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In vitro study of intestinal epithelial interaction with engineered oil in water nanoemulsions conveying curcumin

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ABSTRACT

The development of innovative nano-bio-encapsulation systems continues to be an area of intense activity as the demand of improved delivery systems is constantly increasing in several fields including nanomedicine. For this purpose, an important goal is carrying out appropriate engineering of the surface of these nanocarriers to satisfy the organ target features for an effective *in situ* release and elucidate the mechanism of action which most of the time is neglected. Here, an oil-in-water (O/W) nanoemulsion coated with a polysaccharide layer film – i.e. a glycol chitosan modified with a thiol moiety – was used as nanocarrier to convey a promising poorly water-soluble nature based drug, curcumin. The final nanocarrier was completely bio-compatible and bio-stable. We investigated the enhancement of the effect of curcumin loaded in our system across monolayers of intestinal epithelial cells CaCo-2 in Transwell culture. Such *in vitro* platform resulted suitable to evaluate the functionality of the proposed nanocarrier and its adhesion towards the mucosal epithelial layer and, as applicative example, to investigate the anti-inflammatory effects exerted by the encapsulation of curcumin.

1. Introduction

The use of molecules or food ingredients with positive effects on health, prevention and treatment of diseases is increasingly in demand [1]. However, many nutraceuticals are unstable or poorly water soluble and their oral administration results to be limited [2]. The development of suitable carrier systems for oral delivery remains a major challenge for biomedical scientists and bioavailability remains limited even in the case of nano-encapsulation. As a consequence, oral formulations must be designed to preserve the drug from gastric absorption and improve its interaction with the intestinal tract.

An example of a useful nutraceutical whose potentiality is not yet fully exploited is the curcumin. Among the naturally derived therapeutic products, curcumin, [1,7-Bis(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione] is one of the most extensively studied in recent decades, due to its various properties. In many chronic illnesses in which inflammation plays a major role, curcumin has shown various therapeutic potentials such as anti-inflammatory and antioxidant properties [3]. In fact, molecular studies have indicated that curcumin blocks the activation of factors, such as cytokines, a class of enzymes present in human cells and able to trigger the inflammatory response [4]. Despite these attractive properties, curcumin has a very limited bioavailability because of its lipophilic nature and its poor stability in aqueous solutions [5] resulting in a low efficient oral administration [6].

We recently proposed a high performance nanocarrier system, able to load and protect curcumin from degradation and to enhance its bioavailability thanks to a coating with a thiol-modified chitosan [7]. It was demonstrated in a systematic way the impact of some parameters such as size and degree of surface modification by direct evaluation of effects such as bioavailability, in *in vivo* anti-inflammation. However, while *in vivo* studies provide a direct proof of the validity of a drug delivery system, appropriate *in vitro* studies are necessary in order to specifically elucidate the mechanism of passage through the intestinal barrier. Nonetheless, there is an urgent need of highly fast, reliable and cost-effective *in vitro* models to predict intestinal absorption in order to reduce, refine or replace *in vivo* experimentation [8]. The intestine epithelial barrier is the first barrier and the most important location for the nutraceuticals coming from the lumen of the gut. The transport of drugs across the intestinal epithelium may occur by one, or more than one, of four

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different routes: the passive transcellular and paracellular routes, the carrier mediated route and by transcytosis [9]. Here, in order to comprehensively understand the route of the nano-system carrying the curcumin and the biochemical effect of the drug release, an air liquid interface (ALI) of CaCo-2 cells in Transwell was effectively reproduced [10–13] and used as an *in vitro* testing platform. As previously reported [14], the human CaCo-2 cells differentiate, after their polarization in culture, forming monolayers of mature intestinal enterocytes which can be used as a model that reproduce the intestinal barrier for *in vitro* prediction of intestinal drug absorption transport and toxicology studies. By using this model, we were able to demonstrate the interaction route and the biosafety of the proposed nanocarriers – in terms of cell viability and tight junction integrity- as well as the curcumin bioavailability and its antioxidant effect on LPS-treated intestinal mucosa. Taken together, our results demonstrate that the proposed nanocarrier represents a very promising tool for the effective release of unstable and poorly water-soluble drugs.

2. Materials and methods

2.1. Materials

1-hydroxybenzotriazole hydrate (HOBt), N-acetyl-L-cysteine (NAC), Rhodamine B isothiocyanate (RBITC), Paraformaldehyde and Curcumin (from Curcuma Longa (Turmeric), powder, m.w. = 368.38 g/mol) were purchased from Sigma-Aldrich. Glycol chitosan (GC) was purchased from Wako Chemicals. 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) was purchased from Iris Biotech. CaCo-2 cells were obtained from American Type Cell Culture (ATCC, Usa). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco, Life Technologies and supplemented with 10% Fetal Bovine Serum (FBS) purchased from Sigma Aldrich, 1% Penicillin/Streptomycin and 1% L-Glutamine purchased from Lonza and 2% nonessential amino-acids NEEA purchased from Euroclone. Hank's Balanced Salt Solution (HBSS) was purchased from Gibco, Life Technologies. Transwell Permeable Supports (24-well plates with polyester membrane 0.4 μm) were purchased from Corning® Costar. FITC-Dextran was purchased from Molecular probes, Life Technologies. Trypsin was purchased from Lonza. High Pure RNA tissue Kit was purchased from Roche. Transcriptor First Strand cDNA Synthesis Kit was purchased from Roche. SYBR® Green Master mix was purchased from Aurogene (2× SensiFaST SYBR). Primer of TNFα, TGFβ1, IL6 was purchased from Diatech. 3-(4,5-dimetiltiazol-2-thiazolyl)-2,5-difeniltetrazolio for MTT test, NMR solutions and LPS from Escherichia Coli were purchased from Sigma Aldrich.

2.2. Methods

2.2.1. Modification of glycol chitosan with N-acetylcysteine (GC-NAC)

Glycol chitosan (GC) was modified with a thiol moiety, exploiting the EDC/HOBt chemistry, as previously described [15]. Briefly, GC (100 mg, 0.20 mmol) was dissolved in 10 ml of Milli-Q water, HOBt (88,9 mg) was added and pH was at first adjusted to a value of 4 with HCl 1 M to allow complete dissolution. Then, NAC (400 mg) and EDC (1916 g) were added to the solution. The pH was adjusted and maintained to a value of 6.8. The reaction proceeded overnight at room temperature. The product was purified by dialysis four times against water containing 1 w/v% NaCl and acidified with HCl at pH = 3 and four times against water acidified at pH = 3. Finally, the purified product was freeze-dried for 48 h (Freeze dryer CHRIST Alpha 1–4 LSC). Free thiols were determined using a colorimetric assay, the Ellman's test. In particular, after reaction of thiolated chitosan with a DTNB solution at 25 °C for 2 h, absorbance was registered at 412 nm using a Varian Cary Scan 100 Spectrophotometer. GC-RBITC_NAC was obtained with the same procedure, by using GC-RBITC instead of pristine GC.

2.2.2. Modification of glycol chitosan with rhodamine B isothiocyanate (GC-RBITC)

Glycol chitosan (GC, 100 mg, 0.20 mmol) was dissolved in 10 ml of 0.1 M of acetic acid solution. After complete dissolution, a solution of RBITC (7 mg in 700 μl of DMSO) was added dropwise. The reaction proceeded over night at room temperature. Sample was then dialyzed (dialysis tubing with a MWCO of 6–8 kDa) against water till no dye was detected (using Perkin Elmer 2300 Enspire plate reader, λ_{ex} = 555 nm, λ_{em} = 565 nm). Finally, the purified product was freeze-dried for 48 h.

2.2.3. Preparation of the nanocapsules

The nano-encapsulation system consists of an oil-in-water (O/W) nanoemulsion [16] coated with a mucoadhesive chitosan derivative, namely glycol chitosan. Glycol chitosan was opportunely modified with a thiol moiety (Fig. 1A). Oil in water nanoemulsion was obtained by using a high-pressure homogenizer (Microfluidics M110PS), as previously described [7]. Briefly, 5.8 g of surfactant in 24 ml of oil were used and the oil phase was loaded with 100 mg of curcumin. To promote dissolution, the oil phase containing surfactant and curcumin was mixed by alternating a high speed blender (RZR 2102 control, Heidolph) at 60 °C and 500 rpm to sonication with an immersion sonicator at room temperature (Ultrasonic Processor VCX500 Sonic and Materials), according to a process protocol previously reported [16]. Curcumin loaded nanoemulsion was then coated with GC-NAC (glycol chitosan conjugated with N-acetylcysteine) or GC-RBITC-NAC (glycol chitosan conjugated with rhodamine B and N-acetylcysteine) providing curcumin loaded nanocapsules (CLNs). This deposition was obtained by adding 1.5 ml of polymer solution (0.033% (w/v) in acidified water at pH 4) to 1 ml of water suspensions of O/W nanoemulsion (5% wt in acidified water at pH 4) under vigorous stirring. To perform the biological tests, 150 μl of HBSS/DMEM (1:1 v/v) were mixed with 150 μl of the as prepared GC-NAC coated nanoemulsion (2% wt) and placed on CaCo-2 cell monolayer seeded on transwell culture. The final oil concentration is 1% wt, corresponding to a curcumin concentration of 0.042 mg/ml.

2.2.4. Electron microscopy

For the TEM analysis of nanocapsules, samples were first exposed to vapors of an OsO_4 water solution (1% wt) as oil core dopant for not less than 4 h then, about 10 μl of the sample were spread on a copper grid (200 mesh with carbon membrane). After carefully removing the excess of solution grids were left to dry overnight. TEM images were obtained using a TECNAI 20 G2: FEI COMPANY (CRYO-TEM-TOMOGRAPHY, Eindhoven) with a camera Eagle 2HS. The images were acquired at 200 KV; camera exposure time: 1 s; size 2048 × 2048. For SEM and EDX measurements a small drop of solution containing nanocapsules was spread onto the surface of an aluminum stub covered by a glass plate. The sample was then sputter-coated with a thin Pt/Pd or gold layer (10 nm) in a Cressington sputter coater 208HR. The aluminum stub containing the Pt/Pd or gold – coated sample was then placed in a FEG-SEM scanning electron microscope and imaged using 20 kV accelerating voltage. For imaging analysis of cells, samples were first fixed with a sodium cacodylate 0.1 M in glutaraldehyde solution (2.5% wt) at room temperature for 2 h. Sample was washed with a solution of 0.1 M sodium cacodylate – 0.1 M sucrose (3 times in an ice bath for 10 min). Samples were then fixed with OsO_4 (1% wt. in 0.1 M sodium cacodylate – 0.1 M sucrose) and washed again with a solution of 0.1 M sodium cacodylate – 0.1 M sucrose (3 times in an ice