Korean J. Chem. Eng., **26**(1), 131-135 (2009)

Enhanced oral absorption of salmon calcitonin-encapsulated PLGA nanoparticles by adding organic substances

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(*Received 22 April 2008 • accepted 10 July 2008*)

Abstract−Two organic compounds with potential absorption enhancing effects, bile acids and transferrin, were examined by the gastro-intestinal (GI) absorption of therapeutic salmon calcitonin (sCT) as encapsulated by poly(lactide-co-glycolide) (PLGA) for the treatment of osteoporosis. The sCT-loaded PLGA nanocapsules were prepared by O/W emulsification approach. Either additive of a designated content was mixed with sCT dissolved in methanol. For bile acids, their content (0-7.5 mg to sCT 6 mg) was observed to have a substantial effect both on the emulsification process and the encapsulation efficiency. When 1.5 mg of bile acids was added, sCT-loaded PLGA nanocapsules of about 700 nm in diameter and with a fairly high encapsulation efficiency greater than 35% were produced. Accordingly, this formulation gave the most significant hypocalcemic effect in an in vivo experiment with SD rats. On the other hand, a too high bile acids loading resulted in a poor encapsulation efficiency of less than 7%. Two principal roles of bile acids were proposed: emulsifying agent and absorption enhancer. Transferrin, a human glycoprotein of 80 kDa molecular weight, turned out to have potential as absorption enhancer as well.

Key words: Salmon Calcitonin, Poly(lactide-co-glycolide), PLGA, Absorption Enhancer, Bile Acid, Transferrin, Oral Delivery, Encapsulation

INTRODUCTION

Peptide medicinal substances such as calcitonin are generally classified as drugs with a relatively poor oral absorption efficiency, primarily associated with their low stability against endocrine enzymes and their poor permeability across the intestinal epithelium.

A pioneering encapsulation study using polymeric carriers ignited research into a mass of various polymeric drug delivery research efforts [1]. A recent series of articles have reviewed the up-to-date status of polymeric encapsulation to deal with the preparation of drug-loaded polymer particles and their biomedical applications [2,3]. The low permeability of free or encapsulated sCT through the intestinal epithelium has been the main obstacle for the oral delivery. Three mechanisms were proposed to enhance the transport of the associated peptide molecules: (a) mucoadhesion, (b) particle interaction/internalization, and (c) permeation enhancing effect [4].

PLGA or poly(lactide-co-glycolide) is a copolymer used in a host of Food and Drug Administration (FDA) approved therapeutic devices, owing to its biodegradability and biocompatibility. Depending on the ratio of lactide to glycolide, biodegradation characteristics are varied. PLGA has been successful as a biodegradable polymer because it undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid. These two monomers under normal physiological conditions, are by-products of various metabolic pathways in the body [5]. Among various preparation methods, reported was the one by dialysis without any surfactant. The optimal ratio of lactide to glycolide was significantly dependent on the drug loading content [6].

Better mucoadhesion or bioadhesion would be likely to increase the residence time and contact of medicinal molecules with the underlying epithelium, which would consequently increase their chance to be absorbed there [7]. In the light of particle interaction, it has been accepted that colloidal particles with hydrophobic surfaces are generally absorbed better than those with hydrophilicity [8], even though there are some exceptions for PEG [9] or chitosan [10]. Otherwise, factors affecting the interaction or internalization of colloidal particles with intestinal epithelium have not yet been well clarified. Following is a summary of recent research results regarding the absorption improvement of calcitonin or other peptide molecules.

Among various absorption enhancing substances, chitosan has been examined most intensively. Chitosan-coated nanostructures such as particles or capsules, were reported to modify the trans-epithelial resistance to result in an improved absorption [11]. In a pulmonary delivery study of calcitonin, surface-modified PLGA nanosphere with chitosan turned out to improve adsorption by mucoadhesion and opening of the tight intercellular junctions [12]. In addition, reduced elimination rate and sustained effect were observed as well.

Recently PEGylation has gathered substantial attention as a tool for absorption enhancement [13,14]. Nanocapsules using chitosan chemically modified with PEG were tested via Caco-2 cells for enhanced and prolonged peptide absorption [13]. For a noticeable improvement by chitosan-PEG nanocapsule approach, the PEGylation degree should be greater than 1%. In another study, the beneficial effect of the Lys^{18}-amine specific PEGylation of sCT on its intestinal delivery was examined [14]. Even though there was no signifi-

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cant increase in intestinal permeability over unmodified sCT, there was a substantial increase in resistance to pancreatic peptidases and brush-border peptidases. Various surfactants including bile salts were employed to improve intestinal absorption of sCT-loaded proliposomes [15]. Among them, TDC (taurodeoxycholate) 0.1% proliposome gave 7-11-fold increase in absorption, probably attributed to the formation of lipophilic ion-pair complexes between sCT and NaTDC.

According to Na-glycocholate study, the rank order of absorption of each compound from various administration sites was lung>small intestine≥nasal cavity≥large intestine≥buccal cavity [16]. It turned out that an absorption enhancer works well for an administration route while it affects near zero for another. Characteristics of three NO donors, NOC5, NOC12, and SNAP absorption enhancers for peptide drugs were studied by using a modified Ussing chamber method [17]. Overall, SNAP was the most effective enhancer by giving 3.4-5.6-fold enhancing effect over the control condition in the jejunum and ileum and 11.3-fold improvement in the colon. It was concluded that NO donors possess excellent effectiveness for use as absorption enhancers of peptide drugs even at lower concentrations such as 0.01 nM. The absorption-enhancing effect of sodium caprate (Cap-Na) and dipotassium glycyrrhizinate (Grz-K) combined was investigated by using Caco-2 cells [18]. The enhanced absorption by Grz-K possibly resulted via PKC cellular signaling pathway after penetration into cells according to increasing membrane permeability by Cap-Na. Little mucosal injury and negligible toxicity on Caco-2 cells were observed.

A novel mucolytic agent and a non-ionic surfactant were applied for the absorption enhancement of sCT [19-21]. N-acetylcysteine (NAC) was examined for intestinal absorption enhancement and acute damage on mucosa combined with surfactant Triton X-100. Their combined enhancement effect was determined to be 12.5 times at maximum as high as control, being much greater than classical enhancers such as sodium deoxycholate, citrate, and taurocholate while comparable to the combination of citrate and taurodeoxycholate [19]. The morphological acute damage on intestinal mucosa was reversible. The effect of NAC was investigated for nasal absorption [20]. In this study, laureth-25 was used as no-ionic surfactant. When they were applied combined, the bioavailability upon nasal route increased to be higher than 20%, about three times greater than nasal control. The potential tissue damage in terms of hemolytic activity turned out to be nil or slight. When ethylcellulose was added as a filler, nasal absorption was further improved [21]. A proposed enhancing mechanism was given.

There should be many more substances and/or technology to study for the sCT oral delivery with a better absorption as well as a higher safety. Two organic compounds were examined as absorption enhancer here while our nanoencapsulation approach was presented in detail elsewhere [22]. The primary goal of this study is to demonstrate the enhanced oral absorption of sCT-incorporated PLGA nanocapsules with several organic additives including bile acids and to propose a feasible mechanism.

EXPERIMENTAL

Two organic molecules investigated as absorption enhancer were bile acids and transferrin. Salmon calcitonin was purchased from Bachem AG (Bubendorf, Switzland). PLGA of 50 : 50 molecular ratio and of molecular weight 40,000-75,000 was supplied from Boehringer Ingelheim (RG503H). All other chemicals were of reagent grade and used as received without further purification.

sCT of 6 mg was dissolved in methanol of 240 μL at room temperature. In a separate beaker, PLGA of 120 mg was dissolved in dichloromethane (DCM) of 9.5 mL. These two solutions were mixed to make a clear homogeneous mixture solution. Then, the mixture solution was transferred into, and homogenized in, 1% PVA solution of 200 mL which was under a vigorous agitation. A peristaltic syringe pump (KSD 100, KD Scientific, USA) with a 30-guage needle was used to retain 1.5 mL/min transfer rate. The Ultra-Turrex mixer (T25, IKA, Germany) was employed as high-speed stirrer and homogenizer.

Emulsification occurred as soon as the initial mixture solution got into 1% PVA solution. In most cases, the mixture became slightly cloudy as the emulsion phase was created. Based on a preliminary study, the homogenization speed was fixed at 7,000 rpm, which repeatedly gave submicron-sized PLGA particles. At each homogenization condition, the stirring operation was performed for 5 minutes followed by the addition of DI water of 200 mL.

After an additional 5 minute stirring, the emulsion mixture was stored at 40 °C for an hour to partly remove DCM. The emulsion was pulled by a vacuum for complete removal of residual organic solvents. In this process, the emulsion particles in O phase precipitate and convert to suspension solid particles. The suspension was centrifuged (Combi-514R, Hanil Science Ind., Korea) to collect precipitated substance. The precipitate was washed three times with DI water and freeze-dried for complete removal of moisture.

For capsules containing the bile salt as absorption enhancer, sCT of 6 mg and bile salts of various contents (0.75-7.5 mg) were dissolved together in methanol of 240 μL at room temperature. Further procedure was exactly the same as that for sCT-encapsulated PLGA particles without bile salt. Other enhancers were added exactly the same as described above.

The particle morphology was measured by SEM (S4300-SE Hitachi, Japan), while HPLC (Autochro-2000, YoungLin, Korea) was employed for chemical analysis of sCT and other compounds. The particle size and polydispersity was determined by using PSA (BIC, 90 Plus, USA). Sprague-Dawley rats weighing 210-250 g were selected for our in vivo experiment. They were purchased as 5-7 weeks old and left for adaptation until 8 weeks old. sCT was dissolved in medical saline solution prior to oral administration by using an oral zoned or to intravenous (IV) injection into tail vein. One IU for sCT is 0.2 μg and the unit dose for IV administration to each rat was 500 IU. Oral dose was 12.5 times of IV unit dose, based on the amount of sCT initially started with.

A venous catheter was installed into the jugular vein near the jaw bone. Whenever 0.4 mL of blood was collected for analysis, the equivalent amount of saline solution was restored for prolonged vitality. The collected blood was characterized both for calcium ion concentration and for sCT content. The former was determined via photometry method using Ca^{2+} Kit (FUJI Dry-Chem Slide Ca-PIII, Japan) combined with spectrophotometer (FUJI Dry-Chem 3500i, Japan), whereas the latter was measured by ELIZA method.

RESULTS AND DISCUSSION

Table 1. Comparison of particle size and encapsulation efficiency at various bile acids contents: A typical example

Classification	Added amount of bile acids (mg)		
	no bile acids	1.5	7.5
Particle size (nm)	1,021±151	698±42	245±15
Encapsulation efficiency (%)	38.6±3.5	35.4±7.1	6.3±1.6

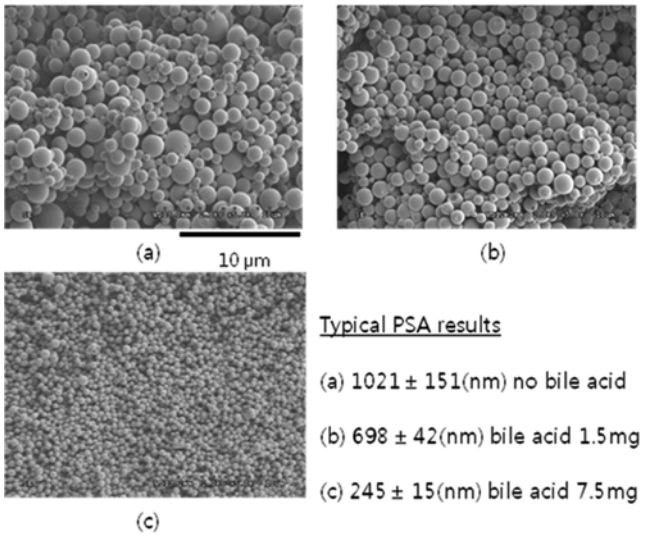

Fig. 1. The effect of bile acids presence on PLGA particle size: A typical example.

Table 1 and Fig. 1 show a typical example of the effect of bile acids addition to sCT-encapsulated PLGA particles on the particle size and the encapsulation efficiency. All experiments were performed at 7,000 rpm homogenization speed. In all cases, the particles were close to spherical shape. As the amount of added bile acids was increased, the sCT-PLGA particle size was decreased to a large extent. With a normal O/W emulsification process, particles of about one micron were produced. When 7.5 mg of bile acids was added to 6 mg of sCT, the average size of the prepared particles went down to about 245 nm with an outstanding size distribution.

Bile acids whose major constituents in human beings are cholic acid and chenodeoxycholic acid are steroid acids found predominantly in mammals' bile. They are known to serve multiple functions, one of which stands for emulsifying lipids and fat soluble vitamins in the intestine. It is also known that 20-30 grams of bile acids are secreted into an human adult intestine daily and about 90% of excreted bile acids are reabsorbed by active transport in the ileum and recycled. This is referred to as the 'enterohepatic circulation'. In this regard, bile acids are strongly expected to give a certain enhancing role for the oral absorption of drug substances.

On the other hand, the emulsifying effect of bile acids was not necessarily favorable to the encapsulation. The encapsulation efficiency is designated as the percentage of sCT amount encapsulated within PLGA matrix compared to the sCT amount initially added to prepare sCT-incorporated PLGA particles via O/W emulsification approach. Up to 1.5 mg bile acids addition, there was no significant decrease in encapsulation efficiency. The encapsulation efficiency, however, was reduced to less than 7% as the bile acids were added at 7.5 mg, the maximum loading in this study.

The above results indicate that bile acids are very active as emulsifier during the emulsification process, but not active as interfacial barrier in preventing sCT molecules from diffusing into W phase. Since sCT is soluble in water as well as in methanol, it would migrate for partition from O phase to W phase to a certain extent. Nevertheless, it is not clear whether the loss of sCT molecules into W phase took place during the emulsification process or during the evaporation process.

It has been reported that a smaller particle would be absorbed better intestinally because of favorable mucosal uptake through tight junctions between enterocytes, so-called paracellular transport [23]. With a very high homogenization speed such as 15,000 rpm, sCT-PLGA nanocapsules of less than 200 nm in diameter were produced. This approach, however, brought some problems in terms of poor polydispersity and extremely low encapsulation efficiency.

Fig. 2 presents schematic cross-sectional views of sCT-PLGA particles with and without bile acids. When the mixture of sCT and

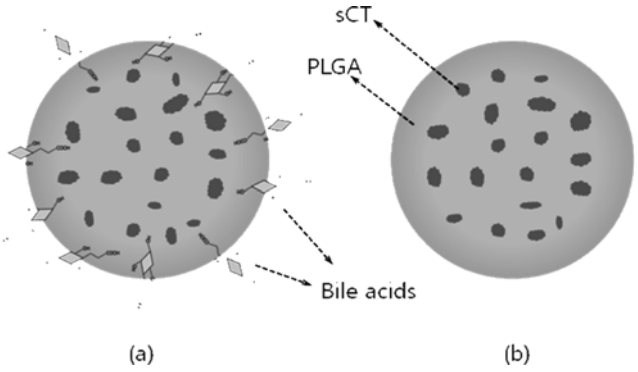

Fig. 2. Schematic cross-sections of sCT-encapsulated PLGA particles: (a) with bile acids and (b) without bile acids.

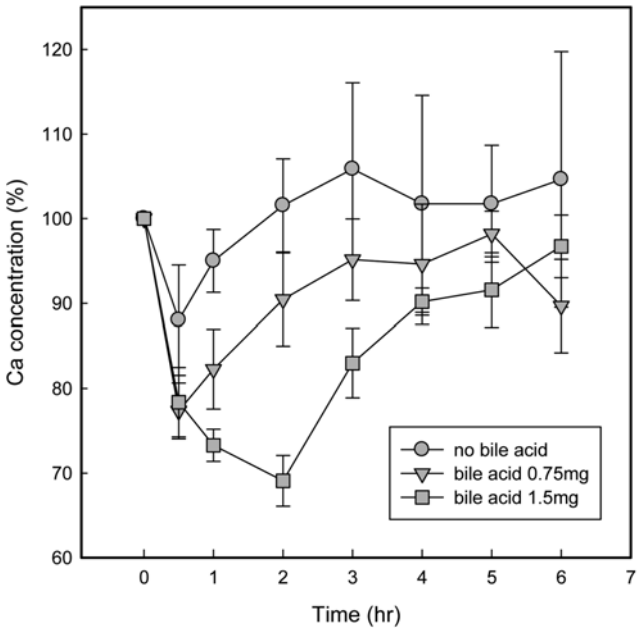

Fig. 3. The hypocalcemic effect of bile acids: (a) control (no bile acids), (b) 0.75 mg bile acids, and (c) 1.5 mg bile acids.

PLGA was emulsified as O phase in water, they would have made a homogeneous solution. As organic solvents (DCM and methanol) were removed from the emulsion during evaporation, Both sCT and PLGA precipitated to form particles of dispersed solid mixture. Since the mass ratio of PLGA to sCT is rather large (~33), PLGA should take up a continuous phase as shown in Fig. 2(b). When bile acids were added to sCT in solution, the agent would have played an emulsifying role and would be localized near a particle's surface as illustrated in Fig. 2(a).

Fig. 3 illustrates the effect of bile acids presence on the therapeutic action. The hypocalcemic activity of orally administered sCT was expressed in the reduction of Ca ion concentration in plasma as standardized to initial (@ time=0) Ca concentration. A reduction in bone resorption by sCT action would result in a lowered calcium level in plasma. When sCT-PLGA particles without bile acids were administered, the calcium ion concentration was initially decreased. It moved up back to ~100% right after two hours. The hypocalcemic effect seemed more profound at bile acids of 1.5 mg than at that of 0.75 mg. Each data point was obtained by averaging data from 2-3 separate measurements. Even with substantial magnitude of error bars, there was apparent difference in hypocalcemic effect. When particles of 1.5 mg bile acids were taken, the calcium level went down to 70%.

Fig. 4 summarizes results from another separate in vivo experiment to elucidate the effect of bile acids content. Among four different loadings of bile acids, sCT-PLGA particles of 1.5 mg bile acids appeared to be the best in terms of hypocalcemic effect. When bile acid loading was 3.75 mg or greater, the decrease in Ca ion concentration in plasma was noticeable deteriorated. This observation could be explained as associated with Table 1, that is, due to poor encapsulation efficiency.

For each in vivo experiment by oral administration to SD rats, as described in 'Experimental', the same dose based on the initial sCT amount was administered. When 7.5 mg of bile acids was added to 6 mg of sCT, sCT-PLGA particles were produced with excellent size uniformity and small size such as 250 nm in diameter. But since the encapsulation of sCT was not as effective, the resulting in vivo results were far from desirable. At this moment, 1.5 mg of bile acids to 6 mg of sCT could be claimed as the optimal loading. Supplementary experimentation should be carried out for more reliable findings.

Fig. 5 similarly shows the effect of transferrin presence in sCT-incorporated PLGA nanocapsules on therapeutic action. Transferrin is a glycoprotein of about 80 kDa molecular weight in blood plasma, especially for iron ion delivery via the reversible binding with two specific high affinity Fe(III) binding sites [24]. When a transferrin

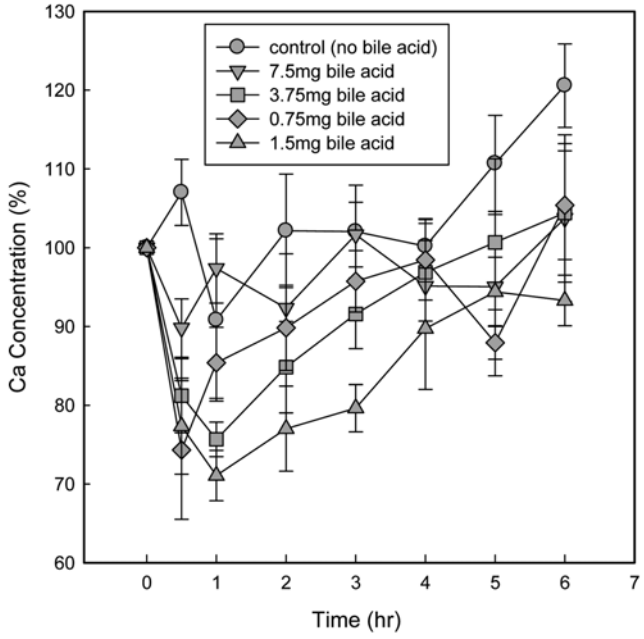

Fig. 4. The hypocalcemic effect at various levels of bile acids addition: 0.75-7.5 mg bile acids.

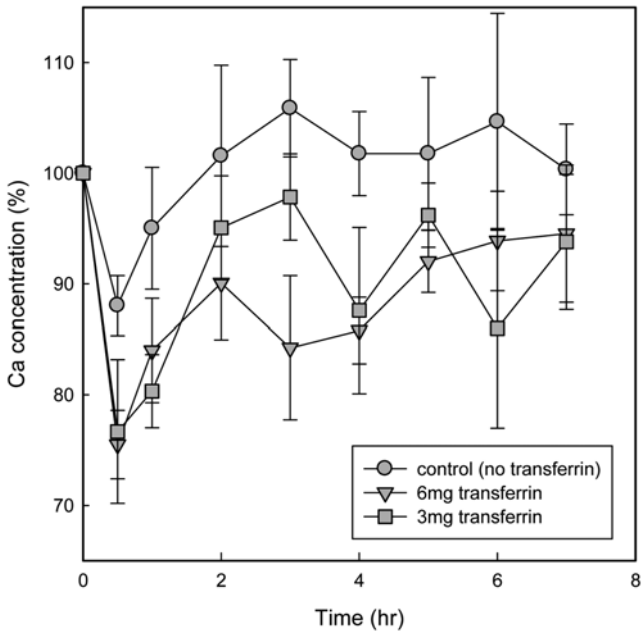

Fig. 5. The hypocalcemic effect of transferrin: (a) control (no bile acid), (b) 3 mg transferrin, and (c) 6 mg transferrin.

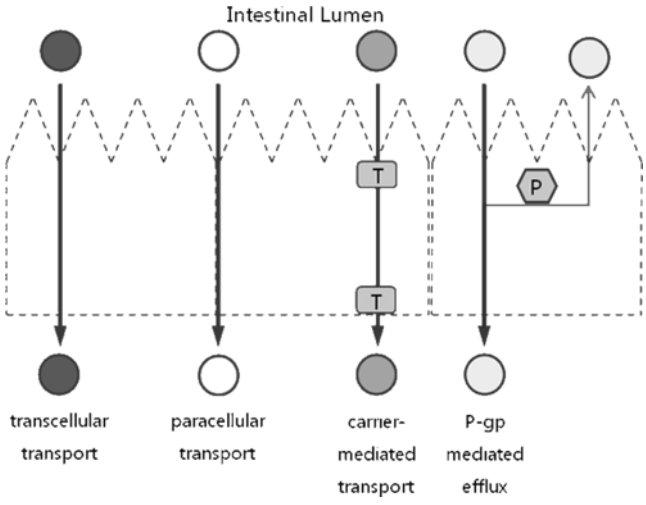

Fig. 6. Principal intestinal membrane transport mechanisms: transcellular transport, paracellular transport, carrier-mediated transport, and P-gp mediated transport.

loaded with iron encounters a transferrin receptor on the surface of an intestinal cell, it binds to it and is consequently transported into the cell in a vesicle. Transferrin also gave enhanced hypocalcemic effect compared to the control data. The sCT-PLGA particle size, however, was not noticeably affected by transferrin loading level. It would be because transferrin is hardly acting as an emulsifier. Besides, the superiority between 3 mg and 6 mg loading in terms of hypocalcemic effect was hardly differentiated as shown in Fig. 5.

According to our preliminary in vivo experiment, sCT-PLGA particles gave definitely much better sustained therapeutic effect than sCT intravenous administration. This implies that the sCT absorption through the GI tract takes place primarily not in the form of molecules but as nanocapsules. As illustrated in Fig. 6, intestinal absorption of drug substances is achieved by four major mechanisms. Among them, transcellular transport and carrier-mediated transport could be associated with our sCT-PLGA nanoparticles with absorption enhancer. Part of enhancing agents' role is bioadhesion or mucoadhesion with/without intestinal receptor, which would prolong the transit time of substances to be absorbed. The other action is known as a temporary widening of intercellular tight junction, which would eventually accelerate transcellular permeation of sCT-loaded PLGA nanoparticles into the blood vessel.

CONCLUSION

The oral absorption of sCT-encapsulated PLGA particles was significantly increased with bile acids added to the formulation when in vivo experiments were performed on SD rats. This enhancing effect of bile acid is possibly associated with two factors: emulsifier action and bioadhesion action. The addition of bile acid to sCT phase tends to lower the emulsion size without a negative effect on the encapsulation efficiency. Just a mere particle size reduction by applying a higher homogenization speed during emulsification was not as effective. An encapsulation efficiency as high as 35.4% was achieved. The optimum bile acid content appears to be 1.5 mg to 6 mg of sCT.

Another major enhancing effect appears to result from the well-known anchoring capability of bile acids onto intestinal cells. Transferrin seems to have a similar action in this regards. With all data reported here, there are still plenty of points open for further improvement. When all parameters were fully optimized, the oral delivery of sCT toward osteoporosis treatment with enough safety as well as efficacy would have come closer for realization.

ACKNOWLEDGMENT

This research has been financially supported by the Korea Industrial Technology Foundation (KOTEF) and Gyeongnam Techno-Park through the Human Resource Training Project for Regional Innovation since 2006. Authors also appreciate the support in 2007 from Inje University with an Academic Research Fund Program.

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