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Application of sonication and mixing for nanoencapsulation of the cyanobacterium *Spirulina platensis* in liposomes

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Abstract

Encapsulation in the food industry is a process in which one or more ingredients or additives (core) are coated with an edible capsule. The use of liposomes is among the many forms of encapsulation of food ingredients. It consists of a sort of microscopic lipid vesicles, where due to the lipophilic and hydrophilic portion of its constituents, substances of various natures can be encapsulated, and the hydrophilic substances stay in the aqueous compartment and the lipophilic are inserted or adsorbed on the membrane. The aim of this work was to apply two different treatments, sonication and homogenization, in the encapsulation of a protein source, such as the cyanobacterium Spirulina platensis, formed out by the thin-film hydration method. Liposomes were prepared using purified soybean phosphatidylcholine and sonicated at 60°C for 30 min or homogenized at 10.000 rpm for 15 min. The average size, encapsulation efficiency and particle morphology were determined. The type of process applied did not differ when obtaining nanometric size particles, however, the liposomes subjected to homogenization had increased medium size compared to liposomes that were subjected to sonication. Morphologically, the liposomes were more uniform when subjected to the homogenizer.

Introduction

Nanotechnology is focused on characterization, manufacturing, handling and application of biological and nonbiological (Sahoo *et al.*, 2007) structures at the nanometric scale. In the food industry, several new nanotechnology applications have become apparent, including the use of nanoparticles such as lipossomes4 that can be used for the encapsulation of food ingredients. Encapsulation involves the merging, absorption or scattering of solid, liquid or gaseous compounds within, or in small vesicles, which can reach the nanometer scale. Combinations of incorporated compounds can be protected against degradation, improving stability and solubility (e.g., solubilization of hydrophobic components in hydrophobic matrices and vice versa) (Klaypradit *et al*., 2008; Jafari *et al.*, 2008). The core is where the active component is found, and this can be composed of one or more ingredients. The retention of these nuclei is governed by its chemical functionality, solubility, polarity and volatilidade (Gharsallaou *et al.*, 2007). The primary purpose of encapsulation is to protect the core material from adverse environmental conditions such as undesirable effect of light, moisture and oxygen, as well as contributing to the increase of the product's shelf life and promote the controlled release of encapsulates (Shahidi an Han, 1993).

According to Azeredo (2005) the choice of the encapsulation method depends on a number of factors, such as particle size required, physical and chemical properties of the core and the wall, application of the final product desired, release mechanisms, production scale and cost. There are several methods that can be used for encapsulation, such as spraying, coacervation, extrusion, drum drying, molecular inclusion, fluid bed, freeze, and inclusion in liposomes.

Liposomes consist of single or multi-layered vesicles involving an aqueous phase into a phospholipids membrane. These vesicles are formed spontaneously when phospholipids are dispersed in an aqueous medium. A portion of the aqueous medium becomes closed lipid membrane which then serves as a particle for controlled release of active material dispersed in the aqueous or lipid phase of the particle (Malheiros *et al*., 2010). The phosphatidylcholines are the most used in studies of liposome formulation, since they have great stability against variations in pH or salt concentration of the medium (Batista *et al*., 2007) in addition to being biocompatible, biodegradable and have detergent action. Due to the presence of lipid and aqueous phase in the structure of the vesicles, liposomes may be used in the encapsulation, delivery and

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release of materials soluble in water, liposoluble and amphiphilic (Khosravi-Darani *et al*., 2007; Mozafari *et al.*, 2008).

The methods of preparing liposomes are numerous and lead to the formation of different types of vesicles in relation to size, structure, encapsulation capacity, retention time of substances, number of lamellae and etc. The preparation of liposomes requires the application of energy to the dispersion of lipid molecules in an aqueous medium, and the main goal of this process is to obtain vesicles with the right size, acceptable polydispersity, elasticity, structure and encapsulation efficiency (Mozafari *et al*., 2008; Mozafari *et al*., 2005). Ordinarily the liposomes obtained by the classical method, i.e, by the lipid film hydration method (Patty and Frisken, 2003) are poorly trained and have a huge variation in size parameters that limit its application both in research and industry.

Bioactive agents encapsulated in liposomes can be protected from digestion in the stomach, and show significant levels of absorption in the gastrointestinal tract, leading to increased bioactivity and bioavailability (Takahashi *et al.*, 2007), and are systems with high biocompatibility and versatility. Accordingly, the liposomes may be used for the encapsulation of Spirulina.

Spirulina platensis is a cyanobacterium used as food by man because of its chemical composition, with a high protein quality and quantity, essential amino acids, minerals, vitamins and polyunsaturated fatty acids. It is considered a GRAS micro -organism (Generally Recognized as Safe) with no toxicity, it is permitted as a food additive by the FDA (Food and Drug Administration) which guarantees its use as a food without risks to health (Parisi *et al*., 2009; Ambrosi *et al*., 2008). Several research (Miranda *et al.*,1998; Herrero *et al.*, 2005; Souza *et al.*, 2006; Mendiola *et al.*, 2007; Bierhals *et al*., 2009) reported the nutritional and functional importance of the compounds present in *Spirulina platensis*. *Spirulina platensis* was used in this encapsulation process as core. The advantages of *Spirulina platensis* encapsulation in liposomes consist of masking compounds of undesirable flavor, slowing changes that may result in loss of flavor, color change or loss of nutritional value when applied in formulations, and also permit release directly in the organism. The objective of this work was to apply two different treatments in the encapsulation process of a protein source such as *Spirulina platensis* cyanobacteria, in order to reduce and standardize the size of the liposomes formed by the methodology of the lipid film hydration.

Materials and Methods

Obtaining Spirulina platensis

Cyanobacterium *Spirulina platensis* LEB -18 strain isolated from the Mangueira lagoon (Morais *et al.*, 2008) was used and supplemented with 20% (v/v) Zarrouk (Costa *et al*., 2004) medium to maintain the production of inoculum and biomass. The pilot plant for the production of *Spirulina* sp. was located near the shore of Mangueira Lagoon (33°302 133 S; 53°082 593 W) and consisted of raceway tanks that differed in dimension and volume depending on their purpose. We used one inoculum tank (4.0 m long × 1.0 m wide × 0.50 m high) with a surface area of 3.87 m^2 and a working volume of 1.000 l and three production tanks (13.0 m long × 3.0 m wide × 0.50 m high), each with a surface area of 37.10 m^2 and a working volume of 10.000 l. All the tanks were lined with glass fibre and covered with a greenhouse structure constructed from transparent polyethylene film. All of the tank cultures were agitated by one paddle wheel rotating at 18 rpm 24 h per day. The volume of culture media was maintained by the periodic addition of MLW to compensate for evaporation; approximately 12 l/d per 10.000 l tank was added over the course of the experiment (Costa *et al.*, 2004).

Purification of soybean lecithin

The pure soya lecithin was purchased in the local market in the form of powder, and purification was performed according to Mertins *et al.* (2008). Firstly 50 g of the same was dissolved in 250 mL of ethyl acetate (Synth®). Then slowly with stirring, 10 mL of distilled water was added, thereby occurring formation of two phases. The supernatant was separated and the lower phase discarded. The lower phase with a gel aspect was dispersed in 150 mL of acetone (Synth®), forming agglomerates that were broken up using a glass rod. Then, the acetone was decanted off and a new portion of 250 ml of acetone was added, repeating the grinding process. The precipitate was filtered under vacuum and dried in an oven at approximately 60°C and cooled in a desiccator, giving a final mass of 40 g, thus obtaining purified phosphatidylcholine(Mertins *et al*., 2008).

Encapsulation of Spirulina platensis

The encapsulation process was performed according to the lipid film hydration method, according to Malheiros *et al.* (2010) The source of lipids used for the preparation of liposomes was phosphatidylcholine purified from soybean lecithin. First, 1 g of phosphatidylcholine was dissolved in 10 ml of chloroform (Synth®) in a round bottom flask,

after its complete dispersion, the organic solvent was removed on a rotary evaporator until a lipid film was observed deposited on the walls of the flask. Traces of organic solvent were removed by storing the flask for 18h in vacuum desiccator. The resulting lipid film was dispersed by adding 20 ml of phosphate buffer pH 7.0, 0.2M containing 2 g of Spirulina platensis dissolved. The mixture in the flask was subjected to a temperature exceeding the transition phase (60ºC). Later, the liposomes were subjected to distinct treatments of sonication using ultrasonic bath (40 kHz, 700 Unique USC) at 60°C for 30 min or homogenization (Ultra-Turrax T25 digital) at 10,000 rpm for 15 min.

Encapsulation efficiency

The encapsulation efficiency, i.e the encapsulated mass of Spirulina in the liposome was evaluated in an indirect way. First 1 mL of the liposome and 2 ml acetone were placed in centrifuge tubes, due to lecithin being insoluble in this solvent. The samples were centrifuged at 14,000 rpm for 30 min at 3°C, resulting in two phases. The supernatant containing the unencapsulated sample was removed and placed in an oven at 36°C until complete evaporation of the solvent. In the dry material remaining crude protein analysis was performed, indirectly determining the amount of unencapsulated sample that solubilized in acetone. The protein analysis was performed by classic Kjeldahl (Instituto Adolfo Lutz, 2008) and the results expressed as % (m/m) of protein, using the conversion factor of 6.25. The material that remained in eppendorf after removal of the supernatant was resuspended em1mL Triton X-100 0.06% (v/v), homogenized by vortexing, and the protein content determined (Li *et al*., 2010). The encapsulation efficiency (EE) was calculated according to Equation 1.

$$EE\ (\%) = \frac{\text{Total protein of Spirulina - Spirulina free}}{\text{Total protein from Spirulina}} \times 100$$

where:
Total protein from Spirulina encapsulated = protein content of Spirulina Total encapsulated
Free Spirulina = Spirulina supernatant
Spirulina inside the capsule = process homogenization and sonication process

Average particle size

To determine the average particle size the technique of dynamic scattering of light (Teixeira *et al*., 2008) was used through the equipment with a wavelength of 632.8 nm, Spectra-Physics model 127 coupled to a BI -200M version 2.0 goniometer and digital correlator BI - 9000AT from Brookheaven Instruments (Teixeira *et al.*, 2008), and sample preparation according to the methodology described by Assis *et al.* (2014).

Lyophilization of encapsulated material

The samples were subjected to lyophilization in a bench freeze dryer FD Model 5505 using a temperature of -50°C to 500 μHg pressure for 24 h to obtain a dry powder for analysis in electronic scanning microscopy. Prior to this process the samples were frozen in an ultra- freezer at -80°C for 48 h.

Scanning electron microscopy

For microscopic analysis, the lyophilized liposomes were resuspended in methanol (Synth®) treated in ultrasonic bath for 20 min and deposited on a silicon substrate. The substrates were attached to the sample holder of the microscope using a carbon ribbon and then metallized with gold for 3 min and 5 mA of current, in a Sanyu Electron Quick Coater Model SC-701. The images were obtained on a Shimadzu SSX - 550 model microscope, which has vacuum and is coupled with EDS (Mertins *et al*., 2008; Li *et al*., 2010).

Statistical analysis

All determinations were performed in triplicate and the results were analyzed with ANOVA and Tukey's test at the 5% level of significance, with the aid of Statistica® (2004) software, version 7.0.

Results and Discussion

The protein content of Spirulina used for the encapsulation was of 60.36% on a dry basis and moisture content of 11.48%. The protein content of Spirulina liposomes have gone through the process of homogenization and sonication, obtained the following results for crude protein% (m/m): 1.21% Total Spirulina encapsulated; 0.12 and 0.26 and 1.09% Spirulina supernatant and 0.95% Spirulina inside the capsule, respectively for liposomes homogenized and sonicated liposomes (Figure 1). The encapsulation efficiency was determined according to equation 1.

The liposomes subjected to homogenization showed higher encapsulation efficiency (90%), compared to liposomes subjected to sonication (79%). Morais *et al*. (2003), using the method of reverse phase evaporation for the preparation of liposomes of casein hydrolysates, obtained encapsulation efficiency between 56% and 62%, determined by indirect method of consecutive centrifugation. Assis et al. (2014) obtained as a result 87% and 92% of encapsulation efficiency for the methanol and ethanol

Table 1. Average size and polydispersity of fresh Spirulina liposomes prepared using homogenization and sonication

Sample	Determinations		
	Average size (nm)	Index Polydispersity	Encapsulation efficiency (%)
Homogenized Liposomes	303,97±1,07[a]	0,248±0,01[b]	90±0,07[a]
Sonicated Liposomes	279,53±1,85[b]	0,291±0,01[a]	79±0,05[b]

Same letters in the same column indicate no significant difference by Tukey Test (α <0.05).

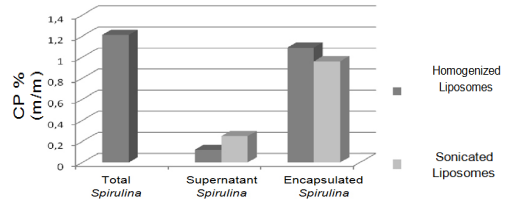

Figure 1. Protein content of Spirulina liposomes have gone through the process of homogenization and sonication. CP: Crude Protein

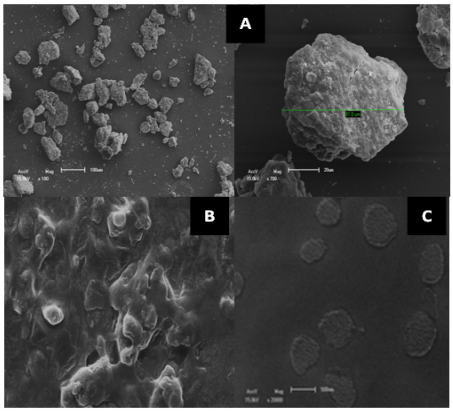

Figure 2. Scanning electron micrograph of Spirulina in natura (A); Spirulina Liposomes undergoing homogenization (B) Spirulina Liposomes subjected to sonication (C).

extracts of Spirulina. Were *et al*. (2004) using the lipid film hydrating method and sonication for the encapsulation of nisin, an antimicrobial peptide, obtained encapsulation efficiency higher than 54%. Under the conditions applied in this study, the percentages obtained for the liposome encapsulation efficiency that were homogenized and sonicated are considered satisfactory. The use of liposomes was investigated for the encapsulation of proteins (Picón *et al*., 1994; Caddeo *et al*., 2008), and other food ingredients (Marsanasco *et al.*, 2011; Nemen and Lemos-Sena, 2011), and various methods for the development have been proposed in the literature. Depending on the method and characteristics of the liposome and the sample, various types of structures can be designed with different features and encapsulation rates.

The average size and polydispersity of the liposomes that went through the process of homogenization and sonication are shown in Table 1. According to Table 1, application of the homogenizer or sonication did not differ in obtaining liposomes in the nanometer range, however, it can be seen that liposomes that have passed through the sonication process, had a smaller average size (279.53 nm) compared to liposomes which were subjected to homogenization (303.97 nm). The polydispersity index, which provides information about the homogeneity of the distribution of sizes was low (<0.3) for all samples, indicating the formation of monodisperse systems (Nemen and Lemos-Senna, 2011) or narrow range of sizes.

Usually, liposomes are classified according to their structure in multilamellar vesicles (> 400 nm), large unilamellar vesicles (80 nm -1 mM), small unilamellar vesicles (20 - 80 nm), giant unilamellar vesicles (> 1 mM), and multivesicular vesicles (> 1 mM) (Gómez-Hens, 2005). The choice of the method of hydration of the lipid film for the preparation of liposomes was due to this being a simple and inexpensive method that requires no specialized equipment, and is very effective in laboratory scale (Santos and Castanho, 2002), however, it is known that this method produces multilamellar vesicles that need further processing to standardize the size and structure of the particule (Jesorka and Orwar, 2008; Sharma and Sharma, 1997). Among the alternatives used to solve the problem of high distribution of types and size of the liposomes obtained is the use of ultra - sonication and stirring in homogenizers. Various authors (Were *et al.*, 2003; Fan *et al*., 2007; Malheiros *et al.*, 2012), use sonication as the method of further processing of hydration of the lipid film for use in food formulations as possible, thus this procedure being the most common than the use of homogenizers, however, despite this producing smaller vesicles it has disadvantages such as low rate of encapsulation and is poorly suited for industrial production.

The micrographs of Spirulina in natura and Spirulina liposomes obtained by sonication and the use of the homogenizer are shown in Figure 2. Through the photomicrographs it is observed that Spirulina in natura showed irregular shape and size in the micrometer range. The liposomes subjected to sonication also have irregular shape, however, the liposomes that were homogenized showed more regular shape and apparently organized cylindrical structure. The scanning electron microscopy was used to characterize the surface of lipid nanostructures, in order to observe evidence on the dispersion of nanoparticles in the lipid matrix. Barbosa *et al.* (2002), in microscopic analysis of lipospheres containing casein hydrolysate prepared by the method of reverse phase evaporation and subsequent treatment in the homogenizer revealed spherical morphology of these,

however, particle size was of about 3.8 μm.

Conclusions

The results suggest that the homogenization process as a final treatment for the preparation of liposomes containing a protein source, such as *Spirulina platensis* may be a favorable option for obtaining particles in the nanometer size scale and with high efficiency encapsulation, however, further deepening the research on the mode of action, applicability and stability of these structures is necessary. Although the process of sonication is the most commonly applied for the preparation of liposomes, it was found that the homogenization may become an option for final treatment, to result in a size of lipospheres significantly higher than that obtained in sonication.

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