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Preparation of Smart Probiotic Solid Lipid Nanoparticles (SLN) for Target Controlled Nanofood

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ABSTRACT

Ultrasonication was employed to prepare solid lipid nanoparticles (SLN) for smart probiotic nanoparticles as a nanofood. The model probiotic material, lactocin from *Lactobacillus plantarum* (CBT-LP2), was incorporated into SLN. The CBT-LP2 loaded SLN (CBT-LP2-SLN) were spherical in the photograph of scanning electron microscope (SEM). The particle size measured by laser diffraction (LD) was found to be 97.3±8.2 nm. Zeta potential analyzer suggested the zeta potential of LP-SLN was −29.36±3.68 mV in distilled water. The entrapment efficiency (EE%) was determined with the sephadex gel chromatogram and high-performance liquid chromatogram (HPLC), and up to 90.59% of nanofood was incorporated. Stability evaluation showed relatively long-term stability with only slight particle growth (P>0.05) after storage at room temperature for 4 weeks. Therefore, ultrasonication is demonstrated to be a simple, available and effective method to prepare high quality SLN loaded probiotic material.

(**Key words** : lactocin, smart solid lipid nanoparticles, target controlled nanofood, ultrasonication, probiotic material, *Lactobacillus plantarum*)

INTRODUCTION

Solid lipid nanoparticles (SLN) are particles made from solid lipids with a mean diameter between approximately 50 and 1,000 nm. SLN are an alternative colloidal carrier system for controlled nutrient delivery (1~3). Compared to other particulate carriers the SLN have more advantages for nutrient delivery system, such as a good tolerability (4) and biodegradation (5), a high bioavailability (6), a targeting effect (7), and no problems with respect to large-scale production and sterilization.

In recent years, the studies on SLN have markedly increased (8, 9), but only a few investigations have been studied regarding the incorporation of effective components of probiotic materials into SLN.

Probiotics are defined as living microorganisms that exert beneficial effects on human health (10). They are effective in shortening the duration of infectious diarrhea in children, and preventing antibiotics-associated diarrhea (11, 12). Probiotics have been shown to prevent a relapse of postoperative pouchitis in ulcerative colitis (13).

Lactic acid bacteria often produce low-molecular-weight, antibacterial peptides that are collectively referred to as bacteriocins (for a recent review, see reference (14). Since many of these compounds exhibit bactericidal activity against potentially pathogenic food-borne bacteria, such as *Listeria monocytogenes*, they have become the focus of considerable research, which is principally aimed at the development of bacteriocins as biopreservatives. Several bacteriocins have been shown to have *in vitro* efficacy for reducing the numbers of viable cells of *Listeria monocytogenes* and other potential pathogens in various food products (15), while nisin, from *Lactococcus lactis*, has been used extensively over the past 30 years to prevent spoilage of a variety of different foods (16~19).

We isolated *Lactobacillus plantarum* CBT-LP2 and *Lactobacillus plantarum* CBT-LP3 from *kimchi*, *Pediococcus pentosaceus* CBT-PP1 from goat's milk, and *Lactococcus lactis* CBT-P7 from cow's milk. From this study, the antimicrobial effects of this probiotics culture were evaluated using *in vitro* and *in vivo* models of food-borne pathogens *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *S. enteritidis*. In *S. enteritidis*, *E. coli* O157:H7 infected mice, PCCM decreased the viable bacteria found in the feces and decreased the mortality rate. (20). This effective component of the probiotic material has high lipophilicity and becomes excellent candidate for SLN

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encapsulation. By using this nutrient delivery system, a high bioavailability, a targeting effect and an intravenous administration are possible.

Since DM Kim showed that a new type of food called firstly the name of 'nanofood', which means nanotechnology for food, and the encapsulated materials can be protected from moisture, heat or other extreme conditions, thus enhancing their stability and maintaining viability applications for this nanofood technique have increased in the food (21～28).

In the present report, CBT-LP2 loaded SLN (CBT-LP2-SLN) for target controlled nanofood were prepared by ultrasonication, and the physicochemical characterizations of the particles produced by this method were studied.

MATERIALS AND METHODS

1. Materials

CBT-LP2 (99.9%) was purchased from Cellbiotech Co. Ltd., Korea. Stearic acid (obtained from Sigma-Aldrich, USA) was used as lipid materials of SLN. Soybean lecithin was obtained from Central Soya Co. LTD., USA. Sephadex gel-50 was purchased from Sigma-Aldrich, USA. Methanol (HPLC grade) and absolute alcohol was purchased from Samahun Co. Ltd., USA. Glycerin (obtained from Amoy Glycerin Industry Co., Ltd., USA) was used as a coemulsifier in water phase.

2. Preparation of CBT-LP2-SLN

The CBT-LP2, stearic acid and soybean lecithin were weighed with electric balance (BP-121S, Sartorius Ltd., Germany) precisely and were dissolved in absolute alcohol in water bath at 70℃. An aqueous phase was prepared by dissolving glycerin in distilled water. The resultant organic solution was rapidly injected through an injection needle into the stirred aqueous phase (80℃). The resulting suspension was stirred at 80℃ for 2 h continually. The CBT-LP2-SLN original suspension was then ultrasonicated for 300s using Ultrahomogenizer (Heidolph Electro, Kelhaim Co. Ltd., Germany). The resulting dispersion was then allowed to cool at room temperature and was filtered through a millipore filter (0.45 μm) in order to remove any titanic granules from the probe. Samples were kept at 4℃.

3. Scanning Electron Microscopy (SEM)

The morphology of the CBT-LP2-SLN was observed by scanning electron microscopy (SEM). The samples were stained with 2% (w/v) phosphotungstic acid for 30s and placed on copper grids with films for viewing. The nanoparticle surface appearance and shape were analysed by SEM. Samples were prepared by finely spreading concentrated nanoparticle dispersions over slabs and by drying them under vacuum. The samples were then coated in a cathodic evaporator with a fine gold layer and observed by SEM using a JSM-6400 scanning electron microscope (JEOL, Tokyo, Japan).

4. Average Diameter and Zeta Potential

Laser diffraction (Mastersizer 2000, Malvern Instruments, UK) and zeta potential analyzer (Zetasizer Nano, Malvern Instruments, UK) were used to study the particle size and zeta potential of SLN. Three samples of CBT-LP2-SLN were prepared according to the method described previously. Each sample was diluted with distilled water until the appropriate concentration of particles was achieved, and each sample was measured three times to calculate the average diameter and zeta potential (n=9).

5. Entrapment Efficiency (EE%) of CBT-LP2-SLN

Chromatographic condition: Chromatographic column: Spherisorb ODS C_{18} (250 mm × 4.6 mm, 5 μm); mobile phase: methanol/aether/ethylamine (100/1/0.05, v/v/v); flow rate: 1.0 mL min^{-1}; column temperature: room temperature; detection wavelength: 282 nm.

The controlled solution (1.25～25 μg mL^{-1}) was prepared by dissolving CBT-LP2 (precisely weighed) in mobile phase. The amount of CBT-LP2-SLN penetrated into the receptor compartment was determined with the high-performance liquid chromatogram (HPLC) described previously. The integral calculus of the chromatographic peak area (A) was recorded as Y-axis, and the concentration of CBT-LP2 (C) as X-axis. Drug recovery was calculated from the following equation. Nutrient recovery=(analyzed weight of nutrient in SLN/theoretical weight of nutrient loaded in system)×100%.

The CBT-LP2-SLN suspension was separated by Sephadex gel-50 column chromatography. The concentrations of CBT-LP2 in the suspension (n_1) and free drug (n_2) were assayed by HPLC after dilution with methanol. EE% could be achieved by the following equation.

$$EE(\%)=(n_1-n_2)/n_1\times 100\%$$

6. Evaluation of Stability

CBT-LP2-SLN were stored at room temperature. Particle

sizes were determined after 1, 2 and 4 weeks to evaluate their stability.

7. Statistical Analysis

Results were reported as arithmetic mean values ± standard deviation ($\bar{\chi}$±S.D.). Statistically significant differences were determined using Student's *t*-test with $P<0.05$ as a minimal level of significance.

RESULTS AND DISCUSSION

1. CBT-LP2 Investigation

The electron microscopy micrographs of CBT-LP2-SLN were shown in Fig. 1. The shape of SLN was spherical. The particle size was approximately from 80 to 200 nm. However, in order to obtain more precise information on the size distribution, more careful analyses were performed as follows.

2. Average Diameter and Zeta Potential

An adequate characterization of the solid lipid nanodispersion is a necessity for the control of the quality of the product. Laser diffraction (LD) is one of the most powerful techniques for routine measurements of particle size (1). The average diameter of CBT-LP2-SLN measured by LD (Mastersizer 2000) was 157.3±8.2 nm (n=9). The drug loading SLN showed a considerable small particle size.

The measurement of the zeta potential allows predictions about the storage stability of colloidal dispersion. In general, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electric repulsion (1). The zeta

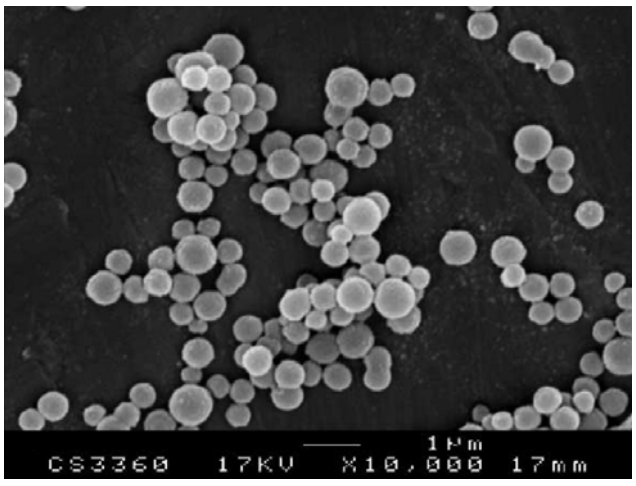

Fig. 1. SEM photograph of CBT-LP2-SLN (×10,000).

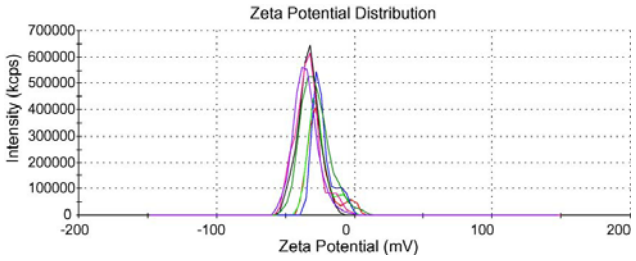

Fig. 2. Zeta potential distribution of CBT-LP2-SLN (n = 9).

potential distribution of CBT-LP2-SLN was showed in Fig. 2. The mean zeta potential was −29.36±3.68 mV (n=9). Therefore, this method had gained a relative high stability and good dispersion quality.

A sufficient high-energy input was necessary to break down the droplets into the nanometer range. A finer dispersion could be obtained by inputting higher energy such as the elevated production temperature, higher stirring rate, longer emulsification time, stronger ultrasound power and so on. Besides the production parameters, lipid matrix, surfactant blend and also viscosity of lipid and aqueous phase influenced the outcome of the procedure (29).

The ultrasonication is the dispersing technique which was initially used for the production of solid lipid nanodispersions (7). This method is widespread and easy to handle. However, metal contamination has to be considered if ultrasound is used. In the present study, CBT-LP2-SLN were prepared by ultrasonication, and the millipore filter could remove any titanic granules from the probe effectively.

3. EE of CBT-LP2-SLN

Many different drugs had been incorporated in SLNs (30) and (31). The prerequisite to obtain a sufficient loading capacity was a sufficiently high solubility of the drug in the lipid melts (32). Relative higher drug EE% was one of the major advantages of SLNs. The amount of drug to be incorporated into the delivery system is dependent on the physicochemical properties of drug and the preparation process.

The regression equation of CBT-LP2 was A=18526C−8342.2. The assay was linear (r=0.9999) in the concentration range 1.25~25 μg mL^{-1}. The percentage recoveries ranged from 98.72% to 101.80%, and the mean was 99.46%. Up to 90.59±1.26% of CBT-LP2 was incorporated in SLN prepared by ultrasonication. Therefore, it was revealed that SLNs produced by this method could achieve high drug incorporation for

Table 1. Particle sizes of SLN after 1, 2 and 4 weeks of storage at room temperature (n=9)

Sample	Average diameter±S.D. (nm)		
	1 week	2 weeks	4 weeks
CBT-LP2-SLN	97.3±8.2	98.5±6.3	102.1±7.2

lipophilic effective components extracted from probiotic materials like CBT-LP2.

4. Stability

Table 1 gives the data of particle sizes of CBT-LP2-SLN after 1, 2 and 4 weeks of storage at room temperature. CBT-LP2-SLN suspension showed sufficient long-term stability with only slight particle growth (P>0.05) after storage at room temperature for 4 weeks. There was also no visible aggregation in system during storage.

CONCLUSION

In the present study, the model probiotic material, CBT-LP2, was incorporated into SLN for target controlled nanofood by ultrasonication. This method was demonstrated to be a simple, available and effective method to prepare SLN loaded probiotic materials, which are small, steady and highly incorporated. A great deal of lipophilic effective components extracted from the probiotic materials can be incorporated in SLN by this method, and it may be a novel approach to the modernization of probiotic materials.

The ability to control the particle size of nanofood materials is of primary importance not only for determining nanofood product properties such as taste, aroma, texture, and appearance, but also for determining the release rates of the carried bioactive compounds and ultimately how much is absorbed into the body, and hence the overall efficacy of the compounds. In the case of CBT-LP2-SLN have to be released from the matrix to allow absorption by the intestinal wall, while nanoparticles may improve absorption of the probiotic materials either by adsorbing to the GI walls to prolong residence time or by direct uptake by the intestinal epithelium (27).

In addition, owing to multiple functional groups in the primary sequences of polypeptides and the resulting diversity of chain folding structures, food proteins can be exploited to create different interactions with nutraceutical compounds and subsequently form three-dimensional networks to incorporate and protect these compounds in a matrix and deliver them to the site of action in the active form. It can be foreseen that, with improvements in manufacturing technologies, new strategies for stabilization of fragile nutraceuticals, and development of novel approaches to site-specific carrier targeting, nanofood-based materials will play an important.

Probiotic materials entrapment into SLN with this nanoprecipitation method is currently under investigation and results will be presented in a forthcoming paper. Finally, it is assumed that the overall process could become a worthwhile option for target controlled nanofood using by smart probiotic SLN production, even at an industrial scale.

We did not directly show any application which is the matter of companies collaborating, and a start-up company is also licensing our nanofood techniques. Obviously the most appealing but also most demanding applications are in the area of drug delivery. Besides delivery to certain sites and issues, the capsules once uptaken by biological cells could play a role of artificial organelles, where encapsulated protected enzymes can cross talk to cell cytoplasm and/or report outwards what happens within the cell. We address readers to description of the concept with preliminary data (20). Nevertheless, at present, one should be aware that there is a long way ahead to arrive at an efficient tumor therapy with these, albeit promising, systems.

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