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Original Article

Microspheres as carriers for lipase inhibitory substances to reduce dietary triglyceride absorption in mice

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ABSTRACT

The present study intends to use microspheres as a delivery system of chlorogenic acid (CGA) to investigate the influences of CGA microspheres on dietary fat absorption and fecal triglyceride excretion in a mice model. Microspheres have an average particle size of about 53.3 μm . Results indicated that the microspheres were capable of gradually releasing the preloaded CGA into the surrounding medium. Their bioadhesive property might help prolong the gastrointestinal transit time in mice, and render a better mixing and contact between CGA and triglyceride. Consumption of CGA microspheres resulted in a significantly higher level of fecal triglyceride (119–144%) as compared with the corresponding control groups. A microsphere would be a desirable vehicle for CGA to improve its efficacy along the intestine.

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1. Introduction

A direct relationship between the incidence of overweight or obesity and the ingestion of dietary triglyceride (TG) has been proved [1,2]. Pancreatic lipase is responsible for the hydrolysis of 50–70 % of total dietary TG to produce free fatty acids and monoglycerides [3,4]. Suppressing TG hydrolysis and absorption by inhibiting lipase activity was thus an effective way to prevent obesity [5,6]. Some natural bioactive compounds such as catechins, tannins, and chlorogenic acids (CGAs) have been

reported to show pancreatic lipase-inhibiting activity [6,7]. Their ability to reduce dietary TG hydrolysis could be enhanced by immobilizing those bioactive ingredients with different carriers [8–10].

Carriers such as microspheres, nanoparticles, and liposomes might offer smart approaches for the delivery of active ingredients by immobilizing them to the carrier particle and regulating their release and absorption [8]. The release of a delivery system is affected mainly by the microstructure and material properties of carriers [10]. Microspheres were believed to be potential carriers for drugs, cell delivery, and

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tissue regeneration as a scaffold [9]. Their ability to be widely distributed throughout the gastrointestinal tract was considered to be one of the advantages to improve drug absorption and reduce side effects [11].

Making microspheres with materials that offer bioadhesive characteristics would be desirable in rendering the carriers capable of attaching to mucosal surfaces, and hence to prolong the residence time and improve the physiological availability of bioactive ingredients [12]. The presence of hydrophilic groups, such as hydroxyl, carboxyl, and amino groups, in a polysaccharide structure enhances its bioadhesion to biological tissues by forming noncovalent bonds. It was a useful strategy to improve physiological availability of bioactive ingredients [13]. Several studies have introduced the use of some natural polysaccharides such as alginate, starch, and chitosan as carrier materials due to their nontoxic, biodegradable, biocompatible, and bioadhesive properties [14].

The present study intends to use microspheres as a delivery system of CGA. Influences of CGA microspheres on dietary fat absorption and fecal TG excretion were evaluated in a mice model. The microspheres were characterized in terms of morphology, particle size, moisture content, bulk density, and flowability. *In vitro* collapse process and CGA release profile of the CGA-microspherical carriers were also discussed.

2. Methods

2.1. Preparation of microspheres

Microspheres were prepared according to the method described by Anandharamakrishnan et al [15], with some modifications. The feed solution, which was prepared by corn starch (Sigma-Aldrich Corp., St Louis, MO, USA) at a concentration of 5 g in 95 mL of water, was atomized through a two-fluid nozzle. The atomizer was used at a compressed air pressure of 275.8–413.7 kPa. The flow rate of the feed solution was set at 5 mL/min. After the spray-freeze process, the frozen particles were collected and lyophilized. The dried microspheres were stored in a dry cabinet at room temperature for future use.

2.2. Characterization of microspheres

Microspheres were examined by a scanning electron microscope (SEM) for surface morphologies and size distribution (TM-1000; Hitachi High-Technologies Corp., Tokyo, Japan). Size distribution results were analyzed by the particle size distribution analysis software (Mac-View ver.4.0; Mountech Co. Ltd, Tokyo, Japan). Moisture content (g/100 g) was determined according to Association of Official Agricultural Chemists (AOAC) method 934.01 [16]. Bulk density (g/L) and tapped density (g/L) of microspheres were measured according to the method of Chau et al [17], with slight modifications. Tapped density was calculated as mass divided by the final volume of the microsphere sample. Powder flowability was characterized by Hausner ratio, which is calculated by dividing tap bulk density by loose bulk density [18].

2.3. Preparation of CGA microspheres

According to the method described in the “Preparation of microspheres” section, CGA extract (purity 45%), which was obtained from Healthmate Co. Ltd. (Changhua, Taiwan), was added and homogenized with the feeding starch solution at a 4% (w/w) level. After the processes of atomization and drying, dried samples were stored in a dry cabinet at room temperature for further use.

2.4. Determination of CGA content in CGA microspheres

CGA microspheres were stirred in a dissolution media at a ratio of 1:1000 (w/v) on a rotating plate (250 rpm) at 37°C for 60 minutes. Subsequently, the CGA microspheres were homogenized at 10,000 rpm (T18 basic; IKA, Staufen, Germany) for 3 minutes to disrupt the structure of microspheres to release the active ingredient. The mixture was centrifuged at 2400g (Universal 320R; Hettich, Tuttlingen, Germany) for 10 minutes. The supernatant was filtered using a 0.45 µm polyvinylidene fluoride (PVDF) syringe filter (Acrodisc, Pall Corporation, Deland, FL, USA). The total CGA contents in the filtrate were determined spectrophotometrically at 324 nm (Genesys 10S UV-Vis; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to AOAC method 957.04 [16].

2.5. Dissolution profiles of microspheres

Microspheres were stirred in a dissolution media at a ratio of 1:50 (w/v) and incubated at 37°C on a rotating plate (100 rpm). At different time points, an aliquot of solution was collected and lyophilized. The dried microsphere samples were observed by a SEM.

2.6. In vitro determination of CGA release

The release of CGA from the CGA microspheres was measured by the method of Bae et al [19]. Briefly, CGA microspheres were mixed with a phosphate buffer (pH 7.2 ± 0.2) at a ratio of 1:50 (w/v), and then stirred at 37°C for 120 minutes on a rotating plate (100 rpm). After being centrifuged at 24g for 1 minute, the supernatant was filtered using a 0.45 µm PVDF syringe filter. The content of CGA in the filtrate was analyzed spectrophotometrically at 324 nm by AOAC method 957.04 [16].

2.7. Diets and experimental design

The study protocol was approved by the Animal Care and Use Committee of National Chung Hsing University. Forty-eight male ICR mice (Blw:CD1, BioLASCO, Taiwan) weighing 29.8 ± 3.6 g were obtained from the National Laboratory Animal Center of Taiwan. Animals were individually housed in a stainless cage in a room maintained at 22 ± 2°C with 12-hour light/dark cycles. Animals were allowed free access to food and water during the experimental period. In the present study, there were six diet groups: one normal chow (NC) control group and five high-fat diet groups, which included high-fat (HF) control, microsphere plus low dose of CGA (CGA-L), microsphere plus high dose of CGA (CGA-H), microsphere

embedded with low dose of CGA (mCGA-L), and microsphere embedded with high dose of CGA (mCGA-H). After an acclimation period of 7 days, animals were divided into eight weight groups of six each. Animals in each weight class were randomly assigned to one of the six diet groups.

Compositions of the NC diet and five high-fat diets are shown in Table 1. The NC control group was fed chow 5001 (PMI Nutrition International, Brentwood, MO, USA), which contained crude lipid (4.5 g/100 g), crude protein (23.0 g/100 g), and carbohydrate (47.5 g/100 g). All high-fat diets contained total fat (23.5 g/100 g) and were prepared by mixing 19.4 g soybean oil with 80.6 g of chow diet [20]. The CGA-L and CGA-H groups (microspheres without CGA embedment) were used for their corresponding test groups in which the microspheres were preloaded with low and high doses of CGA (mCGA-L and mCGA-H, respectively). Animals in the CGA-L and mCGA-L groups received a low dose of CGA (90 mg/kg body weight) each day, while those in the CGA-H and mCGA-H groups were given a high dose of CGA (180 mg/kg body weight). The CGA dosages of 90 mg/kg and 180 mg/kg were approximately equivalent to dosages of 438 mg/d and 876 mg/d, respectively, for a 60 kg adult [21]. The feeding experiment was carried out for 7 days. Food intakes and body weights were recorded daily. During the last 3 days of the feeding period, feces were collected, weighed, and dried.

2.8. Determination of fecal TG content

Following the method of Folch et al [22], with slight modifications, fecal TG was extracted from the dried feces with a mixture of methanol:hexane:deionized water (1:2:0.8, v/v). The lipophilic layer was collected and dried under a nitrogen stream (TurboVap LV; Biotage AB, Uppsala, Sweden). After mixing with 10% Triton X-100 (Sigma-Aldrich Corp.), the fat content in the crude fat was measured using a commercial TG kit (Fortress Diagnostics Ltd, Antrim, Northern Ireland, UK).

2.9. Statistical analysis

Data were subjected to one-way analysis of variance and Duncan's multiple range tests using SPSS version 20 statistics

software (SPSS Inc., Chicago, IL, USA). Data are presented as means \pm standard deviation. Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Characterization of microspheres

Morphological features of the microspheres, as shown in the low-magnification SEM image (Fig. 1A), revealed that the microspheres have a uniform spherical shape. In Fig. 1B, the high-magnification SEM image presents the shape of a single microsphere. The higher-magnification SEM image in Fig. 1C shows that there are numerous pores on the surface as well as in the inside core of the microspheres and the diameter of a pore size is around 1–2 μm . Particle size and size distribution of the microspheres were influenced by different factors including formulation of solution, concentration of solution, airflow rate, and solution feed rate [23]. Semyonov et al [24] have reported that the airflow affected the size of the particles more than the flow rate of feeding solution.

Different characteristics of the microspheres including moisture content, bulk density, tapped density, and flowability are given in Table 2. Moisture content is one of the important characteristics of powder and has a great influence on its storage stability. The low moisture level (3.32 g/100 g) of the microsphere powders was desirable for prolonging their stability. The microspheres that exhibited a porous microstructure had a low bulk density (13.4 g/L) (Table 2), and the volume occupied by 1 g of untapped microspheres was up to 74.2 mL. Bulk density of the microspheres in this study was lower than those of other spherical carriers (up to 200 g/L), as reported by some other authors [23]. Flowability of powders could be classified based on the Hausner ratio. Particles having a Hausner ratio larger than 1.25 are considered to have poor flowability [18]. Microsphere powders are considered to have poor flowability as the Hausner ratio is up to 1.71 (Table 2). Awasthi and Kulkarni [25] have demonstrated that particle size and morphology significantly influenced the flow characteristics of powder. Fine particles < 100 μm in size tended to

Table 1 – Compositions of the normal chow diet and the experimental high-fat diets.

	Normal chow diet ^a		23.5% fat high-fat diet ^b		23.5% fat high-fat diet (microsphere plus CGA)		23.5% fat high-fat diet (microsphere embedded with CGA)	
	NC	HF	CGA-L	CGA-H	mCGA-L	mCGA-H		
Food intake (g/d)	5.5	5.5	5.5	5.5	5.5	5.5		
Oil intake (g/100 g diet)	4.5	23.5	23.5	23.5	23.5	23.5		
Protein intake (g/100 g diet)	23	19	19	19	19	19		
Carbohydrate intake (g/100 g diet)	47.5	38.5	38.5	38.5	38.5	38.5		
CGA consumption (mg/kg bw)			90	180	90	180		

bw = body weight; CGA = chlorogenic acid; CGA-H = microsphere plus high dose of chlorogenic acid group; CGA-L = microsphere plus low dose of chlorogenic acid group; HF = high-fat group; mCGA-H = microsphere embedded with high dose of chlorogenic acid group; mCGA-L = microsphere embedded with low dose of chlorogenic acid group; NC = normal chow group.

^a Chow contained crude protein (23.0 g/100 g), crude lipid (4.5 g/100 g), and carbohydrate (47.5 g/100 g).

^b HF group was used as a control for the corresponding CGA-supplemented groups including low doses (CGA-L and mCGA-L) and high doses (CGA-H and mCGA-H).

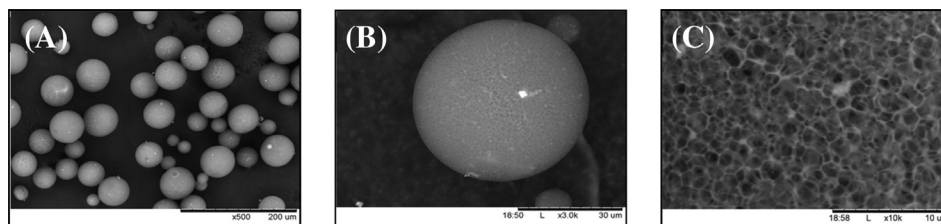


Fig. 1 – SEM images of microspheres at different magnifications: (A) 500 \times , (B) 3000 \times , and (C) 10,000 \times .

be more cohesive and were therefore less freely flowing, whereas larger, denser particles tended to be freely flowing. In the present study, particle size distribution of microspheres mostly ranged from 14.0 μm to 158 μm (Fig. 2). Diameters at 10%, 50%, and 90% of the cumulative size distribution were 28.4 μm , 63.5 μm , and 134.7 μm , respectively. A small particle size might in part explain the poor flowability of the dry microsphere samples.

3.2. In vitro dissolution and releasing profiles of CGA microspheres

Active ingredients are loaded into the carriers by two approaches, including preloading of active ingredients during the preparation of carriers or loading the active ingredients into the carriers after their formation. In the present study, the active ingredient CGA was preloaded into the microspheres during the preparation process of the carriers; hence, the CGA component could be well dispersed inside the microspheres. It should be noted that the solubility of the CGA powder was good and could be dissolved within 5 minutes in an aqueous medium. The CGA content in the CGA microspheres was 194 ± 2 mg/g of microsphere. As compared to the microsphere shown in Fig. 1B, the SEM image (Fig. 3A) indicated that preloading of CGA did not affect the spherical shape and porous structure of the microsphere.

Dissolution profiles of CGA microspheres in a phosphate buffer, pH 7.2, are shown in Fig. 3. When the CGA microspheres were placed in an aqueous medium, the hydrophilic microspheres swelled. The starchy microspheres dissolved gradually, and apparent dissolution was observed around the pores. Fig. 3B shows that the microspheres maintained the distinct three-dimensional structure for the first 40 minutes. The pore size on the microspheres increased from ~ 1 μm to ~ 6 μm after being soaked for 40 minutes. Starch materials in

the carriers contributed a firm framework structure, which could dissolve gradually in the aqueous medium [26,27]. As the soaking time increased, the microspheres continued to collapse gradually (Fig. 3C and 3D). It rendered the microspheres with the ability to be applied as a dissolvable carrier in the intestine.

The preloaded ingredients could be released from the carriers in different ways, for example, through osmotically driven burst mechanism, pore diffusion mechanism, and erosion or degradation of carriers [10]. Once the microspheres were placed in an aqueous medium, they swelled and their structure collapsed gradually. The preloaded components would be released slowly and continuously into the aqueous medium. Fig. 4 illustrates the *in vitro* release of CGA from the CGA microspheres in a phosphate buffer, pH 7.2. After 40 minutes, the CGA microspheres have released about 53% of the total amount of CGA being preloaded. The results demonstrated that approximately 98% of CGA would be released at the 2nd hour. The preloaded CGA inside the core of the microspheres could be effectively released into the surrounding medium during their dissolution and structural collapse. The porous structure of carriers could generally lead to efficient dissolution and release their preloaded components effectively compared to nonporous carriers [28].

A microspherical carrier in general could be designed to release some preloaded active components continuously into its surrounding medium over a desired period of time, the length of which was dependent on the targeted functions of those components. To decrease the gastrointestinal absorption of dietary TG, the CGA microspheres were expected to release a majority of their CGA in the upper small intestine to

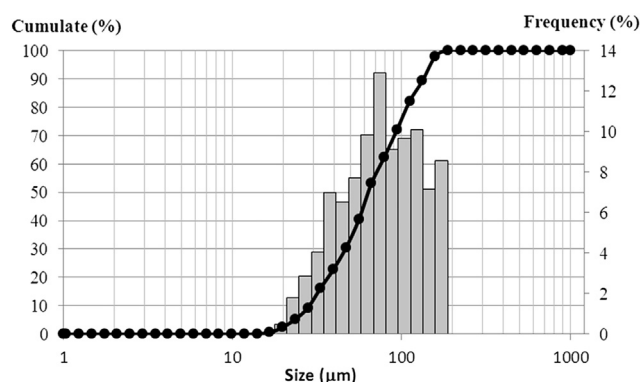


Fig. 2 – Particle size distribution of microspheres.

Table 2 – Characterization of microspheres.

Characteristics	Microspheres
Moisture content (g/100 g)	3.32 ± 0.47
Average particle size (μm)	53.3 ± 32.3
Bulk density (g/L)	13.4 ± 0.1
Tapped density (g/L)	23.0 ± 1.1
Hausner ratio	1.71 ± 0.08

Data are presented as mean \pm standard deviation; $n = 3$.

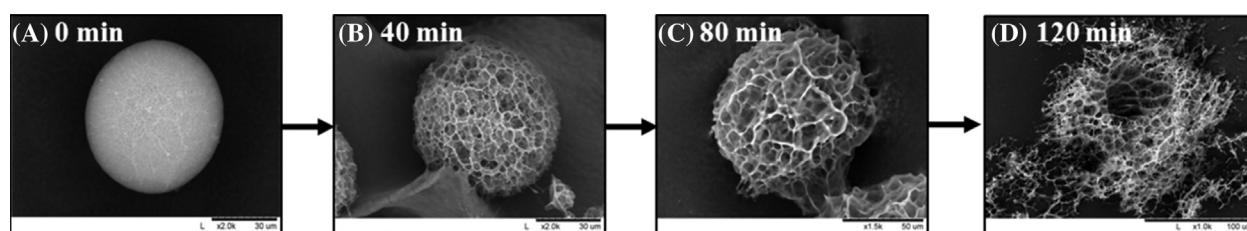


Fig. 3 – *In vitro* dissolution profiles of CGA microspheres. SEM images indicated the dissolution profiles of CGA microspheres in a phosphate buffer (pH 7.2) within 120 minutes. CGA = chlorogenic acid; SEM = scanning electron microscope. (A) dissolution profile at 0 minute; (B) dissolution profile at 40 minutes; (C) dissolution profile at 80 minutes; (D) dissolution profile at 120 minutes.

enable more direct contact between TG and CGA. According to the results of the above *in vitro* dissolution and release tests, the microspheres were believed to be capable of gradually releasing the preloaded CGA into the surrounding medium in the small intestine within 120 minutes.

3.3. *In vivo* evaluation of CGA microspheres

After the acclimation period, there were no differences in the initial body weights (30.4–34.2 g) of mice among the six dietary groups. Throughout the whole experimented period, all animals remained healthy and active. The diet intakes were comparable among the six different groups. No changes in dietary intake were observed. During the experimental period, all groups fed high-fat diets had significantly ($p < 0.05$) higher body weight gains (192–248%) than those of the NC control group (2.5 ± 0.4 g) due to their higher fat intake. The body weight gains in the CGA-L, CGA-H, mCGA-L, and mCGA-H groups (5.6 ± 0.5 g, 5.1 ± 0.9 g, 5.1 ± 1.1 g, and 4.8 ± 0.7 g, respectively) were seldom reduced (by -10% to -23%) as compared with that of the HF control group (6.2 ± 1.0 g).

As shown in Fig. 5A, there were no significant differences in the fecal dry weights between the HF control and the CGA-L groups. However, the CGA-H, mCGA-L, and mCGA-H groups had significantly ($p < 0.05$) higher fecal dry weights (ranging from 116% to 129%) than the HF control group. The fecal TG excretion (Fig. 5B) in all four CGA-ingested groups (i.e., CGA-L,

CGA-H, mCGA-L, and mCGA-H) were significantly ($p < 0.05$) higher (1.3–2.4-fold) than that in the HF control group, with the highest fecal TG output in the mCGA-H group. These results demonstrated that consumption of CGA at dosages of 90 mg/kg and 180 mg/kg body weight markedly ($p < 0.05$) increased the fecal TG outputs in animals in a dose-dependent manner. Narita et al [29] demonstrated that CGA had an

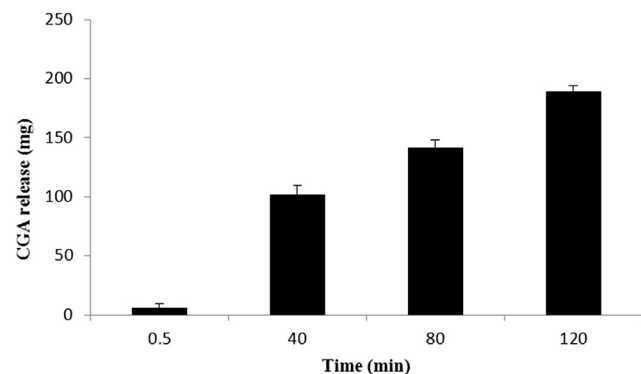


Fig. 4 – CGA released from CGA microspheres within 120 minutes. CGA microspheres were preloaded with CGA at a level of 194 mg/g. CGA = chlorogenic acid.

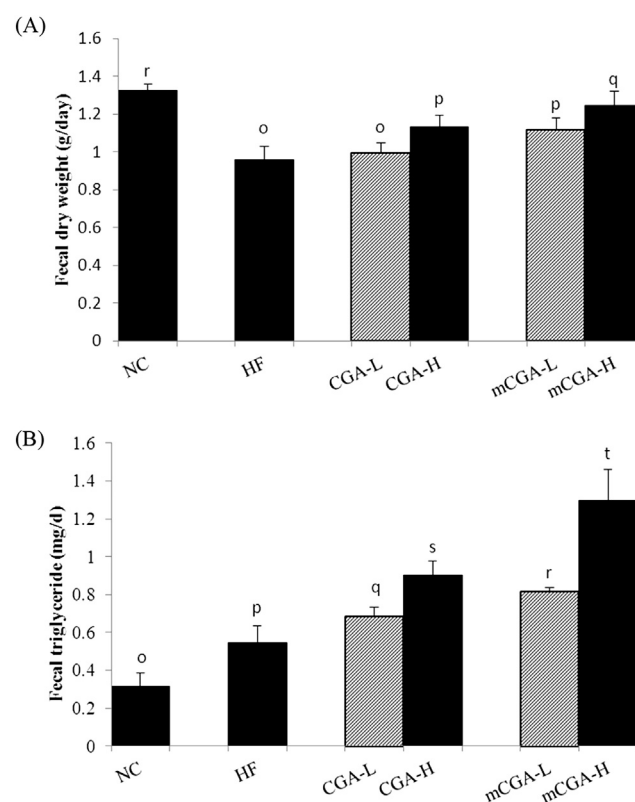


Fig. 5 – Effects of different diet groups on (A) fecal dry weights and (B) fecal triglyceride excretion. Bars with different letters (o–t) are significantly different (Duncan, $p < 0.05$). CGA-H = microsphere plus high dose of chlorogenic acid group; CGA-L = microsphere plus low dose of chlorogenic acid group; HF = high-fat group; mCGA-H = microsphere embedded with high dose of chlorogenic acid group; mCGA-L = microsphere embedded with low dose of chlorogenic acid group; NC = normal chow group.

inhibitory effect against the pancreatic lipase catalysis of TG emulsions, leading to a decrease in dietary fat absorption as well as an increase in TG excretion into feces.

As shown in Fig. 5B, the daily fecal TG outputs of mCGA-L and mCGA-H groups were significantly ($p < 0.05$) higher (119% and 144%, respectively) than those of the CGA-L and CGA-H groups. Based on these findings, it was inferred that porous microspherical carriers could release CGA gradually and enhance fecal TG excretion by preventing rapid clearance of soluble CGA from the gastrointestinal tract. As polyphenols might have poor inhibiting pancreatic lipase activity resulting from their instability under digestive conditions and rapid metabolism followed by excretion [6,30], it was also believed that the bioadhesive property of starchy microspheres could prolong their gastrointestinal transit time and thereby result in better mixing and contact between TG and CGA. Microspheres would be desirable vehicles for CGA to be active along the intestine.

In sum, it was concluded that microspheres could be explored as suitable carriers for CGA to improve its physiological availability. The microspheres were capable of gradually releasing the preloaded CGA into the surrounding medium. Their bioadhesive property might also prolong their gastrointestinal transit time, and render better mixing and a better contact between TG and CGA. After the consumption of CGA microspheres, the gradual release of CGA along the intestine resulted in a significantly higher level of fecal TG as compared with the corresponding control groups.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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