of EGCG intestinal permeability have been demonstrated. Overall, these results confirm that encapsulation of EGCG into a suitable delivery vehicle can increase its Papp across Caco-2 monolayers and enhance its intestinal permeability, thus being suitable for the oral delivery of EGCG. The lipid NPs here described offer many advantages to be used as a nutraceutic due to their easy and low cost production method, which make it easily scalable to the food and pharmaceutical industry. Despite the promising results obtained with the developed nanovehicles one should take into account that when inside the gastrointestinal tract, NLC may suffer lipolysis and be disintegrated. However, one way to circumvent this is phenomenon is to administer the NLC into a gastro-resistant capsule thus minimizing the possible degradation that might occur in the gastric environment.

In conclusion, this nanomedicine-based strategy can therefore be employed as a safer and efficient carrier of EGCG, increasing its pharmacokinetic properties by oral route and ultimately improving its pleiotropic health benefits, which makes it a promising delivery vehicle for the prevention and management of several diseases.

Declarations

Author contribution statement

Andreia Granja: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ana Rute Neves, Marina Pinheiro: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Célia Sousa: Conceived and designed the experiments.

Salette Reis: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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