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PRODUCTION, CHARACTERIZATION AND STABILIITY OF LIPOSOMES CONTAINING CREATINE[1]

PRODUÇÃO, CARACTERIZAÇÃO E ESTABILIDADE DE LIPOSSOMAS CONTENDO CREATINA

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ABSTRACT

Creatine is a compound of three amino acids that is widely used as an ergogenic resource in sports, because it has the ability to form Creatine Phosphate (PCr) in the mitochondria and increase ATP resynthesis. Liposomes are nanoscale lipid vesicles used to increase the stability, viability and bioavailability of molecules such as drugs. In this work, creatine-containing liposomes were obtained by the ethanol injection method, producing liposomes with an average diameter of 124.5 nm, a polydispersity index of 0.141, and a zeta potential of -3.9 mV. Stability studies showed that the creatine-containing liposomes are stable for up to 90 days when stored at room temperature (± 25 ºC).

Keywords: Bioavailability, Nanotechnology, Liposomal Vesicles.

RESUMO

A Creatina é um composto de três aminoácidos muito utilizada como recurso ergogênico nos esportes, por ter capacidade de formar Creatina Fosfato (PCr) na mitocôndria e aumentar a ressíntese de ATP. Lipossomas são vesículas de lipídios, em escala manométrica, utilizadas para aumentar a estabilidade, viabilidade e biodisponibilidade de moléculas como fármacos. Nesse trabalho lipossomas contendo creatina foram obtidos pelo método de injeção de etanol, produzindo lipossomas com diâmetro médio de 124,5 nm, índice de polidispersão de 0,141 e potencial zeta de -3,9 mV. Estudos de estabilidade mostraram que os lipossomas contendo creatina são estáveis por até 90 dias, quando armazenados em temperatura ambiente (± 25 ºC).

Palavras-chave: *Biodisponibilidade, Nanotecnologia, Vesículas Lipossomais*

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INTRODUCTION

CREATINE

Creatine is one of the most popular nutritional ergogenic resources among high-performance athletes. Its structure is composed of three amino acids, and its use is due to the fact that it accelerates and increases the resynthesis of adenosine triphosphate (ATP) in the mitochondria (KREIDER *et al.*, 2017). Studies consistently show that supplementation of this compound increases its concentrations at the intramuscular level, which may help explain the improvements observed in high-intensity exercise performance, leading to greater training adaptations (KREIDER *et al.*, 2017).

Around two thirds of intramuscular Creatine is in the form of phosphocreatine (PCr), with the remainder being found in the form of free Creatine (KREIDER; JUNG, 2011). Creatine is synthesized mainly in the liver and kidneys, from arginine, glycine and methionine, by the enzyme arginine-glycine amidinotransferase (AGAT) and guanidinoacetate (GAA), which is then methylated by guanidinoacetate N-methyltransferase (GAMT), using S-adenosyl methionine to form Creatine (PADDON; BORSHEIM; WOLFE, 2004). Most Creatine is found in skeletal muscle (~ 95%) with small amounts found in the brain and testes (~ 5%).

Creatine's main metabolic role is its combination with an inorganic phosphate group (IP) to form Creatine Phosphate (CrP) through a reversible enzymatic reaction of Creatine Kinase (CK). As ATP is degraded into adenosine diphosphate (ADP) and IP to provide free energy for metabolic activity, the free energy released from the hydrolysis of CrP into Cr + IP can be used as a buffer to resynthesize ATP, maintaining the availability of ATP in the cell (BENDER; KLOPSTOCK, 2016; KREIDER *et al.*, 2017).

LIPOSOME

Due to the need to produce drugs with a more specific action and less capacity to develop adverse effects, nanotechnology has emerged as an interdisciplinary area that allows for the production, characterization, handling and application of structures on a nanometric scale (KLOSTERGAARD; SEENEY, 2012). It can be used in different areas, including medicine, the pharmaceutical and cosmetics industries, environmental sciences, diagnostics, among others (SHAFFER, 2005; SAHOO; PARVEEN; PANDA, 2007, HALEEM *et al.*, 2023).

Liposomes are products of nanotechnology and were produced by Alec Bangham over 50 years ago, when he was researching phospholipid vesicle systems, which later became known as liposomes (BANGHAM; STANDISH; WATKINS, 1965). Today they are produced and widely disseminated throughout the world, with different methodologies depending on the objective and application.

Work is still underway to improve this structure, especially in terms of encapsulation efficiency (GIORDANI *et al*., 2023; KUMBHAM *et al*., 2023).

This type of structure is basically a spherical, biocompatible, biodegradable, non-toxic vesicle made up of one or more lipid bilayers (phospholipids) wrapped around an aqueous core (BRANNON-PEPPAS, 1993), and has the advantage of being able to encapsulate hydrophobic, hydrophilic and amphiphilic compounds. Because they have a closed structure, they improve the stability of drugs and increase their bioavailability (HUANG *et al*., 2005).

Another influencing factor is the type of lipid used in the formulation, i.e. the surface charge of the liposomes, which depends on the charge presented by the lipid. They can be cationic liposomes (positively charged), anionic liposomes (negatively charged) or neutral liposomes (uncharged). The choice will depend on the type of target site that the liposome needs to reach, for example cationic liposomes are the most commonly used for gene therapy, as DNA has an effective negative charge (DUA; RANA; BHANDARI, 2012).

MATERIALS AND METHODS

This study was carried out at the Franciscan University, in the Nanotechnology Laboratory. The water used was of high purity (Milli-Q Millipore® - USA) and the reagents used to produce the creatine-containing liposomal vesicles were all of analytical grade (PA). To obtain the creatine-containing liposomes (LCr), the ethanol injection method was used, according to the protocol of Justo and Moraes (2005), with some adaptations detailed below.

PRODUCTION LIPOSOME CONTAINING CREATINE

The liposomes were prepared by adding an organic/oily phase (OF) to the aqueous phase (AF). The OP contained 400 mg of phospholipid (Lipoid S-100, Sigma-Aldrich®, Germany), 20 mg of polyethylene glycol (PEG 2000, Sigma-Aldrich®, Germany), 75 mg of cholesterol (Sigma-Aldrich®, Germany) and 20 mg of vitamin E (Sigma-Aldrich®, Germany), solubilized in 20 mL of ethanol in a 250 mL Erlenmeyer flask, which was placed in an ultrasonic bath (Ultrasonic Cleaner, UNIQUE®) for 10 minutes to solubilize the components. The constituents of AF were added to a 250 mL beaker: 250 mg of the active ingredient (Creatine - Sigma-Aldrich®, Germany), 65 mg of polysorbate 80 (Tween 80, LabSynth®), 38 mL of saline-phosphate buffer (PBS pH= 7.4) and 12 mL of water. The AF was placed on magnetic stirrers in a water bath at a temperature of ± 35 ºC. The OF and AF were then kept for a further 10 minutes in a water bath, under constant stirring, at a temperature of ± 35ºC. The OF was then slowly poured over the AF. At the end, a homogeneous mixture with an opalescent white color was obtained. The solution was kept stirring for another 10 minutes and then placed in a

250 mL flask and taken to a rotaevaporator (Yamato - RE801, Japan) to remove the organic solvent (ethanol). After removing the solvent, the liposomes obtained were packed in amber bottles. For the stabilization studies, part of the formulation was kept in a refrigerator (5 ± 2 ºC) and the other at room temperature (25 ± 2 ºC) for up to 90 days.

CHARACTERIZATION OF THE FORMULATION

The liposomes were characterized in terms of mean particle diameter (MD), polydispersity index (PDI), zeta potential (ZP), pH and morphology.

Determination of mean particle diameter and polydispersity index

The mean particle diameter and IPD of the liposomes were determined using the dynamic light scattering technique on the Zetasizer® nano-ZS model ZEN 3600 (Malvern Instruments, Malvern, Worcestershire, England). The samples were diluted in ultrapure water (500 times, v/v) and the results expressed as the mean ± standard deviation (SD) of three repetitions.

Zeta potential and pH

Zeta potential was obtained using the electrophoretic mobility technique on the Zetasizer®, Nano-ZS model ZEN 3600 (Malvern Instruments, Malvern, Worcestershire, England). The liposomes were diluted 500 times (v/v) in 10 mol L^{-1} sodium chloride and the results expressed in millivolts (mV) from the mean ± standard deviation of three determinations.

The pH of the formulations was measured using a potentiometer (DM-22, Dimed®), previously calibrated (buffer solutions pH 4.0 and 7.0). The results were expressed as the mean ± standard deviation of three measurements of each sample.

Morphology

Morphological characterization was carried out using scanning electron microscopy (SEM: Sigma 300 VP, Carl Zeiss, Germany) connected to a Gemini and Schotky type field emission filament (FEG). To obtain the images, it was used in high vacuum mode with an excitation voltage of up to 1 kV. To prepare the sample, 200 uL of liposome containing creatine were deposited on double-sided carbon tape and fixed to the sample holder, placed in a desiccator for 12 hours to dry.

Stability of the formulation

To assess stability, the parameters MD, PDI, ZP and pH were measured at 0, 15, 30, 60 and 90 days after the formulations were produced and stored in refrigeration and at room temperature, as mentioned above.

RESULTS AND DISCUSSIONS

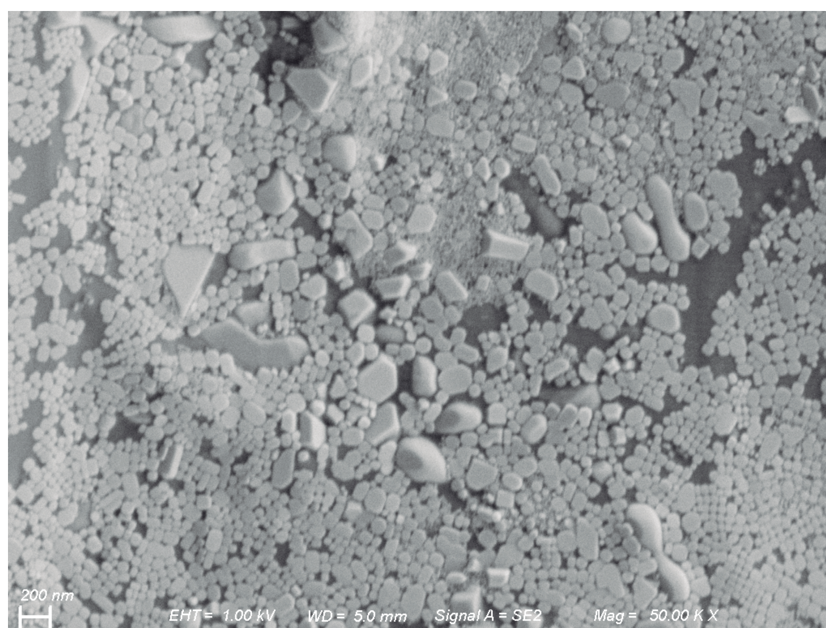
The formulation obtained had an average size of 131.5 ± 3.93 nm, with IPD of 0.142 ± 0.03, ZP of -3.82 ± 1.21 mV with pH 7.69 ± 0.03. These results show that the method used was suitable for the production of liposomal vesicles containing creatine, one of the objectives of this work. The data presented in Table 1 refers to the stability results over a period of up to 90 days, in two different storage forms.

Table 1 - Stability studies of liposomes containing Creatine (LCr), n=3.

Days	Storage	Mean Diameter (nm)	Polydispersion Index	Zeta Potential (mV)	pH
0	-	131.5 ± 3.93	0.142 ± 0.03	-3.82 ± 1.21	7.69 ± 0.03
15	Room temp.	127.2 ± 1.99	0.108 ± 0.02	-11.25 ± 6.52	7.67 ± 0.01
	Refrigeration	127.0 ± 2.43	0.119 ± 0.02	-3.82 ± 1.22	7.64 ± 0.03
30	Room temp.	130.1 ± 4.21	0.126 ± 0.03	-5.38 ± 0.94	6.58 ± 0.09
	Refrigeration	134.2 ± 3.34	0.157 ± 0.03	-9.49 ± 4.36	6.55 ± 0.03
60	Room temp.	132.7 ± 3.02	0.133 ± 0.02	-5.88 ± 0.76	6.51 ± 0.05
	Refrigeration	133.9 ± 3.40	0.143 ± 0.03	-3.45 ± 1.27	6.48 ± 0.10
90	Room temp.	127.6 ± 1.78	0.102 ± 0.02	-5.67 ± 0.66	6.51 ± 0.03
	Refrigeration	125.2 ± 3.37	0.110 ± 0.01	-3.65 ± 1.17	6.69 ± 0.18

The liposomes obtained are unilamellar, large, with sizes between 80 nm and 150 nm. The MD, PDI and ZP for all liposome formulations showed no significant difference during the 90-day storage period, both at room temperature and when stored under refrigeration. A decrease in pH was observed in LCr from 30 days onwards, at both temperatures, but this did not compromise the stability of the formulation. Figure 1 shows the Scanning Electron Microscopy (SEM) image of the creatine-containing liposome.

Figure 1 - Scanning electron microscopy of liposomes at 50,000x magnification**.**



Source: authors, 2021.

The liposomes produced in this work had a spherical and homogeneous morphology, with a size of less than 200 nm, as can be seen in figure 1. According to the Zeta Sizer analysis, the average diameter obtained was 124 nm and falls within the category of large vesicles, which range in size from 80 nm to 1 µm (ALAVI; KARIMI; SAFAEI, 2017). It is not only the average diameter that defines a nanoparticle; some authors describe the properties that the particle presents and that strengthen the nanostructure principle (VERT *et al.*, 2012; LEE; YUN; PARK, 2015).

The particle polydispersity index is a parameter used to describe whether there is a heterogeneous or homogeneous population of particles and considers different values for each type of nanoparticle. In the case of liposomes, a homogeneous or monodisperse sample is considered when the PDI value is less than 0.300 (PUTRI *et al.*, 2017). All the liposome samples in this study had a PDI of less than 0.200 during the 90-day stability period, confirming that the particle population is homogeneous.

The pH of the LCr formulations showed a reduction over the course of 90 days, observed both at room temperature (6.43 ± 0.05) and at refrigeration temperature (6.54 ± 0.06). There is no data in the literature describing these active ingredients nanoencapsulated in liposomes and evaluating their pH. Creatine as a supplement, in addition to its ADP buffering role, has the characteristics of preventing a decrease in pH in the muscle cell, reducing the feeling of fatigue (OLIVEIRA; AZEVEDO; CARDOSO, 2017). However, given a liposome suspension, this characteristic does not seem to be reproduced as it would in a living organism.

The zeta potential is an indirect analysis, which checks the difference in electrical potential, usually in millivolts, between the dispersed particles and the colloidal suspension (ISO, 2017). The further away from zero charge, the greater the stability of the particles in the medium in which they

are arranged, with a value in millivolts above 10 being considered the beginning of good stability (KUMAR; DIXIT, 2017). Despite this relationship of the higher the ZP, the better, all the liposome formulations tested here showed a value of less than -10 mV.

CONCLUSION

Liposomes with Creatine produced in this work showed stability within 90 days. The size is within what is recommended for it to be called a nanometric structure, as all the average diameter analyses showed sizes of less than 140 nm. Overall, the liposome production method presented here is easy, fast and efficient.

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