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Mucoadhesive curcumin nanospheres: Biological activity, adhesion to stomach mucosa and release of curcumin into the circulation

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ABSTRACT

Although mucoadhesive drug carriers for the gastro-intestinal tract (GIT) have been reported, the mucoadhesive property and drug release characteristics have never been evaluated separately, whilst the adherence of the carriers to the surface of GIT has not been directly visualized. Here, a monopolymeric carrier made from ethylcellulose (EC) and a dipolymeric carrier made from a blend of methylcellulose (MC) and EC (ECMC) were easily fabricated through a self-assembling process and yielded the highest reported curcumin loading of ~48–49%. Both curcumin loaded ECMC (C-ECMC) and curcumin loaded EC (C-EC) particles showed an *in vitro* free radical scavenging activity and a dose-dependent *in vitro* cytotoxic effect towards MCF-7 human breast adenocarcinoma and HepG2 hepatoblastoma cells in tissue culture. The *in vivo* evaluation of their adherence to stomach mucosa and their ability to release curcumin into the circulation were carried out through quantification of curcumin levels in the stomach tissue and in blood of mice orally administered with the two spheres. Direct evidence of the adherence of the C-EC and C-ECMC particles along the mucosal epithelia of the stomach is also presented for the first time through SEM images. The mucoadhesive property of self-assembled C-EC nanoparticles is discussed.

1. Introduction

Over the last decade, the imparting of mucoadhesion to drug delivery through the use of nanocarriers has been an important mechanism in designing drug delivery systems to improve the buccal, nasal, oral, ocular and vaginal administration of drugs to the mucosal epithelia of the mouth, nose and lungs, upper gastro-intestinal tract (GIT), eyes and vagina [1–5]. Recently, the colon, *via* the rectum, has been reported as a mucosal surface that may serve as a potential site for the non-systemic delivery of drugs through nanocarriers [6]. Along with reports of new coating surfaces, various mucoadhesive polymers have been proposed [7–10] and a greater understanding of the nature of mucus and the mucosal tissue of various organs has been pursued [11–14]. Although the oral delivery of drugs remains the most favored route of administration, only a limited number of mucoadhesive nanocarriers for the GIT have been shown to significantly improve the bioavailability of drugs *in vivo* [15–18]. In addition, the slight gains in drug uptake have been assumed to be due to mucoadhesion whereas, in fact, the actual adherence of the nanocarriers at the surface of the GIT without any chemical modification (for observation purposes) has never been directly visualized, despite the fact that this is an important concept that requires confirmation and optimization for each delivery vehicle.

Curcumin, [1,7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione], a major yellow pigment obtained from the rhizomes of turmeric (*Curcuma longa* L.), is commonly used as a spice and food-coloring agent, and has recently been mooted as having potential uses in medicine, especially as an anti-cancer drug and other health related aspects. Because of its numerous pharmaceutical effects and its inherent nontoxicity, curcumin has been the focus of investigations into the potential treatment of various human disorders. For example, clinical trials of curcumin are currently being conducted in patients with pancreatic cancer, multiple myeloma, rheumatoid arthritis, cystic fibrosis, inflammatory bowel disease and psoriasis, amongst other disorders [19,20]. However, this natural pigment molecule not only degrades easily [21,22], but it also shows a low bioavailability when administered orally [23,24]. *In vivo* studies have shown that the compound possesses poor absorption, and once absorbed has a rapid

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metabolism [25] and fast elimination [24,26], which all lead to a poor bioavailability. Thus, curcumin is an ideal candidate drug for encapsulating into mucoadhesive carriers that can offer improved oral bioavailability and sustainability.

This paper reports on the development of two high loading, highly efficient curcumin nanoencapsulation systems using the biocompatible, safe and inexpensive polymers ethyl cellulose (EC) and methylcellulose (MC). The *in vitro* cytotoxic activity to breast cancer and liver cancer cell lines in tissue culture, and the free radical scavenging activity of the encapsulated curcumin were evaluated. Both the mucoadherence and the ability of the adhered spheres to release curcumin into the blood circulation were investigated through quantification of the curcumin levels in the stomach tissue and blood of the mice after oral administration with the curcumin-loaded EC (C-EC) and ECMC (C-ECMC) spheres. Attachment of the nanospheres on the surface of the GIT was also captured through scanning electron microscopy (SEM). In addition, the mucoadhesive property of self-assembled EC spheres was discussed.

2. Materials and methods

2.1. Materials

EC (MW 170000, ethoxy content 48%) and MC (Mn 40,000 with a degree of methoxy substitution of 1.60–1.90) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Curcumin (>98% (w/w) purity) was purchased from ACROS Organics (Geel, Belgium). All other chemicals were reagent grade and were used without additional purification.

2.2. Nanoencapsulation

Nanoencapsulation of curcumin using EC was carried out by solvent displacement through dialysis. For encapsulation at a curcumin: EC ratio of 1:1 (w/w), EC (0.30 g) and curcumin (0.30 g) were co-dissolved in ethanol to a final volume of 100 ml, the solution was then transferred into the dialysis tube (CelluSep T4, MWCO 12,000–14,000, 45 mm flat width, 6.42 ml cm^{-1} volume capacity, Membrane Filtration Products, USA) and dialyzed against distilled water (six changes of 1000 ml water each). The obtained aqueous suspension of curcumin-encapsulated nanoparticles was then freeze-dried and subsequentially subjected to SEM, transmission electron microscopy (TEM) and differential scanning calorimetry (DSC) based analyses. The amounts of curcumin incorporated into the polymeric particles and in the dialysate water were determined using a UV 2500 UV–vis spectrophotometer (Shimadzu Corporation, Japan) at 425 nm, with the aid of a calibration curve. Dialysate-water was subjected to UV/VIS absorption analysis directly, while the freeze-dried nanospheres were first dissolved in ethanol prior to the analysis. The encapsulation efficiency (EE) and loading capacity were calculated as follows:

$$\mathrm{EE}(\%) = \frac{\text{weight of encapsulated curcumin}}{\text{weight of curcumin used}} \times 100$$

$$\text{Loading } (\%) = \frac{\text{weight of encapsulated curcumin}}{\text{weight of curcumin loaded nanopheres}} \times 100$$

Encapsulation of curcumin into dipolymeric ECMC spheres (EC:MC of 1:1 by weight) was carried out by co-dissolving EC (0.15 g) and curcumin (0.30 g) in 75 ml ethanol and separately dissolving MC (0.15 g) in 25 ml water. The two solutions were mixed together and then transferred into the dialysis tube and dialyzed against water as described above. Encapsulation at other EC:MC (w/w) ratios was also carried out similarly but with adjusted EC to MC weight ratios.

DSC thermograms of the C-EC and C-ECMC preparations were acquired at 0–300 °C under nitrogen with a scanning rate of 20 °C min^{-1} using a DSC 204 (Netzsch Group, Germany). SEM based micrographs of each samples were obtained using a JSM-6400 SEM (Jeol, Japan) in which gold coating was performed under a vacuum at 15 kV for 90 s (IB-3 ion coater, Eiko, Japan) and visualization was carried out at an accelerating voltage of 15 kV. TEM images of the spheres were obtained through a JEM-2100 TEM (JEOL, Japan) with an accelerating voltage of 100–120 kV in conjunction with selected area electron diffraction (SAED). The hydrated particle size and zeta potential of particles in water at pH 5.5 or 1.2 (adjusted with HCl) were acquired with a Zetasizer Nano series model (Malvern Instruments, Worcestershire, UK) equipped with a He–Ne laser beam at 632.8 nm (scattering angle of 173°), using <0.1 mg ml^{-1} aqueous nanoparticle suspension in water.

2.3. DPPH radical scavenging assay

DPPH radical scavenging activity was measured as previously described [27]. Briefly, 195 μl of 100 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH, Fluka-Chemika, Bucns, Switzerland) ethanolic solution and 5 μl of each serial dilution of each test compound were mixed in a 96-well plate and the absorbance measured at 515 nm after incubation in the dark for 30 min at room temperature. Residual DPPH free radicals were determined from the absorbance. Ascorbic acid (1 mM) and solvent were used as the 100% and 0% radical scavenging controls, respectively. Samples included C-EC (aqueous suspension), C-ECMC (aqueous suspension), free curcumin (DMSO solution), free curcumin (10% (v/v) aqueous ethanol solution), empty EC particles (aqueous suspension) and empty ECMC particles (aqueous suspension). The curcumin 10% (v/v) aqueous ethanol solution was prepared by dissolving curcumin in absolute ethanol and then diluting to the appropriate concentration with water but with all the final solutions containing the same 10% (v/v) ethanol concentration. Assays were performed in triplicate wells, and the data are expressed as the mean % of the residual radicals.

2.4. In vitro antitumor assay

2.4.1. Cell culture

The MCF-7 Human breast adenocarcinoma cell line and the HepG2 hepatoblastoma cell line were obtained from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL, Life Technologies Inc., NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, UT, USA), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 125 ng ml^{-1} amphotericin B (all antibiotics were from Gibco, Grand Island, NY, USA) in humidified atmosphere of 5% (v/v) CO_2 at 37 °C.

2.4.2. Cell viability assay

Cell viability was determined by the MTT method as previously described [27]. Briefly, cell suspensions in culture medium were seeded at 1×10^4 cells in 96-well plates (100 μl/well), and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. After 24 h, additional medium (100 μl) containing the test sample was added to each well, followed by further incubation for 24 h, 48 h and 72 h. Then, the culture fluid in each well was replaced with fresh culture media containing 0.5 mg ml^{-1} of (3-[4, 5-dimeylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (MTT, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and incubated for 2 h at 37 °C. Finally, the medium was removed and DMSO was added to the wells (100 μl/well), and mixed thoroughly to ensure all cells were lysed and the released crystals fully dissolved before the absorbance was measured at 550 and 650 nm in a micro plate reader. The difference between the absorbance at 550 and 650 nm was used to evaluate the relative number of viable cells. Assays were performed in triplicate wells and data are expressed as the percent viability compared with control.

2.5. In vitro release of curcumin encapsulated nanoparticles

Release of curcumin from C-EC and C-ECMC particles was measured at 37 ± 0.1 °C using the conditions that simulated the stomach and intestinal environments. Five mg of freeze-dried C-EC or C-ECMC were placed into 100 ml of release medium (simulated gastric fluid (SGF; pH 1.2)) or simulated intestinal fluid (SIF; pH 6.8), with stirring at 100 rpm [28]. After 24 h, each suspension was centrifugally filtered through a membrane (MW cutoff of 100,000) and the obtained liquid was subjected to quantification of curcumin using UV/VIS spectroscopy at 425 nm with the aid of a calibration curve constructed from a series of free curcumin solutions prepared in the same release medium. Also, the filtrate was soaked in ethyl acetate to extract out curcumin and the ethyl acetate layer was also subjected to curcumin quantification by UV/VIS spectrophotometry. The percentage of curcumin released was calculated with the following equation:

$$\text{Release } (\%) = \frac{\text{Released curcumin}}{\text{Total curcumin used}} \times 100$$

2.6. In vivo oral bioavailability of curcumin loaded nanoparticles in mice

2.6.1. Nanoencapsulated curcumin samples

Unencapsulated (or free) curcumin, and the C-EC and C-ECMC aqueous suspensions, with a final curcumin concentration of 0.5% (w/v), were each used for direct oral administration.

2.6.2. In vivo oral administration, blood and tissue collections

In vivo studies were performed in ICR mice, strain ICR/Mlac, with 30 ± 2 g body weight under permission from the animal care and use committee in the Faculty of Veterinary Science, Chulalongkorn University.

One hundred and sixty ICR mice (eight weeks old) of both sexes (National Laboratory Animal Center, NLAC, Nakornpathom, Thailand) were randomly divided into four groups of 40 mice each. The animal groups at 10 mice each were fed with one of distilled water (control group), the unencapsulated curcumin, C-EC or C-ECMC aqueous suspensions at a curcumin dose of 100 mg kg^{-1} body weight by direct oral administration using feeding tube. At 15, 30, 60, 90, 120, 180 and 300 min after administration, the animals in each group were anesthesized by isofurane (Terrell TM, Minrad Inc, PA, USA) inhalation. The blood was collected from the posterior *vena cava* and kept in the heparin anticoagulant. Plasma was separated by centrifuging the collected heparinized blood samples at 4000 rcf for 10 min at 4 °C. The cell free liquid plasma was then subjected to curcumin extraction and quantification. The visceral organs were collected in 2.5% (w/v) aqueous gluteraldehyde, quickly soaked in water before being freeze-dried and subjected to SEM image acquisition.

2.6.3. Extraction of curcumin from tissues

To extract curcumin from the C-EC or C-ECMC spheres that had attached to the stomach tissue and the curcumin in the stomach tissue, the freeze-dried sample (~10–20 mg) was accurately weighed and then soaked in 2.0 ml of ethyl acetate (overnight). The suspension was then vortexed and centrifuged at 8000 × *g* for 10 min to pellet the solid. The liquid was collected, filtered through a nylon membrane filter (0.2 μm pore size) and dried under nitrogen. The solid residue was then solvated in 1.0 ml of methanol and subjected to quantification of the curcumin content by HPLC (see Section 2.6.5 below).

2.6.4. Extraction of curcumin from plasma

Extraction of curcumin from plasma was adopted from previous work [29] with slight modification. Two hundred μl of plasma and 500 μl of ethyl acetate were mixed and vortexed for 60 s, and then sonicated (40 KHz) for another 60 s. The organic layer was filtered, dried and resolvated in 1.0 ml of methanol as above (Section 2.6.3) ready for HPLC quantification of the curcumin levels.

2.6.5. HPLC quantification of curcumin

Quantitative analysis of curcumin in extracts of tissue samples and plasma samples was performed using a Shimadzu HPLC system with a Hypersil ODS-C_{18} column (5 μm, 4.6 mm × 150 mm, Shandon, UK) at room temperature using a 73:27 (v/v) ratio mixture of methanol: 3.6% (v/v) aqueous acetic acid as the mobile phase at a flow rate of 0.8 ml min^{-1} [30]. The eluent was monitored with a UV/VIS detector (SPD-10A, Shimadzu, Japan) at 425 nm. The sample injection volume was 20 μl. Under these conditions the retention time for curcumin was ~2.5–3.0 min. The average amount of curcumin in each sample was determined from triplicate sample injections by integration of the peak area with the aid of a calibration curve, constructed from a series of curcumin standards (0.01, 0.02, 0.04, 0.06, 0.08 and 0.10 ppm curcumin solutions) freshly prepared in methanol.

3. Results and discussion

3.1. Characterization of the C-EC and C-ECMC particles

Two sets of nanoparticles: a single polymeric carrier based on EC and a dipolymeric carrier based on the blend of EC and MC, were prepared by self-assembling of polymers in the presence of curcumin. EE of 91.2 ± 1.7% and 95.4 ± 2.9% at a curcumin loading of 47.7% and 48.8% were obtained for the preparation of C-EC and C-ECMC particles, respectively. SEM images revealed a spherical architecture for both types of nanoparticles (Fig. 1) with estimated average diameters of the dry particles deduced from the SEM images of 281.2 ± 92.9 and 117.1 ± 92.5 nm (mean ± S.D.), for C-EC and C-ECMC, respectively. Dynamic light scattering (DLS) analysis indicated a hydrodynamic diameter of 276.28 ± 8.54 (PDI of 0.19) and 190.15 ± 3.61 (PDI of 0.14) nm for the C-EC and C-ECMC particles, respectively. Comparing between the size of the dry particles and the hydrodynamic size, it was obvious that in water the C-ECMC spheres were seriously swelled while the C-EC particles were not, and this could be explained through the better water solvation of the MC polymer compared to the EC polymer. However, it should be noted here that the size of the dry C-EC particle was a little bigger than their hydrodynamic size, and a large standard deviation (not very well correlated with polydispersity of the hydrodynamic size) was also observed for the dry size. This probably was a result of aggregation during the drying and gold coating process.

The pH value of the suspension was around 5.5 and the zeta potential obtained at this pH value was −30.80 ± 1.23 and −32.45 ± 1.35 mV for C-EC and C-ECMC, respectively, suggesting a likely high stability with minimal aggregation in water medium at this pH. The highly charged surface of the spheres confirmed that during the particle formation by self-assembling process, the polar hydroxyl groups of the cellulose polymer were directed to the spherical surfaces, to have maximum interaction with polar water medium. Since at pH 5.5 the hydroxyl group should not be deprotonated, it is likely that the polarized hydroxyl moieties or the polarized water molecules around these polar moieties are responsible for the high zeta potential value. However, when the pH of the suspension was adjusted to 1.2 by HCl addition, the zeta potential values were only −4.85 ± 1.25 and −1.33 ± 0.90 mV for C-EC and C-ECMC, respectively, indicating spheres with an almost neutral surface charge under very acidic conditions, somewhat akin to the pH of the stomach/SGF.

DSC analysis of the C-EC and C-ECMC particles revealed a curcumin melting temperature of 178 °C for both types of particles, indicating that at the high loading (curcumin:polymer of 1:1 by weight), some of the encapsulated curcumin molecules were probably not in a homogeneous solid solution within the polymer matrix (limited

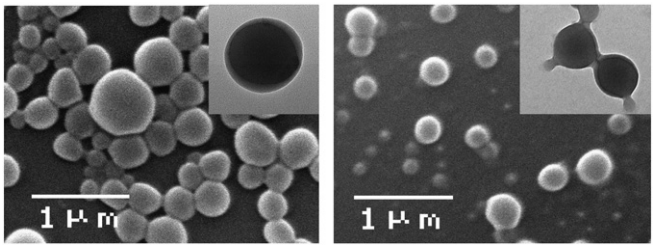

Fig. 1. SEM and TEM (inset) images of C-EC (left) and C-ECMC (right). Micrographs shown are representative of at least ten such fields of view per sample and four independent samples.

availability of hydrophobic moieties from the EC), but rather accumulated at the center of the spheres as curcumin crystalline.

Since EC is insoluble in water, it is likely that upon slow displacement of ethanol by water, EC chains self-assemble into spheres with most of the hydrophobic ethoxyl moieties oriented away from the surrounding water medium, making full contact with the curcumin core, while most of the polar hydroxyl groups of the sugar units orient outwards to have the maximum level of interaction with water molecules. In the case of the ECMC spheres, during the self-assembling process, MC chains would be trapped along with the EC molecules through entanglements, and so the spherical walls would be composed of the ECMC polymer-blend. Some of the MC chains must also be left in the aqueous medium because of their relatively good water solubility, and these chains act as stabilizers for the polymer-blend-spheres. The good water solubility characteristic of MC contributed to the more severe swelling nature of the ECMC spheres comparing to the unswelling nature of the EC spheres.

Evaluation of the curcumin encapsulation at other EC:MC weight ratios revealed that to get particles with a stable morphology in aqueous medium, the amount of MC should not exceed 50% (w/w) of the total polymer mass used. At an MC content of 0–50% (w/w), the curcumin loading capacity and the EE were not significantly affected by the MC:EC ratio.

3.2. Antioxidant activity of C-EC and C-ECMC

The free radical scavenging activity of curcumin is well known. Thus, it was of interest to see if the two encapsulated curcumin preparations still possess such activity. To this end, here, the scavenging of DPPH radicals was monitored, and both the C-EC and the C-ECMC particles showed obvious radical scavenging activity. Their antioxidant potency was then compared to that of free curcumin. Since curcumin possesses limited solubility in water, a 10% (v/v) aqueous ethanol solution was used as the solvent for the free curcumin aqueous solution. In addition, a free curcumin solution in DMSO was also tested. Thus, four curcumin samples, free curcumin in 10% (v/v) aqueous ethanol, free curcumin in DMSO, C-EC and C-ECMC, were compared (Fig. 2). Kolmorgov–Smirnof test confirmed a similar normal distribution for all the four groups. Therefore, a paired *T*-test was used to compare them. The result indicated that the radical scavenging activity of the aqueous suspension of C-ECMC was in the same range to that of the free curcumin solution in DMSO ($t = 1.368$, $df = 9$, and Sig. $= 0.102$) and was significantly higher than that of the free curcumin in water ($t = 3.029$, $df = 9$, and Sig. $= 0.007$). Moreover, C-EC was significantly less superior in scavenging free radical than C-ECMC ($t = 3.478$, $df = 9$, and Sig. $= 0.0035$) and free curcumin in water ($t = 2.714$, $df = 9$, and Sig. 0.008). It should be noted here that the EC and ECMC spheres (no curcumin) showed no radical scavenging activity. The result clearly indicated that upon encapsulation into ECMC carriers, free radical scavenging activity of curcumin can be significantly improved even when used in a mostly aqueous media. This implied that, compared to the free curcumin, the curcumin inside the ECMC particles could make a better contact with the DPPH radicals and scavenged them. This increased antioxidant activity, however, was not observed in C-EC. The small size and the swelled nature of C-ECMC, probably directly increased the contact probability between the encapsulated curcumin and the DPPH radicals, resulting in the higher activity of the C-ECMC comparing to C-EC and free curcumin.

3.3. In vitro cytotoxicity to cancer cells

MCF-7 Human breast adenocarcinoma cells and HepG2 hepatoblastoma cells were exposed to free curcumin solution (in DMSO), free curcumin solution in 10% (v/v) aqueous ethanol, aqueous suspension of C-EC and aqueous suspension of C-ECMC, at various curcumin concentrations and various exposure times (Fig. 3).

A one way ANOVA analysis with PostHoc test (Duncan) at the $p<0.01$ significance level of the MCF-7 results indicated that the free curcumin in DMSO was cytotoxic to the cells in a dose-independent fashion ($F = 2.603$, and Sig. $= 0.095$), while the free curcumin in DMSO, and the C-EC and C-ECMC particles, showed a dose-dependent cytotoxic activity. At the longest incubation time (72 h) and the highest concentration (100 μM), C-ECMC was the most cytotoxic compared to the other three groups ($F = 8.289$, and Sig. 0.000). The dose-independent cytotoxicity of free curcumin in the 10% (v/v) aqueous ethanol is not surprising since curcumin possesses very limited solubility in water, and so increasing the curcumin concentration would not actually increase the curcumin availability to the cells. In contrast, in the case of C-EC and C-ECMC or the curcumin solution in DMSO, increasing the curcumin concentration directly led to a greater availability of curcumin to the cells. It is very interesting to see that at similar curcumin concentration, the C-ECMC always outperformed the free curcumin in DMSO.

In the case of the HepG2 cell line, the free curcumin in DMSO and C-ECMC were cytotoxic to the cells in a dose-dependent fashion, while the free curcumin in water and C-EC showed a dose-independent cytotoxic activity. At the highest concentration tested (100 μM) and at the longest incubation time (72 h), free curcumin in DMSO and C-ECMC were significantly more cytotoxic than free curcumin in the 10% (v/v) aqueous ethanol solution and C-EC ($F = 27.353$, and Sig. $=$ 0.000). The result clearly indicated that by encapsulating curcumin into ECMC carriers, the anticancer activity of curcumin in aqueous medium towards HepG2 cells could be increased to the same level as that of curcumin solution in DMSO.

The above results clearly indicated that the type of the carrier significantly affected the *in vitro* cytotoxicity of the curcumin towards these two cancer cell lines in tissue culture. This is likely to be related to the ability to transfer the curcumin into the cells from the particles, which would be related to both the particle size (C-ECMC spheres are smaller than C-EC spheres) and the polymer materials used.

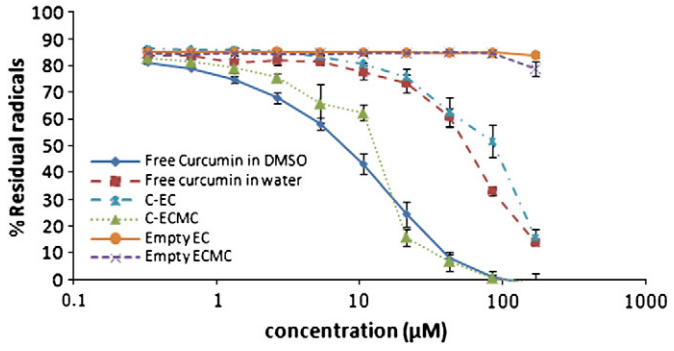

Fig. 2. The 1, 1-Diphenyl-picryldrazyl (DPPH) radical scavenging of free curcumin, C-EC and C-ECMC. Data are expressed as the mean ± S.D. of three independent experiments.

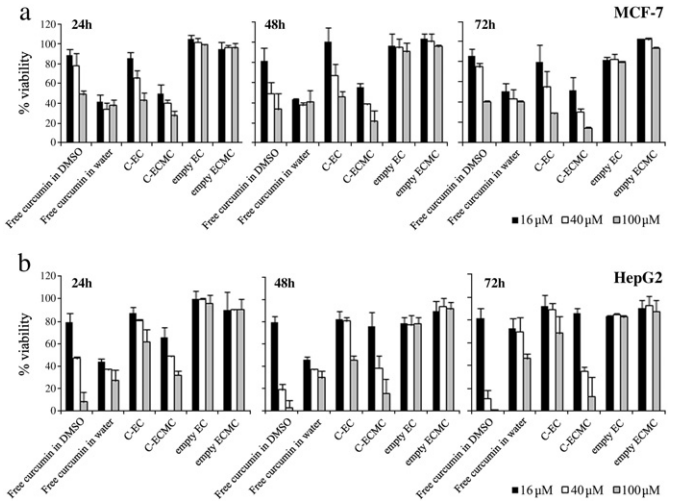

Fig. 3. Effect of free curcumin and the two encapsulated curcumin preparations (C-EC and C-ECMC) on proliferation of MCF-7 (a) and HepG2 (b) cell lines as assessed by the MTT (3-[4,5-dimeylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay after incubating the cells with various concentrations of test compound for 24 h, 48 h and 72 h. Data are expressed as the mean ± S.D. of three independent experiments.

3.4. In vitro release

The release of curcumin from C-EC and C-ECMC nanoparticles in the SGF (pH 1.2) or SIF (pH 6.8) was determined *in vitro*. The results indicated only a minimal release (less than 10%) of the encapsulated curcumin after 24 h for both carriers (Fig. 4). This result agrees well with the fact that curcumin shows only minimal solubility in aqueous media. In fact, the encapsulation of curcumin into EC or ECMC spheres did not alter the structure of curcumin, therefore, it was not surprising to see that the compound still possessed poor solubility in the two aqueous media. Nevertheless, this *in vitro* release result indicated that curcumin was released a little faster from the C-ECMC nanoparticles than from the C-EC ones at pH 1.2.

3.5. Oral bioavailability

The C-EC and the C-ECMC (1:1 (w/w) ratio EC:MC) were compared for their ability to attach to the mucosal layer of the GI tract and their ability to deliver curcumin into the blood circulation.

The *in vivo* pharmacokinetics study indicated that the mice administered with free curcumin showed a significantly lower amount of curcumin in their blood than those administered with C-EC and C-ECMC, respectively (Fig. 5). In addition to the Kruskall–Wallis analysis that indicated that the three groups were significantly different from each other, a Kolmogorov–Smirnov test was performed on all the three pairs, free curcumin versus EC, free curcumin versus ECMC, and EC versus ECMC, at each post feeding time. The results indicated that at 30 min all three groups showed significantly different curcumin levels with C-ECMC as the highest (0.7838 ± 0.2438 μg ml^{-1}) and C-EC as the lowest (0.1822 ± 0.1374 μg ml^{-1}). However, at 60 and 90 min, all three groups showed significantly different curcumin levels in the order of C-EC>C-ECMC>free curcumin. At 180 and 300 min, the free curcumin and the C-ECMC groups showed no significant level of curcumin, while the C-EC group gave 0.0886 ± 0.0454 and 0.024 ± 0.0182 μg ml^{-1} curcumin in the blood, at each respective time point.

Since each mouse of the 160 mice used in the experiment (30 ± 2 g body weight) possessed approximately 2.1 ml blood in its body, the total amount of curcumin available at each time point could be approximated from the blood curcumin concentrations depicted in Fig. 5. Bioavailability of curcumin could then be estimated by integrating amount of the curcumin in the blood during the 0 to 300 min post feeding. The estimated amounts of available curcumin in blood were 33, 279 and 118 μg for free curcumin, C-EC and C-ECMC, respectively. It was very obvious that the EC carrier gave an 8.50-fold increased bioavailability compared to the unencapsulated curcumin while the other carrier, ECMC, gave a 3.6-fold increased curcumin bioavailability. Therefore, it could be concluded that i) at a similar oral dosage, the sustainability of curcumin in the blood was significantly longer for C-EC compared with C-ECMC and free curcumin, and ii) an 8.5- and 3.6-fold increase in curcumin bioavailability could be achieved through the use of EC and ECMC carriers (as C-EC and C-ECMC), respectively.

The blood curcumin concentration (Fig. 5) and bioavailability estimated above implied that C-ECMC nanospheres released curcumin into the blood more quickly than the C-EC counterparts, which agrees well with their smaller size and higher degree of swelling in an aqueous environment. It should be noted here that at 120 min, most of the unattached masses had already moved down to the small intestine, thus allowing an easy observation of the stomach surface. Quantification of curcumin levels in freeze-dried stomach tissue samples collected at 120 min post oral administration indicated 0.067 ± 0.018, 0.031 ± 0.015 and 0.0174 ± 0.010 mg g^{-1} for samples from mice fed with C-EC, C-ECMC and free curcumin, respectively. This not only confirmed the better attachment of C-EC over C-ECMC spheres at the stomach mucosa observed by SEM, but also supported our hypothesis on the delay of curcumin elimination from the stomach by the use of the carriers. Thus, it was concluded that the better bioavailability of C-EC over the C-ECMC was related to the better attachment of the spheres at the gastric mucosal layer.

To test this notion, SEM images of the stomach were taken at 120 min post feeding and a high density of particle adherence for C-EC and C-ECMC was clearly observed. The spheres were well embedded within the mucosal layer with minimal aggregation (Fig. 6). The size of the attached C-EC and C-ECMC particles on the gastric surface also agreed well with the size of the two spheres reported above. It was likely that these numerous adhered spheres acted as curcumin reservoirs that continuously released curcumin into the blood circulation. At 120 min post feeding, C-ECMC sphere adhesion was at a lower density than that of the C-EC particles (Fig. 6). The observable nature of both C-EC and C-ECMC spheres in the GIT agrees well with the fact that both EC and MC polymers are un-digestible. Their excellent morphological stability under various conditions has been witnessed in our lab, including that both carriers can withstand freeze-drying, spray-drying (130 °C) and high pressure homogenization (pressure of 200 bar) processes very well.

Since the *in vitro* release experiment indicated a minimal release of curcumin into the SGF, we speculate that the curcumin is directly released from the spheres into the epithelium of the stomach. It was likely that the hydrophobic curcumin molecules crossed the spherical wall and went directly into the hydrophobic lipid bilayer membrane of the epithelium. As such, the concentration gradient of curcumin would be the driving force on this diffusion equilibrium. The absorbed curcumin molecules quickly enter the blood circulation, thus

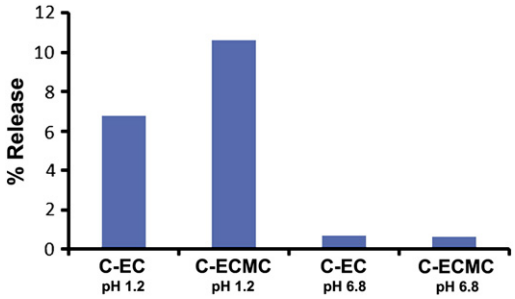

Fig. 4. Release of curcumin from C-EC and C-ECMC nanoparticles in SGF (pH 1.2) or SIF (pH 6.8) prepared according to the USP. The starting concentration of curcumin in either release medium was 25 ppm. Data are expressed as an average of two repeats.

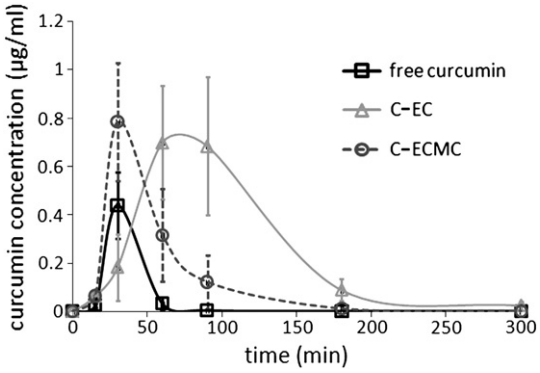

Fig. 5. Curcumin concentration in the blood of mice orally fed at t = 0 with one of free curcumin, C-EC and C-ECMC. Each data point is expressed as mean ± S.D. of five different mice in which three replicates of quantifications were performed for the blood sample from each mouse. Kruskall–Wallis analysis indicated that the three groups were significantly different from each other ($\chi^2 = 13.572$, df = 2, and Sig. = 0.0005).

decreasing the concentration of curcumin at the tissue of contact site. As a result, a curcumin concentration gradient remained in the direction that curcumin was diffused from the spheres into the tissue. Good contact between the spheres and the stomach surface would be an important factor for this transportation to be possible, and the SEM picture (Fig. 6) showed excellent adhesion of both the C-EC and the C-ECMC spheres. The long stay of the curcumin-loaded self-assembled particles (~50% loading capacity, small size and high surface area) on the stomach surface enabled the protected curcumin molecules in the spheres to be continuously diffused from the particles directly into the stomach tissue, and so a high curcumin concentration was observed in the blood for a long time.

Although it has been known that MC is a better mucoadhesive polymer than EC, the result here showed excellent mucoadhesion of self-assembled EC nanospheres. This result seemingly contradicts with the well known water insolubility nature of EC. However, the EC carriers in this work were self-assembled nanospheres. During the self-assembling process, most of the hydrophobic moieties of the EC (ethyl groups) should orient themselves towards the inside of the spheres, away from water medium, while most of the hydrophilic moieties (hydroxyl groups) should be directed to the outer surface of the spheres. This, therefore, makes the obtained EC spheres well dispersible in water despite the fact that the non-self-assembled EC polymer is neither soluble nor dispersible in water. Hydroxyl groups at the outer surface of the spheres can also form H-bonding interactions with mucin, thus enhancing mucoadhesion. In addition, the hydrophilic nature of their outer surface should make the spheres dispersible in the mucous gel layer. With many hydroxyl groups at the surface of the curcumin loaded spheres and their very small size and thus high surface area, the spheres can disperse well in water. Upon contact with the mucosa, hydrogen bonding between the sphere and the mucin protein is very likely to occur. This prolongs the time the curcumin-loaded spheres stay in the stomach and facilitate the contact between curcumin-loaded particles and the stomach tissue. With the spheres in contact with stomach surface, direct diffusion of curcumin from the spheres into the stomach epithelium can occur. The curcumin-loaded spheres which are stuck at the stomach surface, therefore, act as reservoirs of curcumin. In addition, with direct diffusion of curcumin from the spheres into the stomach tissue and into circulation, degradation of curcumin under the very acidic environment of the stomach could be avoided. As a result, an increased bioavailability could be observed. This is very different from the unencapsulated curcumin molecules which by themselves cannot disperse into the mucus layer and thus will be exposed to the extreme acidic condition in the stomach causing the molecules to degrade. Moreover, the hydrophobic curcumin molecules will quickly aggregated into big pieces under the aqueous environment and will be moved down the GIT very quickly.

Previous *in vivo* studies have shown that curcumin possesses a rapid metabolism [25], and fast elimination [24,26]. Therefore, the long sustainability of curcumin observed here implied a continuous supply of the curcumin into the circulation. High blood curcumin concentration during the first three hours post feeding indicated that the stomach was the most likely curcumin absorption site.

With good attachment of the C-EC and C-ECMC nanoparticles on the stomach's surface, only a small fraction of the (unattached) spheres were voided into the duodenum. It was likely that most of the C-EC and C-ECMC particles that had passed into the duodenum were the larger sized aggregated spheres, since microsize-spheres were observed at the surface of the duodenum (Fig. 6).

In summary, the good adherence of C-EC and C-ECMC spheres to the surface of the stomach is a result of the various favorable characteristics of the two carriers, including the hydrophilic nature of their outer surface (good dispersibility in the aqueous medium), the abundance of hydroxyl groups at their outer surface (hydrogen bonding with mucin), the physical stability of the carriers (minimal burst) and their small enough size to be able to escape the motility of the digestive tract. In other words, the nanocarriers were very small compared to the physical structure of the foveolae of the stomach, and so the attached spheres could withstand movement without detachment and/or reattach shortly after detachment, during the motility of the GIT. Once attached, the encapsulated curcumin molecules could diffuse directly to the stomach epithelium and then into the blood circulation beneath. Therefore, the normally fast excretion of free curcumin from the stomach could be retarded through the use of either one of these two nanocarriers.

4. Conclusion

Here two curcumin-loaded nanospheres, C-EC and C-ECMC, were successfully fabricated through a self-assembling mechanism with an excellent loading capacity and EE. The obtained C-EC and C-ECMC possessed a high curcumin loading of close to 50% (wt curcumin/total wt) and were around 300 and 200 nm diameter-spheres, respectively. The self-assembling process likely leads to the organization of the polymer chains into nanospheres with a hydrophilic surface, and so the spheres disperse well in water and showed approximately −30 mV of surface charge in water at pH 5.5. The encapsulated curcumin molecules still possessed free radical scavenging activity and *in vitro* cytotoxicity towards cancer cell lines in tissue culture. When orally administered to mice, as confirmed by SEM and stomach tissue analysis for curcumin, C-EC spheres attached well to the gastric mucosa and, as confirmed by blood curcumin quantification, slowly release curcumin into the blood circulation for up to 3 h. In contrast, it was clear that although C-ECMC particles could attach to the gastric mucosa, the attachment was not as favorable as that seen for C-EC. In addition, C-ECMC released curcumin more quickly than C-EC did *in vitro* under an acidic condition and this could be attributed to the higher swelling nature and smaller size of the ECMC spheres compared to the EC spheres. Although the improvement in the curcumin sustainability in blood attained by the use of C-ECMC nanoparticles was significant compared to free curcumin, it was not as great as that attained with C-EC nanoparticles. Excellent mucoadhesion of the self-assembled EC nanospheres could be explained through the hydrophilic surface of the self-assembled spheres.

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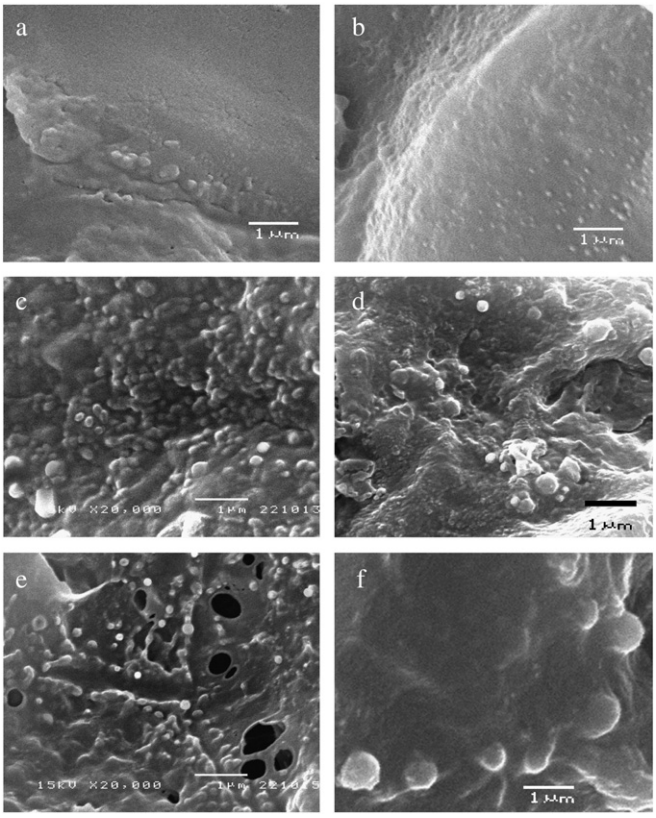

Fig. 6. SEM of stomach (left column, a,c, and e) and duodenum (right column b,d, and f) of mice fed with water (a, and b), C-EC (c, and d) and C-ECMC (e, and f).

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