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Bioaccessibility and cellular uptake of β-carotene in emulsion-based delivery systems using scallop (*Patinopecten yessoensis*) gonad protein isolates: Effects of carrier oil

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Abbreviations:

BC, β-carotene; LCT, long chain triglyceride; MCT, medium chain triglyceride; SGPIs, scallop gonad protein isolate; GIT, gastrointestinal track; MTT, 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; DMSO, dimethyl sulfoxide

ABSTRACT

The emulsion-based delivery systems were structured by using scallop gonad protein isolates (SGPIs) as novel food-grade emulsifiers. The effects of carrier oil including the long chain triglycerides (LCT) and medium chain triglycerides (MCT), on the bioaccessibility and cellular uptake of β-carotene (BC) were investigated. Both LCT and MCT delivery systems remained stable at pH 7-8 but aggregated at lower pH values (3-6) according to the results of light scattering and microscopy measurements. LCT droplets fabricated within SGPIs were digested and released more slowly than MCT droplets during the simulated gastrointestinal tract digestion. The LCT emulsion showed higher BC bioaccessibility (65.5%) than MCT emulsion (23.1%) as a result of the greater solubilization of BC in mixed micelles fabricated from long-chain fatty acids. Moreover, LCT-emulsion produced higher cellular uptake of BC as comparing with MCT-emulsion in intestinal epithelial cells. These results demonstrated that SGPIs could be used as novel food-grade emulsifiers to protect lipophilic bioactive compounds in emulsion-based delivery systems, in which LCT is more suitable to encapsulate and deliver BC than MCT.

Keywords: scallop (*Patinopecten yessoensis*) gonad protein isolates; β-carotene; carrier oil; emulsions; bioaccessibility; cellular uptake

1. Introduction

β-carotene (BC) is one of the most important carotenoids exists in numerous vegetables and fruits [1,2]. As the precursor of vitamin A, it is extensively used as an antioxidant and natural colorant which playing a vital role in human bodily functions [3]. However, the chemical instability, low bioavailability and poor water-solubility of BC greatly restrict its application in numerous aqueous-based beverages and foods [4,5]. Due to the polyunsaturated structure, BC degradation happens rapidly at certain environmental conditions [6]. Nevertheless, these restrictions can be broken by solubilising BC in lipid droplets and homogenising with a water-soluble emulsifier to format emulsion-based delivery systems [7,8].

Owing to a growing number of consumer demands for "healthy" labelled food products, the natural emulsifiers are widely considered in formulating emulsions as comparing with the synthetic surfactants or polymers [9]. Protein is a kind of natural emulsifier with the advantages of strong surface activity, low cell toxicity and good stability [10]. Nowadays, the protein-based emulsifiers applied in food industry are numerously derived from milk, soybean and egg [11]. Many reports have indicated that these emulsion-based delivery systems can be utilized to enhance the bioaccessibility of BC [12,13]. However, due to the dietary preferences and territory restrictions, food manufacturers and consumers are looking for some alternative marine protein sources in the human diet. Basing on some literatures, several marine proteins have already been explored as food-grade emulsifiers [14,15]. For instance, shrimp (*Penaeus vannamei*) heads with a high content of protein has been used to stabilize palm oil-in-water food emulsions [14]. Silver carp (*Hypophthalmichthys molitrix*) protein isolate has displayed certain

emulsifying capacity and foaming capacity [15]. Nevertheless, the research on shellfish, especially scallop proteins as emulsifier to deliver BC is still relatively vacant.

Scallop (*Patinopecten yessoensis*) is an important bivalve extensively distributed in Eastern Asia. In China, scallop production from aquatic breeding has increased to 1.86 million tons in 2016 (FAO 2017). The demand for scallop processing is increasing with the continuous expansion of scallop farming. Scallop gonads are main edible byproduct with high level of protein during the processing of *P. yessoensis* adductor, which are regarded as good sources to develop protein matrix. Our previous studies have shown that protein isolates from scallop gonad (SGPIs) provide high nutritional value and good emulsifying properties when compared with crude scallop gonad or soybean protein isolates. SGPIs contain a mixture of proteins, such as vitellogenin, actin, and a little myosin. These proteins have both hydrophobic and hydrophilic regions on their surfaces [16], and these regions can easily consume oil droplet forming an interfacial coating [10]. As a consequence, it is necessary to study the effect of SGPIs in emulsion-based delivery system for BC.

It has been confirmed that the bioaccessibility and bioavailability of hydrophobic bioactive compounds can be improved by co-existing with digestible lipids, which is impacted by lipid nature [17]. The digested triacyglycerols form monoacylglycerols and free fatty acids (FFA), which are combined with phospholipids and bile acids forming mixed micelles to solubilise and transport the BC molecules to epithelium cells [18]. After absorption, the FFA and monoacylglycerols are recombined together into triacylglycerols, which can be incorporated along with BC into lipoproteins (chylomicrons). Therefore, the type of triacylglycerols will show important effect on the SGPIs-BC emulsion delivery system.

In the current study, the protein isolates were prepared from scallop gonads and further used as emulsifiers. The emulsion-based delivery systems adopting SGPIs as food-grade emulsifiers were structured by using long chain triglycerides (LCT) and medium chain triglycerides (MCT) as the carrier oils. A simulated gastrointestinal track (GIT) and Caco-2 cells model were adopted to investigate the impact of carrier oil on the bioaccessibility and cellular uptake of BC. The knowledge gained from the present study will provide fundamental information to establishing SGPIs-based delivery systems to encapsulate BC for further application.

2. Materials and method

2.1 Materials and chemicals

Fresh female scallop (*P. yessoensis*) was purchased from Dalian Changxing market (Dalian, China) in April 2017. Different carrier oils, LCT (corn oil) was purchased from XIWANG FOOD (Shandong, China). MCT was acquired from Coletica (Northport, NY). BC, lipase from porcine pancreas, mucin from porcine stomach (type II), porcine bile extract, pepsin from porcine gastric mucosa, Fluorescein isothiocyanate (FITC), Nile red, and 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human colon carcinoma cell lines (Caco-2 cells, ATCC number: HTB-37) were acquired from the American Type Cell Collection (Manassas, VA). Cells after 25-40 passages were used in the uptake study. Fetal bovine serum (FBS), DMEM (with 4.5 g/L glucose, L-glutamine, and sodium pyruvate), nonessential amino acid solution, penicillin-streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), trypsin-EDTA and n-hexane were purchased from Fisher Scientific (Agawam, MA, USA). All the other reagents were of analytical grade.

Scallop gonad protein isolates (SGPIs) were prepared from defatted scallop gonad by isoelectric precipitation process [19]. Briefly, defatted scallop gonads were added with distilled water at a ratio of 1:20 (*w/v*) and adjusted to pH 11.5 using NaOH solution, then the mixture was centrifuged at 2000 ×*g* for 20 min. The resulting supernatant was adjusted to pH 3.8 with 0.5 M HCl and centrifuged at 10000 ×*g* for 30 min. Thereafter, the sediment was washed twice with distilled water, neutralised using NaOH solution, and dialyzed for 48 h. Finally, the sediment was freeze-dried and referred as SGPIs. The proximate composition of SGPIs was found to be 86.39±1.2 wt% protein, 0.17±0.02 wt% lipid, 3.27±0.28 wt% ash, 6.27±0.31 wt% moisture and 1.9±0.08 wt% carbohydrate.

2.2. Preparation of BC emulsions

An oil phase was obtained by dispersing BC (0.1%, w/w) into LCT or MCT and sonicating in an ultrasonic water bath (45 °C) for 30 min so that dissolve completely. The aqueous phase was obtained by dispersing SGPIs at 0.25% (w/w) in 5 mM PBS (pH 7.0) and stirring overnight for complete hydration. The aqueous (98%, w/w) and oil phases (2%, w/w) were mixed at a speed of 10,000 rpm for 2 min by using a high-speed blender (M133/128190, Biospec Products, Inc., ESGC, Switzerland). The coarse emulsion was further homogenized using a microfluidizer (M110Y, Microfluidics, Newton, MA) equipped with a 75 μm interaction chamber for five times at 12,000 psi as described previously [20]. After cooling down, the final emulsions were added with the sodium azide (0.02%, w/w) to avoid microbial growth, and stored at 4 °C in dark place.

2.3. Analysis of particle size and ζ-potential

The particle size distributions as well as the mean particle size of samples were determined by dilution with phosphate buffer (5 mM, pH 3.0-8.0) by a laser light scattering equipment (Mastersizer

2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The refractive index values were 1.33 for the aqueous phase, 1.47 and 1.45 for the LCT and MCT oil phase, respectively [21,22]. ζ-potential was measured by a particle electrophoresis instrument (Zetasizer Nano, Malvern Instruments, Worcestershire, UK).

2.4. In vitro digestion of BC emulsions

The simulated gastrointestinal tract (GIT) model was composed of mouth, gastric and intestinal digestion as described previously with slight modification [20,23]. Briefly, the simulated saliva fluid (13.64 mM NaCl, 2.05 mM NH_4NO_3, 2.34 mM KH_2PO_4, 1.35 mM KCl, 0.5mM potassium citrate, 0.06 mM uric acid sodium salt, 1.65 mM urea and 0.65 mM lactic acid sodium salt) containing 75 U/mL mucin (type II) and samples were preheated at 37 °C. In order to initiate the mouth phase digestion process, simulated saliva fluid (20 mL) including mucin (0.03 g/mL) was added to 20 mL of BC-emulsion (2% oil phase) sample. The mixture was agitated by adjusting to pH 6.8 and incubation at 37 °C for 2 min. Then, 20 mL oral digestive sample was mixed with an equal volume of simulated gastric fluid (17.11 mM NaCl and 41.91 mM HCl) including 2000 U/mL pepsin at pH 2.5, and the mixture sample was incubated again for 2 h to simulate stomach phase. Finally, the mixture was added with 1.5 mL of simulated intestine fluid (10 mM $CaCl_2 \cdot 2H_2O$ and 150 mM NaCl), 3.5 mL of bile salts (10 mM) and 2.5 mL of lipase (2000 U/mL). The pH value at 7.0 was maintained using an automatic titration unit (Metrohm, USA Inc.) by titrating 0.2 M NaOH into reaction system at 37 °C for 2 h. The volume of NaOH neutralizing the FFA was recorded. The percentage of FFA released was calculated according to the following formula [24]:

$$\text{FFA}\ (\%) = \left(\frac{V_{NaOH} \times m_{NaOH} \times M_{Lipid}}{W_{Lipid} \times 2} \right) \times 100$$

Where V_{NaOH} is the NaOH volume (mL) titranted to neutralize the FFA generation during the lipid digestion, m_{NaOH} is the NaOH solution molarity (M), W_{Lipid} is the weight of oil exist in the digestion system (0.15 g), M_{Lipid} is the average molecular weight of LCT (824 g/mol) and MCT (500 g/mol) [20]. The digestion of oil-free protein micelles was used as blank.

2.5. Microstructural analysis

Samples (50 μL) were dyed with the solution of FITC (2 μL, 10 mg/mL) and Nile red (2 μL, 1 mg/mL) to acquire green (protein) and red (oil) fluorescence images, respectively. Afterwards, 10 μL of sample was dropped on a glass slide and covered with a coverslip, and then used to characterize the microstructure using either optical or confocal scanning laser microscopy with a 60× oil immersion objective lens and 10× eyepiece lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, US.). The excitation and emission wavelengths were 488 nm and 515 nm for FITC, while 543 nm and 605 nm for Nile red, respectively. All images were captured and assessed using digital image analysis software (NIS-Elements, Nikon, Melville, NY, US.).

2.6. Transformation and bioaccessibility of BC after digestion

After *in vitro* digestion, the digesta samples (20 mL) were centrifuged at 18000 rpm (41657 ×*g*), 4 °C for 50 min, and the yellow supernatants in which containing the solubilized BC as micelle fraction was collected. The concentration of BC in the supernatants was determined on the basis of a procedure described by Zhang et al. with slight modifications [25]. Briefly, micelle fraction was added with the mixture of n-hexane and ethanol (3:2, *v/v*) at ratio of 1:2 (*v/v*). After vortexing (20 s) and centrifuging (940 ×*g* for 5 min), the top layer including the solubilized BC was collected. The bottom layer was extracted repeatedly with the same process until the supernatant getting colorless and transparent. The

collected n-hexane layers were mixed together, and the absorbance of the diluted supernatant was measured at 450 nm using a Microplate Reader (BioTek Instruments, Inc., Winooski, VT) with n-hexane as a control.

The concentration of BC was calculated according to the standard curve: Y=0.0723X+0.0445. The BC concentration in the entire digesta phase was also determined using the same method. The transformation and bioaccessibility of BC were estimated using the following formulas [26]:

$$\text{Transformation}(\%) = \frac{C_{\text{digesta}}}{C_{\text{initial}}} \times 100$$

$$\text{Bioaccessibility}\ (\%) = \frac{C_{\text{micelle}}}{C_{\text{digesta}}} \times 100$$

Here, C_{micelle}, C_{digesta} and C_{initial} are the BC concentrations in the micelle fraction, entire digesta phase, and initial emulsions before digestion, respectively.

2.7. Cytotoxicity of BC-loaded SGPIs emulsions after digestion

The MTT test was used to evaluate the effect of the samples on cell viability and potential cytotoxicity. Caco-2 cells were cultured in DMEM medium including 10% FBS and 100 U/mL penicillin-streptomycin at 37 °C under 90% humidity and 5% CO_2. The cells (1.7×10^5 cells/well) were incubated in a 96-well plate for 48 h to achieve 80% post confluence. The medium was discarded, and cells were incubated with 200 μL of mixed micelle fraction from digesta samples diluted with DMEM (10, 20, 50, 100, and 200 times, i.e., the final concentration of BC was 0.1, 0.05, 0.02, 0.01, and 0.005 μg/mL) for further 24 h. Simultaneously, BC dissolved in THF/DMSO (1:1, *v/v*) solution diluted with DMEM was used as control. After treatment, the DMEM medium was replaced by 200 μL of MTT solution (0.5 mg/mL) and incubated at 37 °C for 2 h. Finally, the supernatant was discarded and added DMSO (100 μL) to dissolve the purple formazan products. Cell viability was measured in the light of

the absorbance at 570 nm using a Microplate Reader and estimated by the formula below:

$$\text{cell viability}(\%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Here, $A_{control}$ is the absorbance of cells incubated with DMEM only, A_{sample} is the absorbance of the cells incubated with samples, and A_{blank} is the absorbance of cells-free wells.

2.8. Cellular uptake of BC-loaded SGPIs emulsions after digestion

Caco-2 cells (3×10^4 cells/well) were seeded in 6-well plates and incubated at 37 °C under 90% humidity and 5% CO_2. The DMEM medium was replaced every two days until approximately 90% confluence of the cell monolayers were observed (11-12 days). Prior to BC analysis, the mixed micelle fraction from digesta samples was diluted 20 times with DMEM to minimize the cytotoxicity of the Caco-2 cells. Meanwhile, BC in THF/DMSO (1:1, *v/v*) solution was also diluted with DMEM to attain the same BC concentration of 0.05 μg/mL as control with final THF concentration less than 0.1%. After incubation for 24 h, the cell monolayer was washed twice with precooled PBS, digested by 3 mL of trypsin solution, and treated with 7 mL of DMEM to inactivate the trypsin. After centrifuged at 4000 rpm (1788.8 ×*g*) for 5 min, the precipitated cells were added 500 μL of precooled cell lysis buffer, and treated by sonicator after incubating on ice for 30 min. Then, cell suspension (400 μL) was extracted with the mixture of n-hexane and ethanol (3:2, *v/v*), and the BC content was analyzed by an Agilent 1100 High pressure liquid chromatography system with DAD UV-vis absorption detector (Agilent, Santa Clara, CA) on the basis of the previous method [20]. BC quantitation range (0.005-0.05 μg/mL) was established for HPLC analysis of BC in mixed micelle fraction and in Caco-2 cell transport, and standard curve was determined as Y=1.1956X-0.0004, R^2=0.9992. The protein content was also

determined by bicinchoninic acid (BCA) assay. All procedures were avoided light exposure and carried out on ice. The cellular uptake of BC was then calculated and expressed as pmol/mg protein.

2.9. Statistical analysis

All values were expressed as means ± SD. One-way analysis of variance was computed using the SPSS software (version 7.5). The differences of the experimental results were analyzed by Duncan's multiple range test with $p<0.05$ as significant.

3. Results and discussion

3.1. Effect of pH on particle stability of different carrier oil-based BC emulsion stabilized by SGPIs

Certain protein isolates extracted from different sources, such as egg, whey, soybean, possess strong surface activity and can form a thin oil-water interface cohesive layer during the emulsification process [25,27]. Our preliminary studies have shown that SGPIs have higher surface activity than soybean protein isolate as confirmed by its emulsion activity and stability (Data not shown). Thus, it can be utilized as a favourable emulsifier for embedding BC emulsion. The aqueous phase pH value during microfluidisation was a critical factor to determine the emulsion stability. Therefore, a variety of emulsions were obtained by microfluidisation of 2% LCT or MCT (0.1% BC) and 98% aqueous phase (0.25 % SGPIs) at pH 3.0-8.0 since this range comprises majority of food applications.

The particle size distributions and mean particle diameters of both LCT and MCT-based BC emulsions are shown in Figs. 1A, 1B and Table. 1, respectively. At the range of pH 3.0-4.0, both emulsions exhibited physical instability. The emulsions had obviously broad particle size distribution coinciding with visible phase separation after microfluidisation (Figs. 1A and B). The mean particle

diameters higher than 10 μm were observed (Table. 1). Furthermore, with the increase in pH value, the mean particle diameters of both emulsions significantly decreased (p<0.05) and tended to be flat when pH reached to 6.0, and particle size distribution at pH 7.0-8.0 showed narrow single peaks within the mean particle size less than 0.35 μm. As shown in Fig. 1C, both emulsions exhibited fairly similar ζ potential-pH profiles altering from moderately positive changes to notably negative charges at pH 3.0-8.0. The zero-charge point was determined to be approximately pH 3.8. Meanwhile, the ζ-potential magnitude of LCT emulsions was always higher than MCT when pH reached above 4.0, and the ζ-potential of emulsions for LCT and MCT was less than -20 mV at pH 7.0-8.0. Furthermore, the oil droplets seemed to be evenly spread throughout the BC emulsions at pH 7.0-8.0 as revealed by optical and confocal microscopy images, while an extensive droplet flocculation was occurred in both systems at pH 3.0-6.0 (Fig. 2). These results suggest that both LCT and MCT emulsion stabilized by SGPIs exhibited good stability under pH 7.0-8.0, and LCT emulsions have a relatively large particle diameter and ζ-potential magnitude than MCT emulsions.

The stability of emulsion exhibited at different pH is attributed to the electrostatic repulsion between the lipid droplets coated by protein [13]. Similar consequences were found in whey protein isolates (WPI) stabilized emulsion. It has been suggested that the WPI-soybean oil emulsion exhibited the greatest phase separation resistance and the emulsions leaded to more stable at pH 7 than pH 3 as the WPI possessed pI at 4.6 [28]. On the contrary, the egg white protein (EWP)-stabilized BC emulsion showed good stability at acid conditions as the EWP had pI at approximately 6.1 and 10.7 for ovotransferrin and lysozyme, respectively [29]. The SGPIs had pI at approximately 3.8, and the oil droplets surface potential was supposed to be rather low at pH 3.0-4.0. The charge on the droplet surface was reduced

by partial protonation of the fatty acids, which lead to the gradual replacement of combined repulsive interactions by the attractive colloidal interaction resulting in the physical destabilization of emulsions [30,31]. However, when the pH was far from the pI, relatively stable emulsions could be observed with mean particle diameters less than 0.35 μm at pH 7.0-8.0. Moreover, the LCT emulsions have a relatively large particle diameter and ζ-potential magnitude than MCT emulsions. The reason for this phenomenon may be due to the changes in the dispersed phase viscosity of the emulsions [32]. Generally, the efficiency of droplet crushing in a microfluidizer increases as the viscosity of the dispersed phase decreases. LCT has a distinctly higher viscosity and contains more anionic impurities than MCT [33]. Therefore, droplet breakup of LCT emulsion becomes less efficient during microfluidisation, which results in the formation of larger droplets and more negatively charged.

3.2. Effect of carrier oil on particle characteristics of BC emulsion stabilized by SGPIs during *in vitro* digestion

In order to adapt to the in vitro digestion and cell uptake, the emulsions were prepared at pH 7.0 for further study. The behavior of BC emulsions by using two different oils (LCT and MCT) were determined as they were subjected to the simulated GIT. The particle characteristics of both emulsions were evaluated at the different GIT stages to offer some information on changes in interfacial characterization. The mean particle sizes and particle size distributions of both LCT and MCT-based BC emulsions stabilized by SGPIs during digestion are shown in Figs. 3A, 3B and Table. 2, respectively. Initially, the BC emulsions containing both triacylglycerol oils had good aggregation ability as well as comparatively small particle diameter (0.35 and 0.31 μm, for LCT and MCT, respectively) with a monomodal particle size distribution. Nevertheless, there was a visible difference

between the particle electrical properties depending on carrier oil type: -43 and -26 mV for LCT and MCT, respectively (Fig. 3C), the reason for the difference in electric charges may be due to anionic or cationic impurities (e.g. phospholipids, mineral ions, or FFA) present within the lipid phase [34]. The oil droplets were evenly distributed throughout both BC emulsions without any significant droplet aggregation as confirmed by confocal scanning laser microscopy (Fig. 4).

After getting through the mouth and stomach stage, the particle diameter distributions of both BC emulsions became bimodal, accompanied by a comparative raise in mean particle size (Figs. 3A, 3B and Table. 2). The extensive oil droplet accumulation was also observed after exposure to oral and stomach stages (Fig. 4), which indicated that some flocculation had happened, especially in LCT emulsions. Meanwhile, there was a palpable decline in the magnitude of negative charge on both oil droplets after exposure to the simulated oral phase, with the surface potential of -17.20 and -13.47 mV for LCT and MCT, respectively. Subsequently, a further decrease in ζ-potential was observed when the emulsions were passed through simulated stomach phase with the surface potential of -1.4 and 0.5 mV for LCT and MCT, respectively (Fig. 3C).

The MCT emulsions exhibited relatively larger particles than LCT emulsions after simulated small intestinal stage, and the particle size distribution were broad bimodal in MCT emulsions (Figs. 3A, 3B and Table. 2). Moreover, the LCT emulsion had more negative charges than MCT emulsion (Fig. 3C). Under small intestinal phase, although the aggregated oil droplet breakup has occurred, the MCT emulsions also contained some relatively large particles (Fig. 4), which is in accordance with the light scattering measurements (Fig. 3B). Overall, all these results indicate that both LCT and MCT-based

BC emulsions stabilized by SGPIs showed exactly alike behavior when they were subjected to the simulated digestion model.247,

It has been reported that emulsions stabilized by proteins are apt to accumulation under stomach phases on account of impairing of electrostatic repulsion, hydrolyzing of adsorbed proteins, and bridging flocculation or depletion induced by mucin [35,36]. Our results draw the similar conclusion as well. Meanwhile, the significant reduction in the negative charge after exposed to the gastric phase, and the ζ-potential nearly close to zero. This phenomenon probably due to the fact that some of the proteins may have been displaced and digested, at the same time some of anionic substances (for example mucin) also adsorb the surface of the protein-coated lipid droplet [37]. Furthermore, our results were coincided with the previous studies which have interpreted that MCT emulsion result in the generation of larger particles than LCT emulsion after small intestine digestion by using Tween 20 and WPI as the emulsifier [38,39]. The highly surface negative charge of the particles in both emulsions were observed in the small intestine stage, which can be ascribed to the chemisorption of anionic colloidal particles to their surface, for instance phospholipids, bile salts, free fatty acids, and peptides [35]. Interestingly, LCT emulsions had pronouncedly higher negative charges than MCT after digestion, which indicated that the fatty acids formed by digestion of LCT were accumulated at the surface of the particles, whereas the fatty acids produced by digestion of MCT still retained in the aqueous phase [38]. Actually, it is hard to confirm the accurate nature of these particles after digestion because of the particle diversity including micelles, undigested protein aggregates, undigested lipid droplets, as well as insoluble calcium salts. The entire particles conduce to the integral signal for calculating the ζ-potential.

3.3. Effect of carrier oil on lipid digestion of BC emulsion stabilized by SGPIs

After GIT model, the impact of oil type on the extent and rate of lipid digestion was assessed by an automatic titration (pH stat) assay. The amount of FFA liberated from the lipid phase was determined during the small intestine stage (Fig. 5A). The profiles of lipid digestion in both LCT and MCT emulsions followed quite similar trends. For LCT emulsions, FFA was swiftly released throughout the first 5 minutes and afterwards more smoothly until a comparatively stable value of around 85% was reached after digestion for 2 h. Whereas, MCT emulsion showed a noticeable increase during the first 5 minutes of reaction, subsequently a more progressive release at a longer digestion time until 110% digestion was observed (Fig. 5A). These results suggest that the lipid digestion extent and rate of MCT emulsion was obviously higher than that of LCT emulsion stabilized with SGPIs.

It has been illuminated that long-chain fatty acid lipids are digested more slowly than medium-chain fatty acid lipids [40]. For instance, LCT (i.e., corn oil) includes appreciably long-chain fatty acids (i.e., C16, C18 and C20), while MCT includes comparatively shorter chain ones (i.e., C8 and C10). The relatively low amount of digested LCT possibly on account of the fact that long chain fatty acids prone to aggregate at the surface of oil-water, consequently lipase was restricted to access into the surface of the droplets [41]. In contrast, the digestive products of MCT-emulsion have high water affinity making the lipase more liable to close to lipid surfaces [42]. Interestingly, the release of total amount of FFA reached to 110% at the end of 2 h of simulated GIT digestion in the MCT-emulsions. The reason for the final amount of FFA released was greater than 100% in MCT-emulsion is attributed to some other components, such as proteins (SGPIs) in the digestion models, which were hydrolyzed and dedicated to the pH-stat process. Moreover, some monoacylglycerols might have been transformed into FFA and glycerol [43], which was not considered in the calculations. Our results are also in accordance with other

latest reports, which have demonstrated a faster digestion of MCT when they are merged into Tween 20, β-lactoglobulin and modified starches-stabilized BC emulsions in comparison with LCT, reaching to 123%, 113% and 120% after 2 h, respectively [24, 33, 43]. As a result, MCT possessing a high specific surface area are easily released from the small intestine leading to quick lipid digestion.

3.4. Effect of carrier oil on transformation and bioaccessibility of BC emulsion stabilized by SGPIs

The transformation refers to the amount of BC that remains in a bioactive form, whereas, the bioaccessibility provides an information of the BC fraction available for absorption in mixed micelle arriving at the small intestine phase. The middle layer of the digesta samples which contained the solubilized BC was collected as the micelle fractions after small intestine digestion.

Our results clearly showed that the transformation, i.e., the fraction of BC retaining in its original form after passing through the GIT was distinctly higher in the LCT-emulsion (45.2%) than in the MCT-emulsions (36.3%) stabilized by SGPIs (Fig. 5B). These results suggest that a greater fraction of the BC in LCT emulsion was not transformed at the stage of small intestine, which may be due to the fact that BC is not subject to the degradation by trapping into the interior of the micelle fractions. Thus, encapsulation of BC in LCT emulsion stabilized by SGPIs gave better protection against chemical degradation. In spite of the fact that the triacylglycerols in both LCT and MCT emulsions were nearly completely digested, the bioaccessibility of BC was significantly distinct. The determined bioaccessibility of BC was approximately 23.1% in the MCT-emulsion while about 65.5% in the LCT-emulsion (Fig. 5B). BC is a highly hydrophobic linear rod-like structure molecule, which is favorable to pass through the entire micelle core for successfully combination with the surfactant

micelles [24]. The long-chain fatty acids are more probably to form micelles with a larger solubilisation ability on account of the larger sizes of their hydrophobic core in comparison with medium-chain fatty acids. Our studies are consistent with some researches showing that BC own a higher bioaccessibility in Tween 80 stabilized emulsion systems when LCT is utilized as a carrier oil rather than MCT [44]. Furthermore, the unsaturation degree of fatty acids can decide the GIT fate of BC as well as its bioaccessibility [45]. Corn oil contains huge amounts of monounsaturated and polyunsaturated long chain fatty acids, which are presumed to be beneficial for BC bioaccessibility. In short, LCT is more effective carrier oil for ensuring a comparatively high BC bioaccessibility than MCT in SGPIs stabilized BC emulsion system since MCT do not form large enough mixed micelle fractions to dissolve BC.

3.5. Cell Toxicity and uptake of BC emulsion stabilized by SGPIs after digestion in Caco-2 cells

After digestion, the BC solubilized within micelle fractions are likely be absorbed in the epithelium cells by different mechanisms such as active or passive transport [46]. Thus, the latent cytotoxicity of the different digested samples on the Caco-2 cells was evaluated. The cells were pretreated with BC-loaded micelles stabilized by either LCT or MCT of different dilution times (DT) for 24 h. As shown in Fig. 6A, no cytotoxicity was found in Caco-2 cells treated with pure BC (dispersed in THF/DMSO) at concentration of 0.1-0.005 μg/mL. The micelle samples included lipid digestion products, protein, as well as GIT components (for instance bile salts). The cell viability was greater than 92% after treatment with both LCT and MCT mixed micelles at any diluted times (Fig. 6A), which indicates the micelles from SGPIs stabilized emulsion and GIT components did not damage the cells within the incubation period, and also did not adversely affect the integrity of Caco-2 cell monolayer. These results were consistent with the preceding study [17], which exhibited that the micelles from quillaja saponins

stabilized VE emulsion consisted of LCT and MCT were both nontoxic and biocompatible with Caco-2 cells. Hence, this model of cell culture was appropriate for evaluating the BC absorption in the native SGPIs-based emulsion delivery systems.

It is supposed that the mixed micelle fractions solubilize the BC and subsequently transport it to the surface of the Caco-2 cells [47]. Therefore, a Caco-2 cell model was utilized to simulate the uptake of the dissolved BC by epithelium cells. After incubation with the samples (DT=20, the final BC concentration was 0.05 μg/mL) for 24 h, the content of BC that had accumulated in cells was measured. The cellular uptake of BC in THF/DMSO (1:1, *v/v*) suspension was 112.0 pmol/mg protein, whereas the cellular uptake of BC in LCT and MCT emulsions stabilized by SGPIs were 1.9- and 1.5-fold increase, respectively (Fig. 6B). These results are consistent with the previous studies which indicated that the cellular uptake values of whey protein isolates, sodium caseinate, and soybean protein isolates loaded BC emulsions were 687, 891, and 452 pmol/mg protein, which were significantly higher than that of the THF/DMSO-BC control (246 pmol/mg protein) [48]. The potential reason for the difference between our results and previously published results is that previous studies mainly focused on the emulsions that were directly applied to Caco-2 cell model without GIT digestion, while the emulsion of our study was applied to Caco-2 cell model after *in vitro* GIT digestion. The LCT resulted in a higher absorption of the BC than MCT in SGPIs-stabilized emulsion, which is similar to the effect of oil type on VE bioaccessibility as reported previously [17]. Actually, there are several reasons for explaining this phenomenon. In the first place**,** the property of the mixed micelle fractions formed is known to lie in the type of fatty acids, which may have affected the BC transport to the cells surface. The mixed micelle fractions include liquid crystalline phases, vesicles, and micelles which may change in their

dimensions and structure, consequently altering their absorption characteristics. In the second place, some fatty acids have the ability to enhance the permeability of cell membrane, which would change the absorption of the hydrophobic bioactive component. As a result, for disparate kinds of lipid digestion products generated by LCT and MCT, Caco-2 cells may have absorbed different amounts of BC. Thirdly, the intracellular processing and absorption way of hydrophobic substance like BC were also influenced by the chain length of fatty acids. Medium chain fatty acids are generally diffused through the epithelium cells rather than being shipped to the endoplasmic reticulum like long chain fatty acids, which are then transformed into triacylglycerols for secreting into the lymphatic system[17, 49]. As a result, more of BC in LCT emulsion was absorbed by the epithelium cells than that in MCT emulsion.

In our study, a comparatively simple HPLC technique was utilized to measure the amount of BC. In our future researches, it would be beneficial to utilize more comprehensive analytical techniques, for instance HPLC-MS/MS, to offer more detailed information in regard to changes in the BC chemical structure throughout the GIT and Caco-2 cells.

4. Conclusion

To sum up, our study reveals that BC-loaded emulsions were produced and stabilized by SGPIs with either LCT or MCT as a carrier oil. Both LCT and MCT delivery systems exhibited favorable stability at pH 7-8 conditions. The particle size of both emulsions increased considerably after passing through the oral and gastric stages, which was ascribe to the aggregation of droplet, while the relatively small particle diameters with lower FFA released but higher transformation and bioaccessibility of BC after exposure to small intestine phase was observed in LCT emulsion than that of in MCT emulsion.

Moreover, the micelles from SGPIs-stabilized both emulsions were nontoxic. LCT was more valid at increasing the absorption of BC in Caco-2 cells than MCT, and they were both significantly higher than that of pure BC. In general, our results indicate that SGPIs-based emulsions fabricated using LCT (i.e. corn oil) as a carrier oil are more appropriate for delivering BC than MCT, which provide significant instructions for designing efficient natural emulsion-based delivery systems for these hydrophobic bioactive compounds.

Acknowledgements

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Figure legends

Fig. 1. Impact of pH on the particle size distribution of different SGPIs-based delivery systems: (A) LCT emulsions and (B) MCT emulsions. (C) Influence of pH on the electrical characteristics (ζ-potential) of different SGPIs-based delivery systems.

Fig. 2. Effect of carrier oil on microstructures of BC emulsions stabilized by SGPIs under different pH conditions: (A) LCT and (B) MCT (scale bar is 1 μm).

Fig. 3. Particle size distribution of SGPIs-stabilized BC emulsions fabricated by different carrier lipids after passing through different stages of a simulated GIT: (A) LCT and (B) MCT. (C) ζ-potential of SGPIs-stabilized BC emulsions containing different carrier lipids after exposure to different phases of simulated GIT. Different capital letters (A-D) compared between different GIT stages are significantly different ($p < 0,05$). Different lowercase letters (a-b) compared between different carrier oils type are significantly different ($p < 0.05$).

Fig. 4. Microstructure of SGPIs-stabilized BC emulsions with different carrier lipids: (A) LCT; (B) MCT after they were exposed to different stages of a GIT.

Fig. 5. (A) Amount of FFA released from SGPIs-stabilized BC emulsions containing different carrier oils using pH-stat in GIT model. (B) Transformation and bioaccessibility of BC in emulsions stabilized by SGPIs with different carrier oils. Data with different letter (a, b) are significantly different ($p < 0.05$).

Fig. 6. (A) In vitro cytotoxicity of SGPIs-stabilized BC emulsions with different carrier oils and pure BC (as control) at different dilution times (DT) on Caco-2 cells determined by MTT assay. (B) Uptake of BC by Caco-2 cell monolayers treated with SGPIs-stabilized BC emulsions with different carrier oils and pure BC at final BC concentration of 0.05 μg/mL. Data with different letters (a-c) are significantly different ($p < 0.05$). Molecular weight of BC is 536.88 g/mol, 1 μg/mL=1.8626×10^6 pmol/L.

Table 1. Influence of pH on the mean particle diameter of different carrier oil delivery systems

	Mean particle diameter (μm)					
Samples	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
LCT-emulsion	19.8±1.28^{a}	25.42±5.79^{a}	2.58±0.06^{b}	0.45±0.03^{c}	0.34±0.03^{c}	0.31±0.01^{c}
MCT-emulsion	10.99±0.18^{a}	23.94±2.92^{b}	1.72±0.05^{c}	0.39±0.02^{d}	0.30±0.06^{d}	0.26±0.02^{d}

Data with different letters in the same row are significantly different ($p < 0.05$).

Table 2. Mean particle diameter of BC-emulsions stabilized by SGPIs with different carrier oil after exposure to different GIT regions

	Mean particle diameter (μm)			
Samples	initial	mouth	stomach	intestine
LCT-emulsion	0.35±0.01[a]	1.92±0.51[a]	15.69±2.26[a]	3.36±0.25[a]
MCT-emulsion	0.31±0.05[a]	0.84±0.48[b]	12.77±1.63[a]	10.81±0.72[b]

Data with different letters in the same column are significantly different ($p < 0.05$).

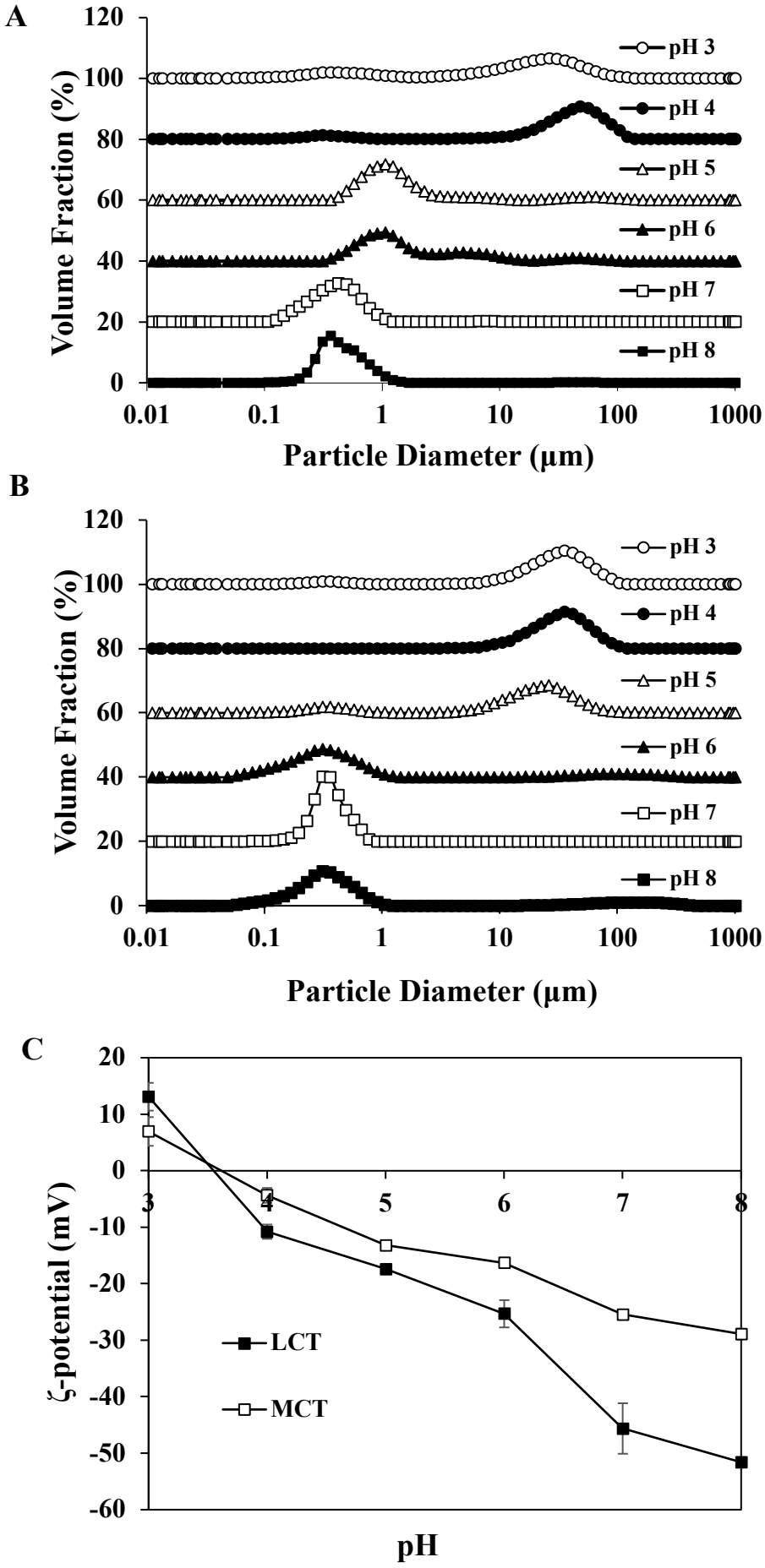

A
Volume Fraction (%)
120
100
80
60
40
20
0
pH 3
pH 4
pH 5
pH 6
pH 7
pH 8
0.01
0.1
1
10
100
1000
Particle Diameter (μm)
B
Volume Fraction (%)
120
100
80
60
40
20
0
pH 3
pH 4
pH 5
pH 6
pH 7
pH 8
0.01
0.1
1
10
100
1000
Particle Diameter (μm)
C
ζ-potential (mV)
20
10
0
-10
-20
-30
-40
-50
-60
3
4
5
6
7
8
LCT
MCT
pH

Fig. 1

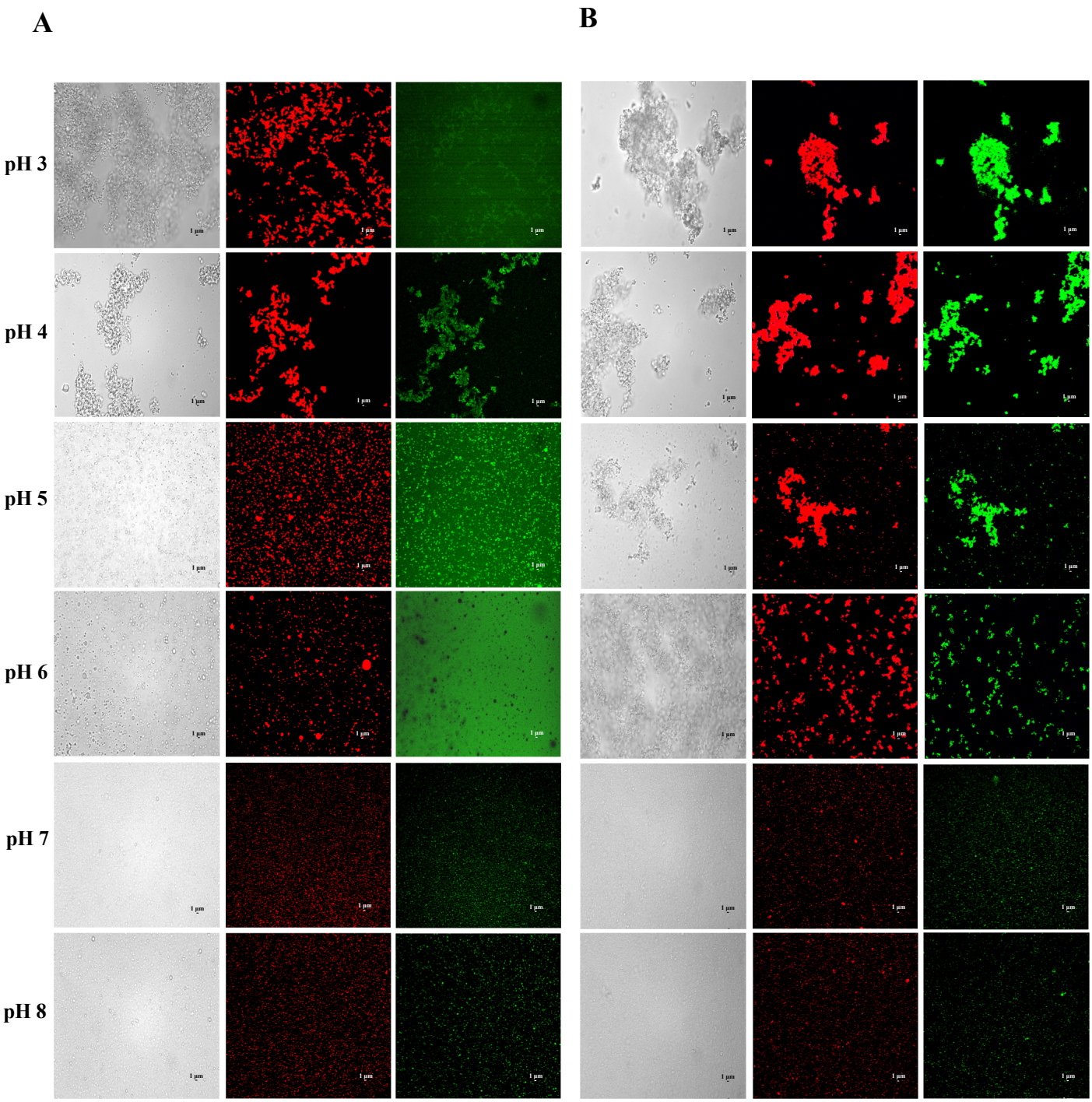


Fig. 2

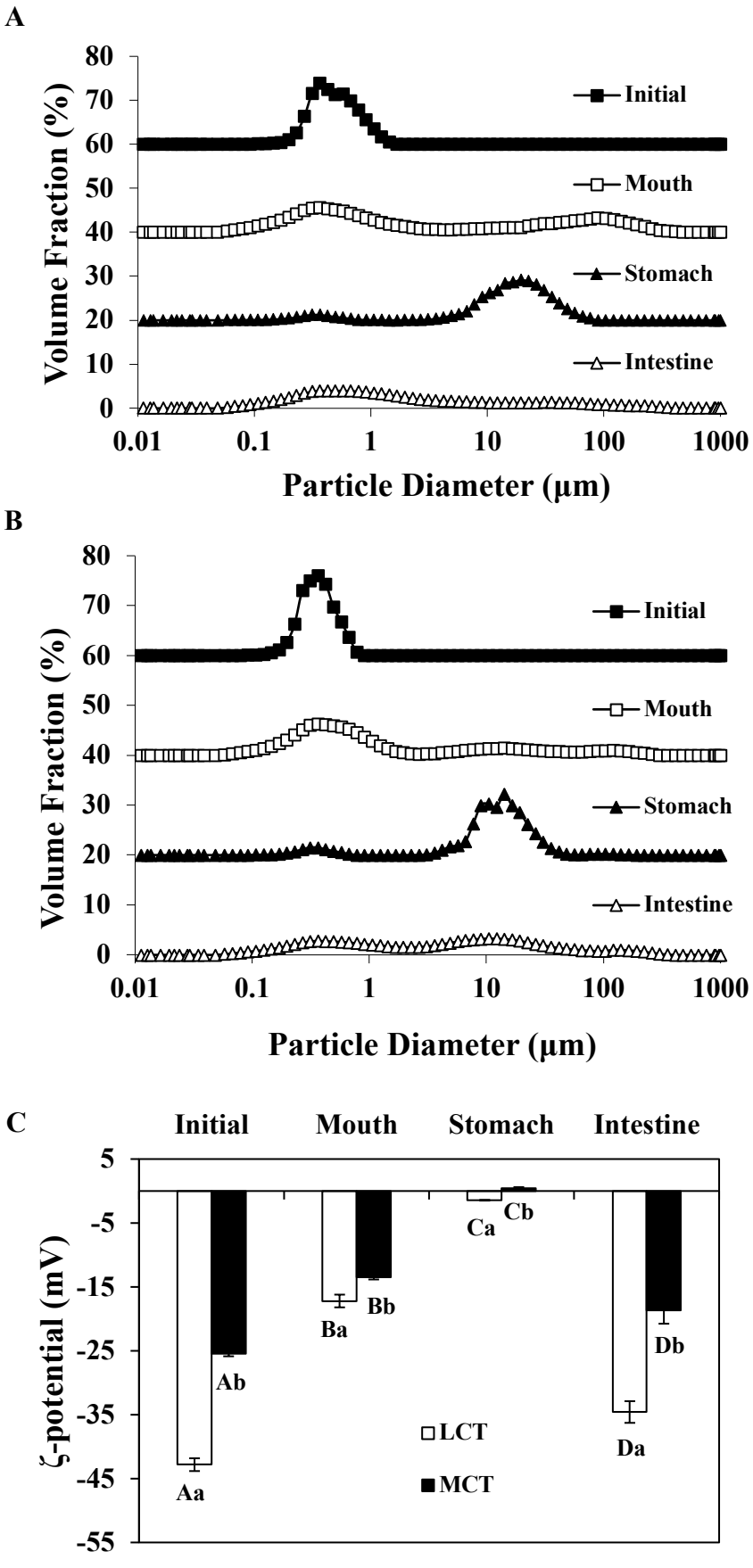

A
Volume Fraction (%)
80
70
60
50
40
30
20
10
0
Initial
Mouth
Stomach
Intestine
0.01
0.1
1
10
100
1000
Particle Diameter (μm)
B
Volume Fraction (%)
Initial
Mouth
Stomach
Intestine
Particle Diameter (μm)
C
Initial
Mouth
Stomach
Intestine
ζ-potential (mV)
5
-5
-15
-25
-35
-45
-55
Aa
Ab
Ba
Bb
Ca
Cb
Da
Db
LCT
MCT

Fig. 3

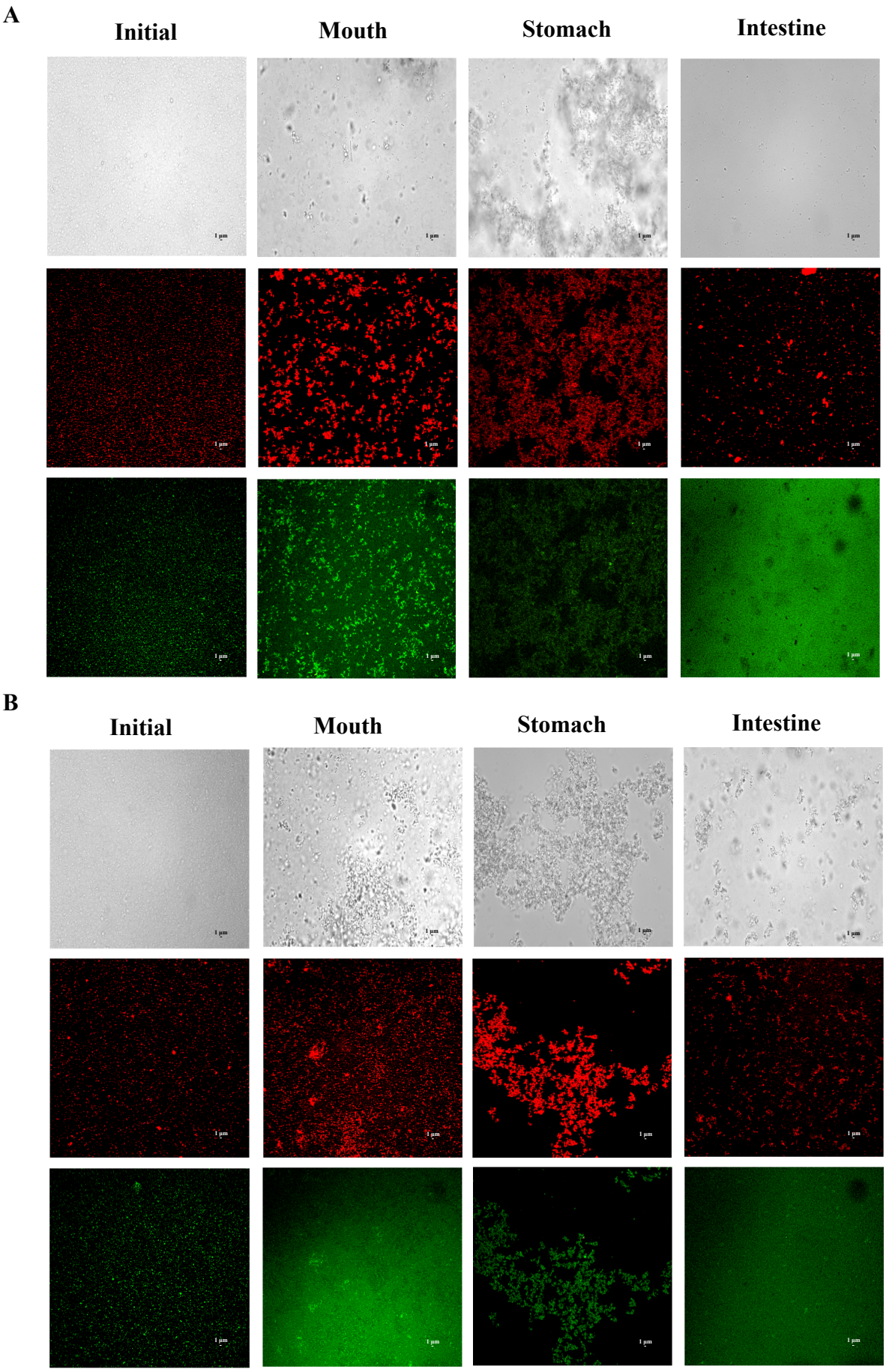
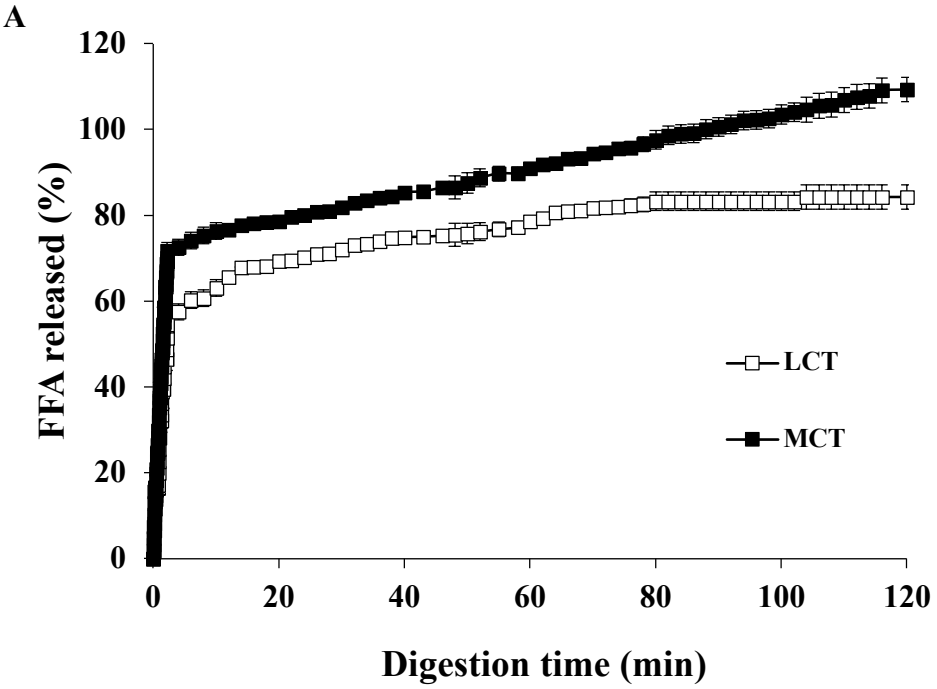

A
Initial
Mouth
Stomach
Intestine
B
Initial
Mouth
Stomach
Intestine

Fig. 4


A
120
100
80
60
40
20
0
FFA released (%)
0
20
40
60
80
100
120
Digestion time (min)
LCT
MCT

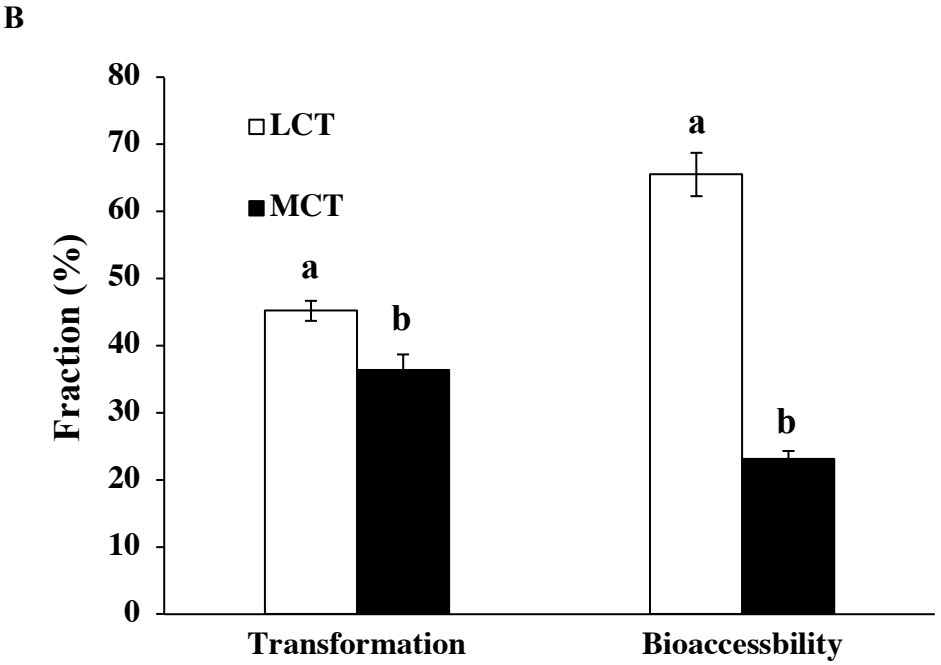

B
80
70
60
50
40
30
20
10
0
Fraction (%)
LCT
MCT
a
b
a
b
Transformation
Bioaccessbility

Fig. 5

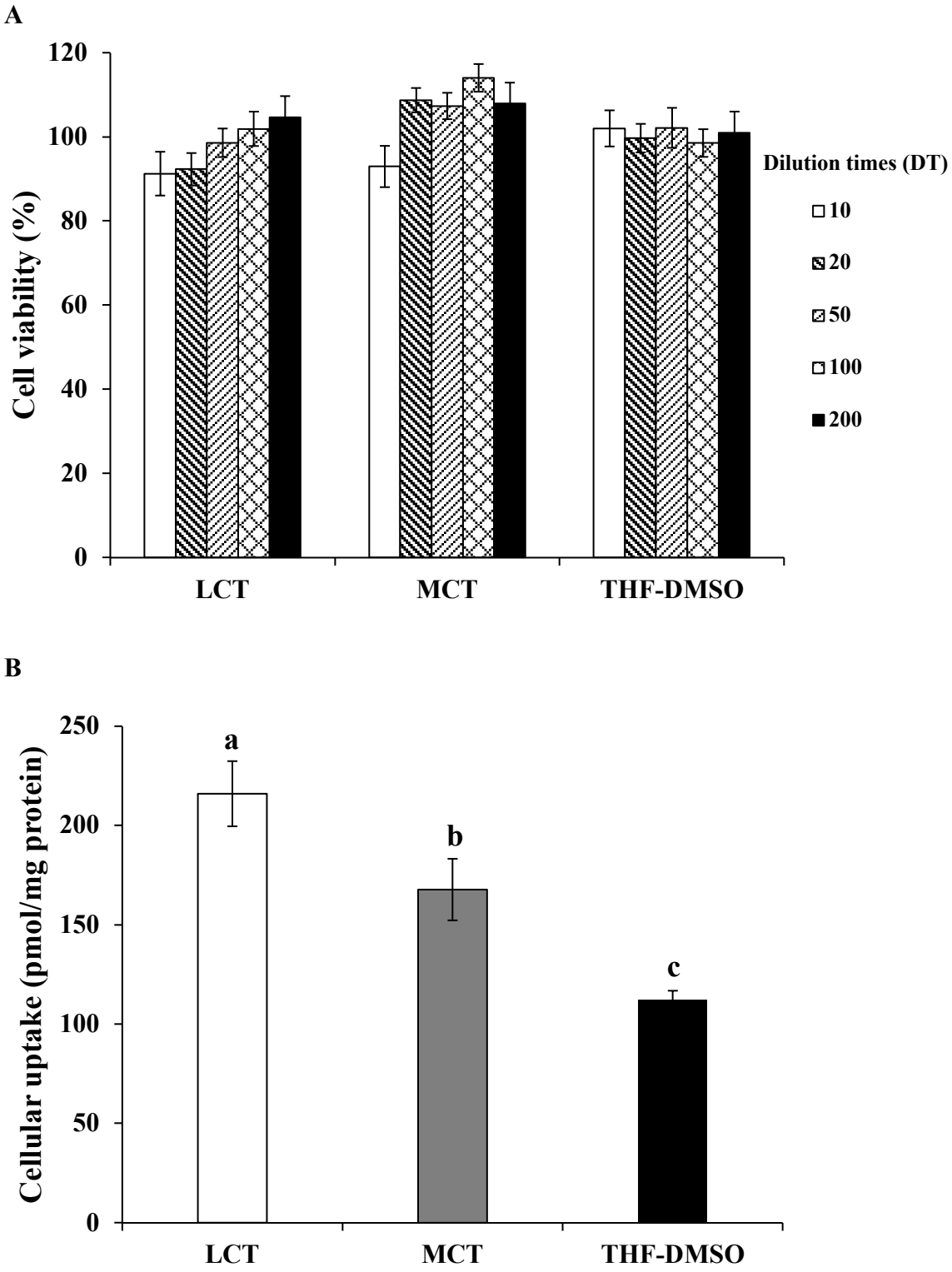
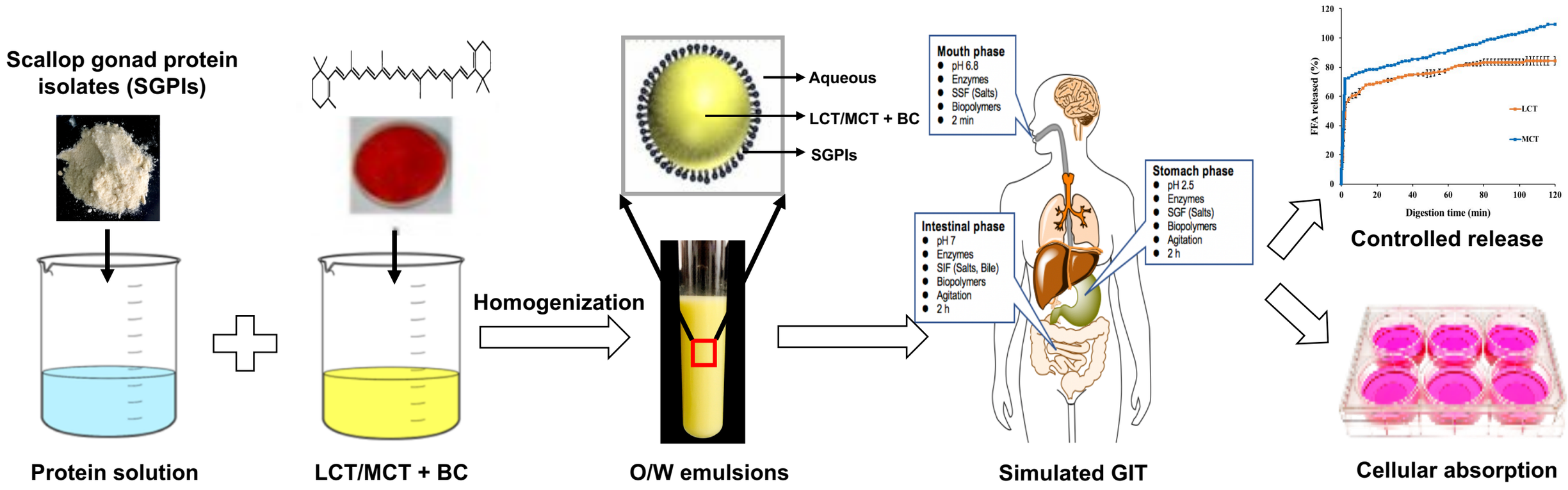

A
Cell viability (%)
120
100
80
60
40
20
0
LCT
MCT
THF-DMSO
Dilution times (DT)
10
20
50
100
200
B
Cellular uptake (pmol/mg protein)
250
200
150
100
50
0
a
b
c
LCT
MCT
THF-DMSO

Fig. 6



GRAPHICAL ABSTRACT