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ORIGINAL ARTICLE

Improved efficacy of appetite suppression by lipoic acid particles prepared by nanocomminution

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Abstract

Purpose: This article was intended to improve the efficacy of alpha-lipoic acid (ALA) for appetite suppression by controlling the particle size and self-polymerization of ALA. *Methods*: ALA was fabricated into micro- and nanoparticles, and the efficacy and in vitro release were investigated. Because of the self-polymerization of ALA into poly[3-(*n*-butane carboxylic acid)propyl]disulfide (PBCPD) by processing heat, low-speed rotation comminution was used to control PBCPD content. *Results*: The ALA particle size initially decreased and then increased after 10 hours of nanocomminution, indicating aggregation related to PBCPD formation. The in vitro release of ALA was significantly reduced by the existence of PBCPD. Interestingly, the reduction was not followed by a decrease in efficacy. Alternatively, the food intake was significantly reduced by ALA particles containing more than 30 mol% PBCPD. *Conclusions*: When the particle size and self-polymerization of ALA were carefully controlled, the efficacy on appetite suppression could be superior to water-soluble ALA salt. The ALA particles might have a composite nanostructure of ALA and PBCPD.

Key words: *Appetite suppression; comminution; lipoic acid; nanoparticles; self-polymerization*

Introduction

Alpha-lipoic acid (ALA, 1,2-dithiolane-3-pentanoic acid) is synthesized in most prokaryotic and eukaryotic cells. In humans, ALA exists in the body as a portion of several multienzyme complexes involved in energy formation and is an essential component of mitochondrial respiratory enzymes[1]. Recently, the pathway of ALA biosynthesis was determined in *Escherichia coli*[2,3].

ALA is clinically used in the treatment of diabetic neuropathy and is also effective in degenerative neuronal disease, atherosclerosis, and acquired immune deficiency syndrome[4–7]. Recently, our group reported that ALA causes profound body weight loss in rodents by reducing food intake and enhancing energy expenditure[5]. However, ALA has two major problems: low bioavailability[8] and high reactivity of its disulfide bonds.

The S−S bond in ALA can be homolytically cleaved by near-ultraviolet light[9] or heat[10,11]. The formed thiyl radicals (S•) induce self-polymerization into a linear chain of disulfides, poly[3-(*n*-butane carboxylic acid)propyl]disulfide (PBCPD). As well as the radicals, thiolate anions (S^-) can attack the disulfide bond, subsequently leading to the self-polymerization of ALA[12,13]. The tendency of ALA to polymerize can be used in the development of liposomes as carriers for drugs[14,15]. Liposomes having PBCPD increased their stability toward aggregation and fusion, where ALA covalently linked to phospholipids was polymerized.

PBCPD undergoes depolymerization back to ALA in basic solutions[11]. The disulfide bond (−S−S−) can be reversibly cleaved in the presence of reducing agents such as dithiothreitol and β-mercaptoethanol[16]. The reactions result in the formation of ALA and PBCPD mixture, and Reed et al. suggested that natural ALA might exist as a mixture[17,18].

Although the effect of PBCPD on bioavailability has remained largely unknown, the previous pharmaceutical

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studies have focused on various salt forms with melting points higher than ALA [63°C (racemic) and 50°C (R form)] to enhance physical stability[19–21]. The soluble salt forms did not satisfactorily improve bioavailability possibly because of the short plasma half-life and low intrinsic bioavailability of ALA[8]. Furthermore, repeated oral administration did not elevate the plasma level to greater than 25 mM[22]. Therefore, novel pharmaceutical approaches are still required to improve the ALA bioavailability for use as an effective appetite suppressant.

It was hard to control the degree of self-polymerization[10], which is sensitive to heat, ultraviolet light, type of medium, and so on. The rate of polymerization can be retarded in a stable solid crystalline form. Consequently, the degree of self-polymerization of ALA can be better controlled in a solid state at a low temperature. Herein, we developed a preparation method of ALA solid particles at a low temperature and explored the effect of particle size and degree of polymerization on the appetite suppression efficacy of ALA.

Solid nanoparticle technologies have been developed for improved bioavailability of poorly water-soluble drugs[23]. This method is based on particle size reduction to increase the dissolution rate, which can be explained by the Noyes–Whitney equation[24]. Various types of mechanical energy, such as high-pressure homogenization, have been used to reduce the drug particle size[25].

Among various solid nanoparticle technologies, nanocomminution has been recognized as a successful technology to fabricate nanocrystals[23,26,27]. Unlike the conventional comminution methods (e.g., hammer or jet mills), an aqueous medium is used for nanocomminution with steric or ionic stabilizers. Nanocomminution at a low temperature was chosen as the method to prepare ALA solid particles of various sizes and degrees of polymerization. In this study, careful control of the processing variables enabled successful preparation of ALA nano- and microparticles with unexpected improvement in bioavailability. Until recently, PBCPD has been recognized as an impurity in pharmaceutical industries; however, this study demonstrates that PBCPD significantly affects the release rate and improves the ALA efficacy of appetite suppression.

Materials and methods

Materials

ALA of Antibioticos S.P.A. (Milano, Italy, volume-averaged particle size: 50 μm) and polyvinylpyrrolidone (PVP, PLASDONE® C-30) of ISP Technologies (Wayne, NJ, USA) were utilized without further purification. HCl, KH_2PO_4, and NaOH for the simulated fluids were purchased from Duksan Pure Chemicals (An-San, South Korea), and water was used after distillation. ALA tromethamine salt (Tris salt) was received from Bukwang Pharm (Seoul, South Korea).

Nanocomminution

Table 1 summarizes the recipes and conditions for three nanocomminution methods to fabricate ALA nanoparticles. Shaking mode was performed by a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK, USA). Equipments for the low- and high-speed rotation were built up in our laboratory. Briefly, a round-neck bottle (30 mL) rotating on a mill and an impellor rotating in a batch chamber (30 mL) were used in the low- and high-speed rotation methods, respectively. The chamber temperature for 4°C nanocomminution was controlled by a refrigerator. As a milling media, 500-μm cross-linked polystyrene (PS) and 1-mm ZrO_2 beads (50%, v/v) were charged in the round-neck bottle and the batch chamber, respectively. The concentration of active pharmaceutical ingredients was 8%, w/w. The temperature change of a medium was measured by inserting a thermocouple into the slurry mixture. ALA particles were in a suspension state.

Particle analyses

The distribution of particle size was analyzed in 150 mL water using a Horiba LA-910 laser light-scattering analyzer with a relative refractive index of 1.3. The agitation speed was level 3 (340 mL/min). Volume-averaged particle sizes were measured after sonication (30 Hz, 40 W) for 1 minute.

The degree of self-polymerization was analyzed using UV/Vis spectroscopy (JASCO V-550; Jasco, Tokyo, Japan).

Table 1. Comparison of the three nanocomminution methods.

Mode of mechanical energy	rpm	Increase rate of temperature (°C/min)/steady-state temperature (°C)	ALA (g)	PVP (g)	Water (g)	Batch chamber size (mL)
Rotation	3000	10/70	0.36	0.06	4.08	30
Rotation	100	Not detectable/4	0.3	0.05	3.4	30
Shaking	4800	60/80	0.03	0.005	0.34	2

The steady-state temperatures were measured at room temperature for the high-speed rotation and shaking, and at 4°C for the low-speed rotation.

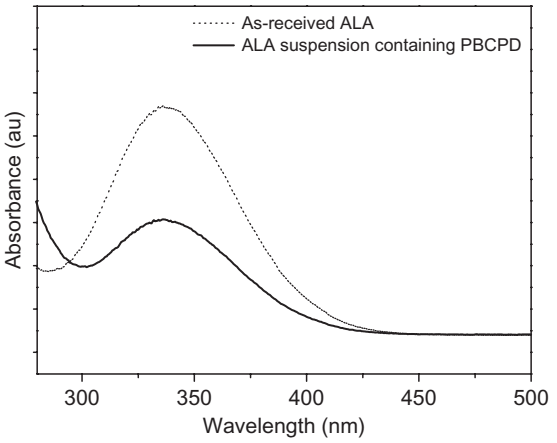

Figure 1. UV-Vis spectrograms of as-received ALA and suspension containing PBCPD (low-speed comminution for 72 hours at 4°C).

Decrease of the absorption peak at 340 nm indicates the destruction of 1,2-dithiolane as shown in Figure 1. Dimethyl sulfoxide (DMSO, 0.05 M) (Sigma-Aldrich, St. Louis, MO, USA) was used as a dissolution solvent. The content of PBCPD was computed from the concentration differences before and after nanocomminution. The relative viscosity of 1%, w/w DMSO solutions of ALA was obtained using an Ubbelohde viscometer at 25°C.

In vitro release tests

Release rates were determined by using the United States Pharmacopeia (USP) paddle apparatus II under a sink condition. Suspension samples (0.2 g) obtained after comminution were released in dialysis tubing cellulose membrane (average flat width 25 mm; Sigma-Aldrich) after activation in water for 6 hours. The release rate of ALA as received was measured at the same PVP concentration. Gastric fluid (no enzyme) was 0.1 N HCl, and intestinal fluid (no enzyme) consisted of KH_2PO_4 (64.7 mM), NaOH (2.2 mM), and distilled water. The volume of dissolution medium was 500 mL (37 ± 0.5°C), and paddle speed was 50 rpm. By calculating the absorbance of 1,2-dithiolane (disulfide ring) at 340 nm, the amount of cumulative ALA was obtained as a function of time (tolerance of ±10 seconds).

Effects of ALA nanosuspensions on food intake

Food intake was measured in male C57BL/6 mice (average weight 20 g, $n = 6$ each). After an overnight fast, various nanosuspension formulae of ALA and water-soluble Tris salt was intraperitoneally (IP) injected (100 mg/kg), and food intake (the mass of food consumed) was measured for 6 hours.

Results and discussion

Comminution modes

In a preliminary experiment, the ALA particles produced by low-speed rotation comminution at 25°C for 10 hours without a polymeric stabilizer were larger than 10 μm. The utilization of PVP resulted in a volume-averaged particle size of approximately 600 nm (SD = 0.6 nm). Among hydroxypropyl cellulose, poly(ethylene glycol-*co*-propylene glycol) F127 and F68, polyethylene glycol, PVP was observed to be the most successful stabilizer for ALA. PVP must most successfully adsorb to the surfaces of fractured particles and provide steric stabilization.

Among the three nanocomminution methods (Table 1) and despite the use of PVP, high-speed rotation and shaking comminution produced ALA particles larger than the original size (50 μm). Because of the mechanical energy, the two methods generated significant processing heat (Table 1), which induced uncontrollable PBCPD generation by self-polymerization, resulting in a swollen gel.

The self-polymerized PBCPD could alter the dispersion stabilization, because of the alterations to the balance between steric repulsive and attractive forces. PBCPD can change the PVP–ALA interaction or cause bridging among the particles, resulting in an increase in particle size. The generation of rubber PBCPD polymer can also prevent the brittle fracture of ALA particles by providing resistance to crack propagation. In addition, the existence of PBCPD could change the adsorption and desorption characteristics of PVP on the nanoparticle surface. To this end, the generation of PBCPD could result in the increase of particle size.

Low-speed rotation comminution did not significantly generate processing heat (Table 1) and was chosen to prepare the ALA particles as the PBCPD content and ALA particle size could be controlled. Despite no significant processing heat, the mean particle size of ALA processed at 4°C (volume-averaged size = 350 nm, SD = ±40 nm) was smaller than at 22°C (410 nm, ±80 nm) (Figure 2). The low processing temperature could suppress self-polymerization and the associated particle size increase.

Particle size reduction and self-polymerization

Figure 3 displays the mean particle size as a function of comminution time at 4°C and 22°C. The particle size gradually decreased with increasing comminution time up to 10 hours; however, the size increased after 10 hours. The increased degree of particle size at 22°C is larger than that at 4°C. This phenomenon can be explained by two counteracting effects: comminution

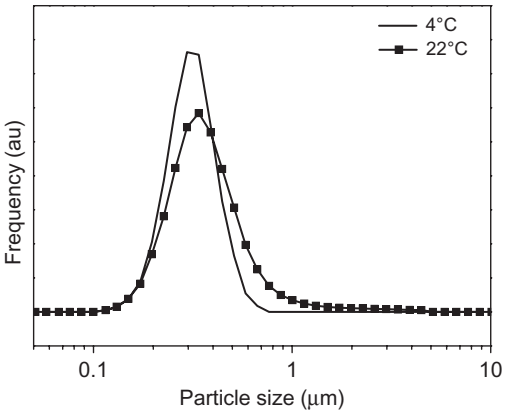

Figure 2. Distribution curves of volume-averaged particle sizes from the processes of two different temperatures (low-speed comminution for 9 hours).

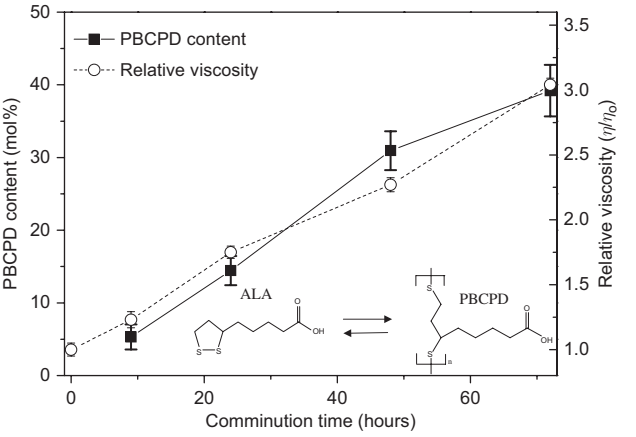

Figure 4. PBCPD content and relative viscosity as a function of comminution time (inset: self-polymerization scheme).

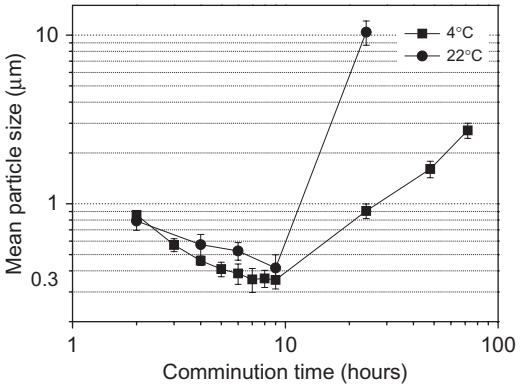

Figure 3. Volume-averaged particle size as a function of comminution time (suspension temperature: 4°C and 22°C, sonication time: 1 minute).

and self-polymerization. Comminution with PVP can effectively reduce the ALA particle size; however, self-polymerization continually progresses and PBCPD accumulates, resulting in an increase in particle size (aggregation). As shown in Figure 4, the PBCPD content and the relative viscosity showed a nearly linear increase as a function of comminution time. Before 10 hours, the content was less than 10 mol%, continually increased to 40 mol%. The increase in the relative viscosity corresponds to an increase in the molecular weight of PBCPD.

The nanocomminution of ALA might be accompanied by the structural development of PBCPD/ALA particles. Comminution energy fractures ALA particles into nanoparticles, and the propagation of cracks usually generates a local temperature elevation. Additionally, attrition between particles and media generates heat. Therefore, PBCPD is most likely to form near the particle surface. The ALA particles of 400 nm obtained by a 10-hour comminution may be covered by a significant thickness of PBCPD, which prevents further particle size reduction. Comminution for longer than 10 hours will lead to particle aggregation through the attractive hydrophobic force between PBCPD. As a result, ALA microparticles obtained by nanocomminution could be different from the as-received ALA particles as the ALA microparticles were aggregates of 400-nm primary ALA/PBCPD composite particles. The ALA phase might remain nanometer size and scanning electron microscopy was attempted; however, distinct contrast between the two phases could not be obtained.

Because of the counteracting effects, 10 hours appears to be the optimum processing time to fabricate the smallest ALA particles. Without generation of PBCPD, the ALA particle size may decrease to less than 350 nm. The particle size and PBCPD content results confirmed the preliminary observations of different comminution modes.

In vitro release of nanosuspensions

The four samples in Figure 4 were compared by in vitro and in vivo experiments. Hereafter, ALA particles including 5, 15, 30, and 40 mol% PBCPD will be denoted as 5-PBCPD, 15-PBCPD, 30-PBCPD, and 40-PBCPD, respectively. The volume-averaged particle sizes were 400, 650, 1200, and 1600 nm, respectively.

Figure 5 shows that the in vitro release was slower in the gastric fluid (Figure 5a) than the intestinal fluid (Figure 5b) because of the acidic nature of the ALA. However, the effects of PBCPD and particle size were consistent for both the relationships. Following the general understanding, the release rate decreased with increasing particle size. The cumulative amount of ALA released at 24 hours also decreased with an increase in

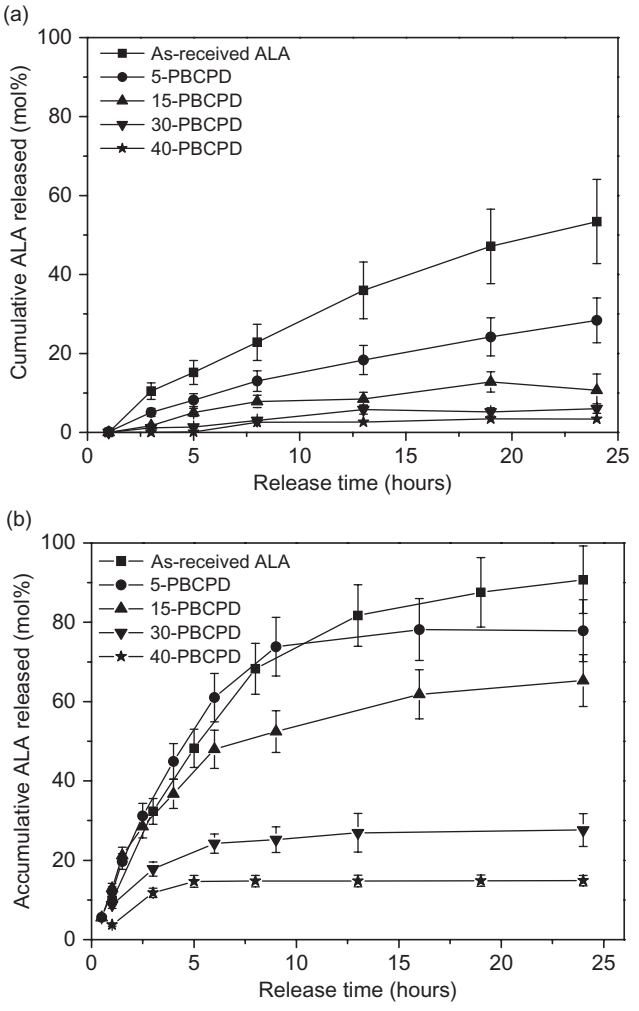

Figure 5. In vitro curves of accumulative ALA released in the gastric fluid (a) and in the intestinal fluid (b). The ALA particles containing 5, 15, 30, and 40 mol% PBCPD are denoted as 5-PBCPD, 15-PBCPD, 30-PBCPD, and 40-PBCPD, respectively.

particle size or PBCPD content. 40-PBCPD had the slowest release rate for both the gastric and the intestinal fluids.

The release rate of ALA suspensions cannot be explained solely by the particle size. In the PBCPD series, the particle size increase was accompanied by an increase in PBCPD content. Thus, the two effects were not exclusive; however, a comparison between the PBCPD series and the as-received ALA shows that the effect of PBCPD content was more dominant than particle size. For example, although the ALA particle size as-received (50 μm) was larger than 40-PBCPD, the particles dissolved faster in both fluids. The effect of particle size must exist; however, the release rate in Figure 5 appeared to reflect the effect of PBCPD content.

The influence from particle size can partially be traced. The initial release rate of as-received ALA was slightly slower than 5-PBCPD (Figure 5b), possibly because of the larger particle size. However, as-received ALA continued to release up to 90% of the initial loading amount after 24 hours whereas 5-PBCPD reached only 80%. The maximum release amount appeared to be smaller than the amount of ALA, which did not self-polymerize. For example, 40-PBCPD included 60 mol% intact ALA; however, the maximum released amount was approximately 10 mol% in the intestinal fluid.

PBCPD chains appeared to act as a diffusion barrier in the ALA release, as it may be difficult for ALA molecules to thread PBCPD chains. The barrier effect would be more distinct with the diffusion of ALA molecules farther inside a particle, resulting in a relatively low maximum release. PBCPD can reversibly depolymerize back to monomeric ALA; however, within the time range studied here, this effect could not be identified. The release results suggest that the formation of PBCPD resulted in the sustained release behavior of the ALA particles.

In vivo food intake

Figure 6 shows the food intake of mice given IP injections of various formulae of ALA nanosuspensions. The efficacy of Tris salt was similar to those of 5- and 15-PBCPD suspensions despite different particle sizes and release rates. In principle, the release rate was much faster than the others due to the simultaneous dissolution of Tris in water. These data suggest that there was no improvement in efficacy with a faster initial release rate. Additionally, the effect of particle size was not evident.

The initial in vitro release rates of 30- and 40-PBCPD were slower than 5- or 15-PBCPD; however, 30- and 40-PBCPD had significantly improved efficacy of appetite

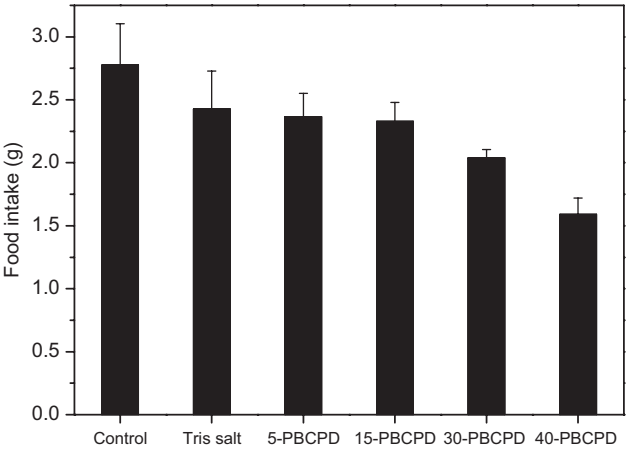

Figure 6. In vivo results of the food intake for 6 hours after the injection (100 mg/kg) of the Tris salt and ALA nanosuspensions. The control group takes no ALA.

suppression. 40-PBCPD was better than the smaller ALA particles, including soluble ALA salt. Contrary to common knowledge, the relatively large particle size and subsequent slow release rate of 40-PBCPD did not deteriorate the efficacy.

The 40-PBCPD microparticles might have composite internal structures of ALA and PBCPD. The internal nanostructure did not show the maximum dissolution rate, although it appeared to be beneficial for maximum efficacy. The unique release characteristics of the composite microparticles may have been the reason for the efficacy improvement. A simple explanation such as sustained release caused by PBCPD or particle size reduction cannot fully explain the results.

The basic conditions can promote depolymerization of PBCPD back to ALA[11]. If the basic conditions of the duodenum and upper intestine could promote the regeneration of ALA from PBCPD, perhaps this is what contributes to the better appetite suppression over 9 hours compared with the immediate release of pure ALA. The in vivo depolymerization of PBCPD could be significant, providing information for future research on mechanisms for improvement and optimization of the ALA formulation.

Conclusions

Nanocomminution successfully produced ALA nano- and microparticles with controlled particle size and degree of self-polymerization to improve its appetite suppression efficacy. Processing heat that induced self-polymerization and increased particle size required careful control during processing. As a result, low-speed rotation comminution at 4°C was chosen to prepare ALA particles. With increasing processing time, particle size initially decreased, followed by an increase after 10 hours. The increase appeared to be caused by self-polymerization, which was linearly dependent on processing time. The in vitro release rates significantly decreased as the content of self-polymerized PBCPD, a diffusion barrier, increased. However, the in vivo food intake results showed better efficacy in systems with higher PBCPD content, which was better than the efficacy of soluble ALA salt. The ALA particles with high PBCPD content appeared to have composite nanostructures of ALA and PBCPD, and the composite structure formation was more advantageous for appetite suppression than simply improving the dissolution rate.

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