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

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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.

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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 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

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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 nanoparticles revealed a high accumulation of anionic carriers in inflamed mullations to the gastrointestinal mucosa. Recent studies have physicochemical or pharmacokinetic parameters [14]. Such 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 bran. Its chemical structure is mainly 1,3,1,6 glycoside linkages. Nutriose was incorporated in liposomes with a high fiber content obtained from wheat starch, formed with dextrin linked with digestible α,1,6 glycoside linkages and non-digestible α,1,2 and α,1,3 glycoside linkages. Due 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 nutrient 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 blottting 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 using a chromatography 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...
source (Xenocs, Grenoble, France) and a 50 focused X-ray beam with 0.1542 nm at a Cu Kα line. The q range was 0.003–0.6 Å⁻¹, where \( q = (4\pi \sin \theta)/\lambda \) is the modulus of the scattering wave vector, \( \theta \) is the scattering angle and \( \lambda \) is the wavelength. All scattering curves, recorded at 25 °C, were reproduced twice, and a representative curve was selected, plotting the scattering intensity \( I(q) \) as a function of the scattering vector \( q \). SAXS patterns were analyzed in terms of a global model using the program GAP (Global Analysis Program) developed by Pabst et al. [24], which provides relevant structural parameters on bilayer-based structures, i.e. vesicles and lamellar phases. From the analysis, the membrane thickness was obtained through the definition \( d_0 = 2(z_H + 2 \sigma_H) \). \( z_H \) and \( \sigma_H \) derive from the SAXS curve fitted with GAP.

2.5. In vitro drug release studies

The in vitro release profile of quercetin from vesicle dispersions was assessed in three different buffered solutions, at pH 2.0 (stomach), pH 5.5 (first tract of the small intestine) and pH 7.0 (large intestine), using a basket USP dissolution apparatus [12]. The formulations were placed in a dialysis tube (see Section 2.2) and immersed in the dissolution medium, thermostatted at 37.0 ± 0.5 °C. Drug release was assessed: for 2 h at pH 2.0, 200 rpm; for 3 h at pH 5.5, 100 rpm; and for 8 h at pH 7.0, 100 rpm. At scheduled time intervals, an aliquot of the medium was withdrawn and refreshed to ensure sink conditions. The drug content in the samples was determined by HPLC (see Section 2.3). All experiments were performed in triplicate.

2.6. Vesicle behavior in gastrointestinal fluids

Since oral formulations are subjected to different pH and ionic strength levels along the gastrointestinal tract, it is important to evaluate the properties of the vesicles under conditions mimicking stomach-to-colon transit. Hence, vesicle average diameter, polydispersity and zeta potential were measured following incubation in the release media with pH of 2.0, 5.5 and 7.0 for 2, 3 and 8 h, respectively, at 37 °C vs. 25 °C, in the absence or presence of sodium chloride (0.3 M), which was used to regulate the ionic strength in buffers. Release studies were also conducted at a fixed ionic strength 0.3 M.

2.7. In vivo quercetin biodistribution and biocompatibility

The in vivo studies adhered to the Principles of Laboratory Animal Care and were approved by the institutional ethics committee of the University of Valencia (code 2014/044/UVEG/008 Tipo2).

Studies were carried out on Wistar male rats aged 8–12 weeks and weighing 230–250 g. The animals were housed in an air-conditioned room at 22 ± 3 °C and 55 ± 5% humidity, with 12 h light/dark cycles, and allowed free access to water and laboratory chow for the duration of the studies. The rats were fasted for 12 h before experiments but had free access to water during this time. 1,2-Dioleolyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (0.035 mg ml⁻¹; Rho-PE) was used to label the quercetin-loaded vesicles [25]. Labeled samples (2 ml) were administered intragastrically to healthy rats by gavage. Rats were sacrificed at 1, 2, 3, 4 and 5 h, and their intestines were removed, stretched slightly and carefully spread on a dissecting board for observation using the In Vivo FX PRO Imaging System (Bruker BioSpin, USA). Rho-PE fluorescence (excitation at 559 nm and emission at 578 nm) was clearly evident, while quercetin fluorescence coincided with that of intestinal lumen content.

To quantify the distribution of quercetin in the gastrointestinal tract, the intestines were removed, divided into segments (i.e. stomach, duodenum, jejunum, ileum and colon) opened by incision, and the solid content was collected. The luminal surface of the segments was gently washed with 3 ml of saline (NaCl 0.9 wt./vol.%), then the muscle was isolated from the mucosa by scraping the segments with a scalpel. The specimens were homogenized in ethanol, shaken for 24 h, centrifuged at 6.2 g for 10 min and then filtered out (0.20 μm). The amount of quercetin present in the different segments and the relative layers was quantified by HPLC with fluorescence detection (see Section 2.3). The results were expressed as the ratio of the fluorescence intensity of quercetin in the segment/layer vs. the initial fluorescence intensity of the vesicle dispersion.

In addition, to assess the biocompatibility of quercetin, the colon and kidneys were excised from healthy rats, dehydrated and fixed with paraformaldehyde, frozen at –80 °C and sectioned using a cryostat (Leica CM1950, Barcelona, Spain). Tissue specimens were stained with hematoxylin and eosin (H&E) and examined under the light microscope on blind-coded samples.

2.8. Induction of colonic inflammation

Chronic inflammation in the rat colon was chemically induced as previously described by Mura et al. [26], with slight modifications. Briefly, rats (at least \( n = 6 \)) were divided into treatment groups \( (n = 6) \), fasted for 12 h with free access to water, then anesthetized with isoflurane. A graduated rubber canula was inserted rectally into the colon, such that the tip was 8 cm proximal to the anus. TNBS (0.13 M) was dissolved in ethanol/water (1:1 by vol.) and instilled (0.5 ml) into the lumen of the colon (day 0).

2.9. Treatment study design

Rats were randomly divided into six groups: group 1 (negative control) were healthy animals; groups 2–6 were TNBS-treated animals receiving PEG400/PBS dispersion (positive control), quercetin in PEG400/PBS dispersion, empty coated vesicles, quercetin-loaded uncoated vesicles and quercetin-loaded coated vesicles, respectively. Based on previous studies in rats, a dose of 9 mg kg⁻¹ day⁻¹ of quercetin was administered by oral gavage once a day for 3 days in the period of the most intense inflammation (days 3, 4 and 5 after TNBS rectal administration, day 0).

2.10. Assessment of colonic injury and inflammation

Colitis development and severity were assessed by a clinical scoring system on a scale of 0–4 by checking weight loss, stool consistency and rectal bleeding for 9 days [27,28]. No weight loss was counted as 0 points, 1–5% as 1 point, 5–10% as 2 points, 10–20% as 3 points and >20% as 4 points. For stool consistency, 0 points were given for well-formed pellets, 2 points were given for pasty and semifomed stools that did not stick to the anus, and 4 points were given for liquid stools that stuck to the anus. Bleeding was scored as 0 points for no blood, 2 points for positive finding and 4 points for gross bleeding. The sum of these scores formed the clinical score, ranging from 0 (healthy) to 12 (maximal activity of colitis).

At day 9 the rats were euthanized with an overdose of sodium pentobarbital (Dolethal®; Vetoquinol, UK, Ltd.), then the abdomen was opened and the distal colon was removed. The samples of inflamed tissue were excised to measure the ratio of distal colon weight to body weight (colon/body ratio), the criteria for scoring the gross morphologic damage (clinical activity score system, CAS) and myeloperoxidase activity (MPO).

The colon/body weight ratio was measured by rapidly excising and opening the colon longitudinally along the mesenteric edge. It was then washed with saline and placed on a glass plate chilled with ice with the mucosal surface upward, and weighed [26].
The ratio of the 8 cm segment distal colon weight to body weight was used as an index of colonic tissue edema.

MPO was used as an index of inflammation. The activity was analyzed according to established methods [26,29–31]. Briefly, colon specimen was added to 750 μl of HTAB buffer (0.5% in 80 mM phosphate buffer pH 5.4) on ice and homogenized. The homogenate was centrifuged (Heraeus Fresco 17 Centrifuge, Thermo Electron Corporation, Spain) at −4°C and 9.6 g for 15 min. Supernatant was incubated with hydrogen peroxide and tetramethylbenzidine. The reaction was stopped with 2 N H2SO4 and the absorbance was measured spectrophotometrically at 450 nm.

2.11. Statistical analysis of data

Results are expressed as the mean ± standard deviation (SD). Analysis of variance and Bartlett’s test for homogeneity of variance were performed using IBM SPSS statistics 20.0 for Windows. Post hoc testing (p < 0.05) of the multiple comparisons was performed by the Scheffé or Dunnet tests.

3. Results

3.1. Vesicle characterization

PEVs were easily fabricated without the help of organic solvents by direct sonication of P90H, cholesterol and quercetin dispersed in PEG400/PBS [30,31]. Afterwards, the vesicle surface was coated with chitosan, then complexed with nutriose, thus obtaining coated vesicles. In this study, uncoated and coated vesicles were used as carriers for the delivery of quercetin to the colon. The coating of vesicles with a polymeric complex was accompanied by an inversion of the ZP from negative values of uncoated vesicles to positive values, whereas the mean diameter did not vary (~130 nm, p > 0.05; Table 1). The PI value increased upon addition of the polymers. Nevertheless, it remained within the range that indicates a good homogeneity of dispersions (<0.27) [32]. The EE was high and similar for both uncoated and coated vesicles (>60%, p > 0.05).

The vesicle structure was evaluated by cryo-TEM and SAXS. As can be seen in Fig. 1, vesicles were spherical and prevalently multilamellar, and their size ranged between 50 and 200 nm. SAXS profiles (Fig. 2) displayed an intense first-order peak and a small second-order peak, confirming the multilamellar structure of vesicles having a higher number of lamellae in the case of uncoated vesicles, as indicated by more intense and sharper peaks. The number of lamellae decreased in the coated vesicles due to the presence of polymers on the surface, while the average size was similar to that of uncoated vesicles. As shown in Table 2, the lamellar spacing (d) underwent a small increase in the presence of quercetin. The coating did not affect this parameter but, similarly to quercetin, led to a reduction in the bilayer thickness (dL). This reduction in quercetin-loaded vesicles was correlated to a decrease in the distance of the head group from the center of the bilayer (zH), while the polar head group (σH) remained constant in all of the formulations. Both quercetin and coating layers caused a marked increase in the water layers (dw).

3.2. In vitro release studies

At pH 2.0 (Fig. 3A), the amount of quercetin released from uncoated vesicles was greater than that obtained from coated vesicles at each time point. Quercetin loaded in uncoated vesicles was released early (after 10 min), and the amount increased linearly up

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>PI</th>
<th>ZP (mV)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty uncoated PEVs</td>
<td>120 ± 4</td>
<td>0.19</td>
<td>−16 ± 2</td>
<td></td>
</tr>
<tr>
<td>QUE uncoated PEVs</td>
<td>130 ± 8</td>
<td>0.21</td>
<td>−11 ± 1</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>Empty coated PEVs</td>
<td>135 ± 7</td>
<td>0.27</td>
<td>+19 ± 3</td>
<td></td>
</tr>
<tr>
<td>QUE coated PEVs</td>
<td>132 ± 6</td>
<td>0.26</td>
<td>+16 ± 2</td>
<td>61 ± 5</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SD (n = 6, at 25 °C).

[Fig. 1. Cryo-TEM micrographs of quercetin-loaded uncoated vesicles (A) and quercetin-loaded coated vesicles (B). Bars represent 100 nm.]

[Fig. 2. SAXS profiles of empty and QUE-loaded uncoated and coated vesicles.]

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>d (Å)</th>
<th>dH (Å)</th>
<th>ZH (Å)</th>
<th>σH (Å)</th>
<th>dw (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty uncoated PEVs</td>
<td>70.6 ± 0.2</td>
<td>68.2 ± 0.6</td>
<td>22.5 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>QUE uncoated PEVs</td>
<td>72.6 ± 0.2</td>
<td>61.0 ± 3.0</td>
<td>19.5 ± 0.5</td>
<td>5.4 ± 0.6</td>
<td>11.6 ± 3.2</td>
</tr>
<tr>
<td>Empty coated PEVs</td>
<td>70.1 ± 0.1</td>
<td>63.0 ± 0.8</td>
<td>21.5 ± 0.3</td>
<td>5.0 ± 0.1</td>
<td>7.1 ± 0.9</td>
</tr>
<tr>
<td>QUE coated PEVs</td>
<td>72.1 ± 0.3</td>
<td>58.8 ± 0.6</td>
<td>19.0 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>13.3 ± 0.9</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SD (n = 3).

3.3. Vesicle behavior in gastrointestinal fluids

Two major variables of the gastrointestinal fluids are pH and ionic strength. They vary greatly along the gastrointestinal tract between fasting and fed conditions, and this can affect the rate at which a drug is released from the dosage form [33]. In man, in both fasted and fed states and under various physiological pH conditions, the ionic strength of the gastrointestinal fluids covers the range of 0–0.4 M [33]. Sodium chloride is in the midrange of the lyotropic series and has the ability to salt out polymers, hence it is often used as the agent for ionic regulation of dissolution media [33,34]. Since oral formulations are subjected to different pH and ionic strength levels along the gastrointestinal tract, it is crucial to evaluate the properties of the prepared vesicular systems under conditions mimicking stomach-to-colon transit.

First, the size of both quercetin-loaded uncoated and coated PEVs was measured as a function of the pH of the release medium (i.e. pH 2.0, 5.5, 7.0; 25 °C). The results, listed in Table 3, show that at first (t0) there was no statistical difference with respect to the same samples analyzed in the medium used to prepare the vesicles (PEG400/PBS; Table 1). On the other hand, as expected, an increase in size was observed as a function of experimental time: a 10% increase was found after 2 h at pH 2 or 3 h at pH 5.5, and a 40% increase after 8 h at pH 7.0. When the samples were analyzed at 37 °C (t0), a variation in the vesicle size was found to depend on the pH of the medium: as the pH increased, the size increased. A further increase was observed after 8 h at pH 7.0, especially in uncoated vesicles.

The PI increased slightly as a function of time and temperature. The ZP of both uncoated and coated vesicles was always positive at pH 2.0, while at pH 5.5 and 7.0 an inversion occurred, unlike the values measured in PEG400/PBS: uncoated vesicles became positive and coated vesicles became negative (compare Tables 1 and 3).

Surprisingly, a quite similar trend was observed for size, PI and ZP of PEVs analyzed in the different media (pH 2.0, 5.5 and 7.0) containing 0.3 M sodium chloride (Table 4). Hence, despite the high ionic strength, the prepared vesicles were able to preserve their structure, even after 2, 3 or 8 h. In particular, it is noteworthy that 8 h incubation at 37 °C and pH 7.0 caused coated PEVs to undergo a ~10% increase in size, while the increase was ~50% for uncoated vesicles.

Overall, the changes in vesicle size as a function of pH, temperature and ionic strength were always moderate and the size never exceeded ~200 nm, denoting a good resistance of PEVs to the gastrointestinal environment. Likewise, the ionic strength of the media did not affect either the rate or the extent of quercetin release (p > 0.05), indicating that pH and time were the major factors influencing drug release from PEVs.

3.4. In vivo quercetin and phospholipid biodistribution in the gastrointestinal tract

To evaluate the gastrointestinal transit of the formulations and to establish their ability to actually facilitate quercetin colon delivery in vivo, vesicles were labeled with a fluorescent phospholipid.
Using quercetin dispersion, at 1–2 h, the drug accumulation was reference to evaluate the efficacy of the vesicles as drug carriers.

<table>
<thead>
<tr>
<th>pH</th>
<th>Sample</th>
<th>Size (nm)</th>
<th>PI</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25 °C</td>
<td>37 °C</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>QUE uncoated PEVs</td>
<td>123 ± 3</td>
<td>115 ± 3</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>133 ± 3</td>
<td>123 ± 2</td>
<td>0.24</td>
</tr>
<tr>
<td>5.5</td>
<td>QUE uncoated PEVs</td>
<td>130 ± 3</td>
<td>148 ± 1</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>145 ± 8</td>
<td>167 ± 3</td>
<td>0.35</td>
</tr>
<tr>
<td>7.0</td>
<td>QUE uncoated PEVs</td>
<td>122 ± 6</td>
<td>162 ± 2</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>197 ± 4</td>
<td>177 ± 5</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SD (n = 6).

(Rho-PE). Quercetin-loaded, Rho-PE-labeled vesicles were administered to healthy rats and their distribution along the alimentary canal was monitored as a function of time (5 h) using the In Vivo FX PRO Imaging System, which allowed the precise anatomical localization of biomarkers. Rho-PE fluorescence was detected only in the gastrointestinal tract, with no fluorescence being seen in other organs or tissues of rats (data not shown), indicating the non-systemic distribution of vesicles and confirming their local delivery. To better visualize Rho-PE distribution in the different tracts, intestine was removed and placed on a board, avoiding the overlapping of segments. Treatment with uncoated vesicles gave similar Rho-PE fluorescence distributions at 1 and 2 h, which reached the maximum intensity along the small intestine at 2 h (Fig. 4). At 3 and 4 h, fluorescence was intense in the ileum and cecum, but faint in the colon. Using coated vesicles, no fluorescence was detected at 1 h and fluorescence was weak and limited to the proximal intestine at 2 h, while at 3 h the intensity increased and was more evident in the small intestine and slightly less in the large intestine, including the cecum and colon. At 4 h, the fluorescence was intense and limited to the ileum, cecum and colon. Unfortunately, quercetin fluorescence overlapped with the autofluorescence of the intestinal content, thus its distribution was quantified by HPLC after extraction from the excised and homogenized tissues (Fig. 5). Quercetin dispersion was used as a reference to evaluate the efficacy of the vesicles as drug carriers. Using quercetin dispersion, at 1–2 h, the drug accumulation was 35–50% in the stomach and 20–35% in the duodenum, and progressively decreased to ~15% or less in the other intestine segments. At 3 h, it was less than 10% in each tract of the gastrointestinal system (Fig. 5A). After treatment with quercetin-loaded uncoated vesicles, ~70% of the drug had accumulated in the stomach at 1 h, with less than 10% in the other tracts; at 2 h, it had decreased in stomach and increased in duodenum (30%), and was ~20% in the jejunum, ileum and colon; at 3 h, it was partially eliminated in all of the intestine (<10%; Fig. 5B). Using quercetin-loaded coated vesicles, drug accumulation at 1 h was high (~30%) in the stomach, less in the small intestine (~20%) and almost nil (~2%) in the colon, and progressively decreased in the stomach and increased in the small and large intestines. At 3 h, ~20% of quercetin was found in the jejunum and ileum, with ~30% in the colon (Fig. 5C).

In addition, the accumulation of quercetin in the different parts (i.e. content, surface, mucosa and muscle) of each segment of the gastrointestinal tract was assessed 3 h after the administration of the dispersion, and the uncoated and coated PEVs (Fig. 5). As can be seen, quercetin was uniformly distributed in the segments' layers and content when it was delivered by both the dispersion and uncoated PEVs (Fig. 5D and E, respectively). On the other hand, as reported above, quercetin accumulation was much higher when coated PEVs were used, and it was mostly localized on the luminal surface and in the muscle (Fig. 5F), indicating that a large portion of the drug reached the deep tissue, while another portion was present on the surface and thus still available for absorption.

Table 3

Average size, PI and ZP of QUE-loaded uncoated and coated PEVs under conditions mimicking stomach-to-colon transit: after 2 h at pH 2.0, 3 h at pH 5.5, and 8 h at pH 7.0, at 37 vs. 25 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Sample</th>
<th>Size (nm)</th>
<th>PI</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25 °C</td>
<td>37 °C</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>QUE uncoated PEVs</td>
<td>128 ± 2</td>
<td>119 ± 3</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>147 ± 7</td>
<td>176 ± 5</td>
<td>0.31</td>
</tr>
<tr>
<td>5.5</td>
<td>QUE uncoated PEVs</td>
<td>111 ± 2</td>
<td>170 ± 2</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>166 ± 9</td>
<td>175 ± 2</td>
<td>0.23</td>
</tr>
<tr>
<td>7.0</td>
<td>QUE uncoated PEVs</td>
<td>114 ± 3</td>
<td>119 ± 4</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>202 ± 8</td>
<td>195 ± 7</td>
<td>0.37</td>
</tr>
</tbody>
</table>
| Values are reported as mean ± SD (n = 6).

Table 4

Variation of average size, PI and ZP of QUE-loaded uncoated and coated PEVs as a function of pH (2.0, 5.5 and 7.0), time (0, 2, 3 and 8 h) and ionic strength (0.3 M sodium chloride) mimicking stomach-to-colon transit, at 37 vs. 25 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Sample</th>
<th>Size (nm)</th>
<th>PI</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25 °C</td>
<td>37 °C</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>QUE uncoated PEVs</td>
<td>114 ± 3</td>
<td>119 ± 4</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>t2h</td>
<td>202 ± 8</td>
<td>195 ± 7</td>
<td>0.37</td>
</tr>
</tbody>
</table>
| Values are reported as mean ± SD (n = 6).
It should be noted that, 4–5 h after the administration of the dispersion, uncoated and coated PEVs, quercetin was no longer detected in the gastrointestinal system.

At the end of the experiment, quercetin was not detected in the liver or kidneys, either. H&E micrographs of the colon and kidney show that tissues were completely preserved, unlike the untreated specimens, confirming the non-toxicity of quercetin and PEVs (Fig. 6).

3.5. In vivo treatment of induced-colitis in rats

Inflammatory bowel disease was induced by intracolonic administration of TNBS. Afterwards, rats were treated for 3 days (on days 3, 4 and 5) with PEG400/PBS or the tested samples. At day 9, the colon was removed and analyzed.

Mucosal and tissue damage was macroscopically evaluated by visual inspection of the internal and external walls of the colon.
Healthy rat colon was intact and pink-colored, with a constant width for the entire length. Colon from TNBS rats treated with PEG400/PBS showed on the external surface the formation of hemorrhagic spots and ulcers, and severe macroscopic edematous inflammation that caused strong enlargements and deformations, with a consequent reduction in length. Internally, colon exhibited congestion and edema of the mucosa, damage of epithelial cells resulting in the appearance of brown-yellowish layers, like burns and necrotic tissues (Fig. 7, right panel). Colon from rats treated with quercetin dispersion, empty coated vesicles and quercetin-loaded uncoated vesicles appeared to be as badly damaged as in TNBS rats treated with PEG400/PBS, while a strong amelioration was evident in those animals treated with quercetin-loaded coated vesicles. The colons of these rats were very similar to those of healthy rats in diameter, length and color, even though small necrotic areas were present (Fig. 7, right panel).

The efficacy of the formulation on the inhibition of TNBS-colitis was quantified by the CAS and the index of colonic tissue edema, expressed as colon/body weight ratio (Table 5). The basal value of the colon/body weight ratio of healthy rats was 5 mg g⁻¹, while in colitic animals treated with PEG400/PBS it was 8 mg g⁻¹ and the CAS was ≈7. After quercetin-loaded coated vesicle administration, rats started to gain weight and have normal stools without bleeding. Indeed, both the CAS (~2.2) and the colon/body weight ratio (~4.3 mg g⁻¹) decreased (p < 0.05) with respect to the values of rats treated with PEG400/PBS. In contrast, after the administration of quercetin dispersion, empty vesicles and quercetin-loaded uncoated vesicles, rats continued to lose weight and had diarrhea throughout the period of the experiment. The clinical activity score and the colon/body weight ratio values obtained from quercetin-treated animals were statistically equal (p > 0.05) to those rats treated with PEG400/PBS, except for animals treated with quercetin-coated vesicles, which gave a lower colon/body weight ratio (p < 0.05).

The colonic damage induced by TNBS was associated with an increase in MPO activity in comparison with non-colitic rats, indicative of a massive neutrophil infiltration that took place in the inflamed tissue (Table 5). The MPO activity increased significantly in rats treated with PEG400/PBS (myeloperoxidase inhibition (MPO I) =0%), while it was low in those rats treated with coated vesicles (MPO I = 75%, p > 0.05), presumably due to the prebiotic effect of nutriose. Further, the administration of quercetin-loaded coated vesicles resulted in a marked reduction of MPO activity, reaching values similar to those of healthy rats, with 90% (p < 0.01) of MPO inhibition.
4. Discussion

Inflammation, especially chronic inflammation, is associated with an overproduction of free radical species and plays a fundamental role in several pathologies, such as cancer, cardiovascular diseases, obesity, inflammatory bowel disease and skin wounds. Natural antioxidant and anti-inflammatory agents, such as quercetin, may be crucial in the treatment of such diseases. Nanoparticulate delivery systems represent an innovative tool to protect quercetin and improve its low bioavailability. Moreover, such systems are the most convenient way to improve quercetin’s colon efficacy. To this purpose, we designed and fabricated vesicles using hydrogenated phosphatidylcholine which exist at body temperature as bilayer vesicles in a gel state [35]. In this state, the vesicle bilayer is more rigid and more stable, and has a superior ability to retain the drug for a long period of time. Further, vesicles were coated with the biocompatible and mucoadhesive polymers chitosan and nutriose, which form a complex that is less soluble than the single polymers in the gastrointestinal fluids (pH 2–7), but is susceptible to enzymatic degradation in the colon. It was

Fig. 7. Representative macroscopic view of external (left) and internal (right) colon tissue (8 cm in length) of rats, healthy and after the induction of colitis with TNBS and treated with different formulations.
demonstrated that both uncoated and coated vesicles were able to maintain their structure and size under different media conditions (pH, ionic strength and temperature). However, uncoated vesicles showed a reduced ability to retain quercetin under the tested conditions compared to coated vesicles, indicating that the polymer complex coating aided the vesicles to retain the drug, thus slowing down its release. Moreover, the polymer coating is expected to undergo selective degradation by the colon microflora, favoring its beneficial proliferation. In this regard, one of the major issues of the local treatment of inflammatory bowel disease is diarrhea, which increases the elimination rate of the formulated drug. Nutriose, thanks to its prebiotic activity, can moderate the rapid elimination of a formulation from the intestinal lumen. Thus, the combination of a controlled-release nanocarrier system with an enzyme-sensitive and prebiotic polymer complex (chitosan/nutriose) represents a promising strategy for quercetin delivery to the colon in the therapy of inflammatory bowel disease. Quercetin-loaded uncoated and coated vesicles were used in this work, and quercetin dispersion served as a reference.

A structural point of view, an important difference between the quercetin-loaded uncoated and coated vesicles was their ZP, which was negative for uncoated vesicles (~−11 mV) and became positive (~+22 mV, data not reported) after chitosan coating, confirming that the positively charged polymer was arranged on the vesicle surface. Afterwards, chitosan-positive charges were only partially neutralized by nutriose (~+16 mV), indicating the electrostatic complexation of the polymer.

SAXS results confirmed that the polymer coating did not alter the closed, bilayered structure of PEVs, even though some modifications were observed. The vesicles were still multilamellar and their size did not increase with coating, because the number of lamellae decreased, leading to an enlargement of the water spaces between the bilayers. A similar behavior was observed after the addition of quercetin, which did not modify the mean diameter of vesicles but caused a small decrease in vesicle lamellarity, a contraction of the lipid bilayer width (2w) and a correlated enlargement of the water layers (dw0). These arrangements indicate an interaction between quercetin and the vesicular bilayers, and the distribution of the polymer complex on the bilayer surface.

In vitro drug release studies at different pH values and times can be predictive of the ability of the formulation to retain and protect the drug in the gastrointestinal tract, releasing it only at a selected time and site. A massive release of drug at acidic pH suggests the need to use a gastro-resistant device (such as gastro-resistant capsules) before administering the formulation in vivo. A rapid release of drug at pH 5–6 indicates a possible use of the formulation for small intestine targeting. A delayed release (>3 h) at pH ~7 underlines a suitable distal intestine/colon targeting. In vitro quercetin release profiles from coated vesicles disclosed a moderate release (~20%) in an acidic environment (pH 2.0) and a delayed release (after 4 h) at neutral pH. The delayed and acidic pH-resistant release of quercetin was partially controlled by P90H vesicles and improved by the polymer complex coating.

Indeed, the P90H bilayer at biological temperature (~37 °C) is in a rigid and poorly permeable gel state that is supposed to preserve vesicle structure, protecting the loaded drug from an acidic environment and providing long drug retention. The initial amount of quercetin released at 2–4 h can reasonably be ascribed to the non-entrapped drug (~35%) and to the entrapped drug located close to the vesicle surface. Clearly, in vivo there exist other important factors that can facilitate drug release, in particular, specific enzymes that digest different specific chemical substances. As is widely known, chitosan and nutriose can be hydrolysed in single monomers only by colon enzymes. Moreover, nutriose hydrolyzation products are used as nutrients by endogenous microflora, which proliferate and facilitate the re-establishment of physiological conditions in damaged tissue [20–22].

The in vivo transit of the drug and the formulation in the gastrointestinal tract was estimated in healthy rats by following the distribution of both quercetin and Rho-PE fluorescence. An imaging study of Rho-PE was carried out immediately after animal sacrifice without washing the removed intestine, while, prior to quercetin quantification, excised intestine was washed with saline to remove the intestinal content (which interfered with drug quantification). The distribution of phospholipids (Rho-PE) did not discriminate between dispersed, adhered or absorbed material, whereas quercetin distribution (after intestine washing) referred only to drug adhered to or absorbed by the intestinal walls. The intestinal transit of food in rats is faster than in humans, and usually lasts 3–4 h. Indeed, in our studies, quercetin was detected up to 3 h and Rho-PE fluorescence up to 4 h, probably because phospholipids interact electrostatically with colonic mucosa, and also after liposome breaking [16]. Neither quercetin nor Rho-PE fluorescence was detected at 5 h. After quercetin-loaded coated vesicle administration, the intestinal distribution of the drug confirmed its selectivity to the colon (30% at 2–3 h), whereas using dispersion or uncoated vesicles the accumulation of the drug was higher in the stomach (~60%) and lower in the small intestine at 1–2 h, with <10% in colon at 3 h. Rho-PE fluorescence was evident in the cecum and colon at 3–4 h (more intensely at 4 h) only after administration of coated vesicles. We can hypothesize that, using this formulation, after 2 h some of the intact coated vesicles stayed in the duodenum and jejunum (where both quercetin and Rho-PE were detected), while free quercetin arrived in and was absorbed by the colon. After 3 h, vesicles were distributed in the small and distal intestine, and were less evident in the cecum and colon because the chitosan/nutriose complex was partially hydrolyzed and vesicles adhered to the colonic mucosa, facilitating quercetin uptake by colonicocytes. After 4 h, the quercetin was completely degraded or metabolized, while Rho-PE remained longer in the intestinal mucosa but was eliminated after 5 h.

Inflammatory bowel disease is an immunological disorder that is caused by the deregulated reaction of endogenous cells to indigenous flora, which causes a chronic inflammatory state of the distal intestine [11]. An in vivo animal model of inflammatory bowel disease cannot completely mimic such conditions, due to the

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**Table 5**

CAS, index of colonic tissue edema expressed as colon/body weight ratio, MPO and MPO I of animals with TNBS-induced colitis.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>CAS (adimensional)</th>
<th>Colon/body (mg g⁻¹)</th>
<th>MPO (ng mg⁻¹)</th>
<th>MPO I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy rats</td>
<td>PEG400/PBS</td>
<td>6.90 ± 1.74</td>
<td>5.10 ± 0.84</td>
<td>137 ± 29</td>
<td>100</td>
</tr>
<tr>
<td>TNBS-rats</td>
<td>Empty coated PEVs</td>
<td>2.74 ± 0.48</td>
<td>8.08 ± 1.13</td>
<td>800 ± 94</td>
<td>0</td>
</tr>
<tr>
<td>TNBS-rats</td>
<td>QUE dispersion</td>
<td>5.58 ± 1.53</td>
<td>6.19 ± 1.83</td>
<td>294 ± 69</td>
<td>75</td>
</tr>
<tr>
<td>TNBS-rats</td>
<td>QUE uncoated PEVs</td>
<td>9.77 ± 2.80</td>
<td>5.92 ± 1.87</td>
<td>545 ± 62</td>
<td>36</td>
</tr>
<tr>
<td>TNBS-rats</td>
<td>QUE coated PEVs</td>
<td>8.64 ± 1.30</td>
<td>2.25 ± 0.42</td>
<td>562 ± 88</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.32 ± 1.26</td>
<td></td>
<td>119 ± 68</td>
<td>90</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of at least six animals. *p < 0.05, **p < 0.01 with respect to TNBS-only treated rats.
complexity of the etiologic factors behind it. Chemically induced models of acute colitis, such as TNBS-induced colitis, reproduce the damage to the intestinal epithelium, the consequent immune response, the decrease in endogenous flora, the alteration of PH and diarrhea, but cannot reproduce the native cause of the pathology, which is the dysfunctionality of the immune system. We used this colitis model due to its good reproducibility and simplicity, and evaluated the macroscopic effects of nanoentrapped quercetin on the protection of colon integrity. The inefficacy of non-entrapped quercetin or uncoated vesicles loaded with quercetin was confirmed by the severe damage to the colon. According to in vivo biodistribution, free or inappropriately formulated quercetin was not able to reach the colon and protect it [36]. In contrast, a suitable formulation, such as chitosan/nutriose-coated vesicles, improved the quercetin accumulation in the colon, allowing it to exert its local antioxidant and anti-inflammatory action there, thereby reducing the colon damage and permitting an important amelioration of the TNBS-colitis symptoms.

TNBS-induced colitis involves the infiltration of colonic mucosa by neutrophils and macrophages, along with an increased production of inflammatory mediators. Inflammation development was quantified as the loss of colon and body weight, CAS and myeloperoxidase activity. It was proved that quercetin, when delivered by chitosan/nutriose-coated vesicles, reached the colon, where it exerted a protective effect against oxidative stress.

5. Conclusions

The results showed that chitosan/nutriose coated vesicles have a promising ability to protect quercetin during its transit through the upper gastrointestinal tract and allow its release in the colonic region. This delivery system remained in the colon as a small and multiple dosage form (liposomes) that spread over the mucosa, facilitating and improving the passage of the drug towards the sub-mucosa and its uptake by the colonocytes. In addition, prebiotic nutriose exerted beneficial effects on TNBS-damaged colon mucosa, favoring the restoration of physiological conditions. Hence, quercetin-loaded coated vesicles represent a suitable, adjunct nutritional tool for chronic intestinal inflammation therapy, and even as a preventive system.

Hence, quercetin-loaded coated vesicles represent a suitable, nutritional tool for chronic intestinal inflammation therapy, and even as a preventive system.

Acknowledgements and Disclosures

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Appendix A. Figures with essential colour discrimination

Certain figure in this article, particularly Figs. 6 and 7 is difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.11.017.

References


