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Therapeutic efficacy of quercetin enzyme-responsive nanovesicles for the treatment of experimental colitis in rats

Ines Castangia [a], Amparo Nácher [b,c], Carla Caddeo [a,*], Virginia Merino [b,c], Octavio Díez-Sales [b,c], Ana Catalán-Latorre [b,c], Xavier Fernàndez-Busquets [d,e], Anna Maria Fadda [a], Maria Manconi [a]

[a] Dept. Scienze della Vita e dell'Ambiente, University of Cagliari, Cagliari, Italy
[b] Dept. of Pharmacy and Pharmaceutical Technology, University of Valencia, Valencia, Spain
[c] Institute of Molecular Recognition and Technological Development, Inter-Universitary Institute from Polytechnic University of Valencia and University of Valencia, Spain
[d] Barcelona Centre for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Barcelona, Spain
[e] Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain

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ABSTRACT

Biocompatible quercetin nanovesicles were developed by coating polyethylene glycol-containing vesicles with chitosan and nutriose, aimed at targeting the colon. Uncoated and coated vesicles were prepared using hydrogenated soy phosphatidylcholine and quercetin, a potent natural anti-inflammatory and antioxidant drug. Physicochemical characterization was carried out by light scattering, cryogenic microscopy and X-ray scattering, the results showing that vesicles were predominantly multilamellar and around 130 nm in size. The in vitro release of quercetin was investigated under different pH conditions simulating the environment of the gastrointestinal tract, and confirmed that the chitosan/nutriose coating improved the gastric resistance of vesicles, making them a potential carrier system for colon delivery. The preferential localization of fluorescent vesicles in the intestine was demonstrated using the In Vivo FX PRO Imaging System. Above all, a marked amelioration of symptoms of 2,4,6-trinitrobenzenesulfonic acid-induced colitis was observed in animals treated with quercetin-loaded coated vesicles, favoring the restoration of physiological conditions. Therefore, quercetin-loaded chitosan/nutriose-coated vesicles can represent a valuable therapeutic tool for the treatment of chronic intestinal inflammatory diseases, and presumably a preventive system, due to the synergic action of antioxidant quercetin and beneficial prebiotic effects of the chitosan/nutriose complex.

1. Introduction

Dietary antioxidant flavonoids have protective and curative effects on several pathologies, such as cancer, diabetes, aging, cardiovascular, autoimmune, neurodegenerative and chronic inflammatory disorders [1–5]. Their potential prophylactic and therapeutic activity is promising as a new prospective in healthcare, and has been intensively studied in the last decades [6,7]. Among flavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the most common in nature and the major representative of the flavonol subclass [8]. It is often linked to sugars as glycosides like rutin (quercetin-3-rutinoside) and quercitrin (quercetin-3-rhamnoside). Quercetin is a potent anti-inflammatory and antioxidant agent that, when administered orally, has protective and beneficial effects on chronic intestinal inflammation [1,9–12].

Ulcerative colitis and Crohn's disease are chronic inflammatory bowel disorders characterized by upregulated formation of proinflammatory mediators and cytokines (e.g. TNF-α, IL-1β) and dysregulated immune responses, resulting in damage of the mucosa and submucosa of the gastrointestinal tissue. These events seem to be caused by an excessive adaptive immune response to luminal bacterial antigens. However, their pathogenesis is not clearly understood and the current treatment typically remains to induce remission of outbreaks and to prevent them during remission [11]. The first-line therapy for patients with inflammatory bowel disease is centered on treatment with high doses of oral immune-suppressant or anti-inflammatory drugs, often complicated by serious adverse effects. The oral administration of appropriately formulated drugs should allow an adequate drug concentration to be reached in the injured colon, which offers an environment of slightly acidic to nearly neutral pH, a relatively long transit time, a low proteolytic enzyme activity and a greater responsiveness to drug absorption. Colon-specific drug delivery systems may

* Corresponding author. Tel.: +39 0706758582; fax: +39 0706758553.
E-mail address: caddeoc@unica.it (C. Caddeo).

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improve drug local bioavailability and prolong their residence time, minimizing adverse effects [13]. In particular, bioadhesive polymer-based nanocarriers can greatly improve delivery to the colon of drugs that are poorly bioavailable due to unfavorable physicochemical or pharmacokinetic parameters [14]. Such systems, after oral administration, can increase the adherence of formulations to the gastrointestinal mucosa. Recent studies have revealed a high accumulation of anionic carriers in inflamed regions of a rat model of colitis [15–17]. Enteric coated nanoparticulate systems offer the advantages of protecting the drug from the gastric environment, carrying it to the colon, delaying its release, extending the distribution surface of the formulation and improving its residence time in inflamed tissue.

In recent years, polymer-coated liposomes have been proposed as promising tools for the targeted delivery of drugs to the inflamed intestinal mucosa. Conventional phospholipid liposomes are hardly used due to their low resistance to gastric pH and enzymatic degradation, but they can be easily protected by a polymeric coating. Chitosan is one of the most widely used polymers for coating liposomes [18]. It is a highly mucoadhesive polysaccharide, is soluble at gastric pH (~2) but insoluble at the pH 5.5 of the small intestine, and passes unaltered to the colon, where it is partially degraded by the local microflora [19]. Due to its solubility, chitosan alone is unable to protect phospholipid vesicles from an acidic environment, but gastric resistance can be achieved by manipulating the vesicle surface by complexing the chitosan. In a previous work, phycocyanin, a natural antioxidant and anti-inflammatory drug, was encapsulated within liposomes coated with chitosan complexed with xanthan gum. This polymeric complex coating was effective in retarding drug release at colonic pH [18].

In the present work, quercetin-loaded polyethylene glycol-containing vesicles were coated with chitosan; afterwards, nutriose was added to complex the chitosan. Nutriose is a water-soluble, branched dextrin with a high fiber content obtained from wheat starch, formed by dextrin linked with digestible α-1,6 glycoside linkages and non-digestible α-1,2 and α-1,3 glycoside linkages. Thanks to this chemical structure, only 10–15% of the polymer is hydrolyzed and absorbed in the stomach and small intestine, the remaining about 85% being progressively fermented in the colon. Another advantage of this starch derivative is its strong prebiotic activity: it acts as a microbial food supplement for host colonic microflora, improving its beneficial enzymatic metabolism and facilitating the rapid restoration of mucosal integrity in patients with inflammatory bowel disease [20–22].

Hence, we combined phospholipid vesicle nanotechnology with a polysaccharide–starch complex to obtain a delayed, enzyme-sensitive and prebiotic system able to deliver quercetin to inflamed colon. Moreover, in the prepared system, the coating layer itself is supposed to act as a colon protector in synergy with quercetin.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (Phospholipon® 90 H, P90H) was a gift from Abaran Materias Primas S.L. (Villaviciosa De Odón, Madrid, Spain) and Lipoid GmbH (Ludwigshafen, Germany). Nutriose FM06®, soluble dextrin from maize, was donated by Roquette (Lestrem cedex, France). Phosphate buffer solution (PBS, pH 7), quercetin (QUE), cholesterol, polyethylene glycol 400 (PEG400) and low-molecular-weight chitosan were purchased from Sigma–Aldrich (Milan, Italy). 2,4,6-Trinitrobenzenesulfonic acid (TNBS), hexadecyltrimethylammonium bromide (HTAB), 3,3′,5,5′-tetramethylbenzidine (TMB), hydrogen peroxide 30% and horseradish peroxidase were obtained from Sigma (Madrid, Spain). All the products and solvents were of analytical grade.

2.2. Sample preparation

Penetration Enhancer containing Vesicles (PEVs) were prepared using P90H (60 mg ml^{-1}), cholesterol (2 mg ml^{-1}), quercetin (5 mg ml^{-1}) and PEG400/PBS (10 vol.%) as the aqueous phase. All components were weighed in a glass flask and left to hydrate overnight. The suspensions were sonicated (5 s on and 2 s off, 30 cycles; 13 μm probe amplitude) with a high-intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK) [23]. Chitosan (50 mg) was dissolved in 10 ml of 0.1% acetic acid aqueous solution (pH 3). To obtain the polymer coating, the vesicle suspension (1 ml) was added dropwise to the chitosan dispersion (1 ml) under stirring at 25 °C; then, the resulting dispersion was added to a nutriose aqueous solution (5 wt./vol.%; 1 ml). Uncoated vesicle dispersion (1 ml) was diluted with 2 ml of PBS in order to obtain the same final phospholipid and drug concentration of coated vesicles: 20 mg ml^{-1} P90H, 0.7 mg ml^{-1} cholesterol, 1.7 mg ml^{-1} quercetin and 1.7 vol.% PEG400/PBS.

Samples were purified from the non-incorporated drug by dialysis against PEG400/PBS mixture using dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands).

2.3. Vesicle characterization

Vesicle formation and morphology were assessed by cryogenic transmission electron microscopy (cryo-TEM). A thin aqueous film was formed by placing a drop of each sample on a glow-discharged holey carbon grid and then blotting it with filter paper. The resulting thin films were vitrified by plunging the grid (kept at 100% humidity and room temperature) into ethane maintained at its melting point, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 transmission electron microscope (FEI Company) using a Gatan cryotransfer (Gatan, Pleasanton, CA), and the samples were observed in a low-dose mode. Images were acquired at 200 kV at a temperature between −170 and −175 °C, using low-dose imaging conditions not exceeding 20 e^{-} $Å^{-2}$, with a CCD Eagle camera (FEI Company).

The average diameter and polydispersity index (PI, a measure of the size distribution width) were determined by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173°. The Zeta potential (ZP) was estimated using the Zetasizer Nano ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering), which measures the particle electrophoretic mobility. Samples (n = 6) were diluted (1:100) with PEG400/PBS (10 vol.%) and analyzed at 25 °C.

Entrapment efficiency (EE), expressed as the percentage of drug post-dialysis vs. pre-dialysis, was determined by high-performance liquid chromatography (HPLC) after disruption of vesicles with methanol (1/100). Quercetin content was quantified by HPLC fluorescence (excitation at λ = 370 nm, emission at λ = 530 nm) using a chromatograph Perkin Elmer series 200 (Barcelona, Spain). The column was a Kromasil C18 (5 μm, 4.6 × 150 mm). The mobile phase was a mixture of acetonitrile, water and acetic acid (80:19.8:0.2 by vol.), delivered at a flow rate of 1.2 ml min^{-1}.

2.4. Small-angle X-ray scattering (SAXS)

SAXS analyses were carried out using an S3-MICRO (Hecus X-ray Systems, Graz, Austria) coupled to a GENIX-Fox 3-D X-ray