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Vol. 10(43), pp. 926-935, 22 November, 2016
DOI: 10.5897/AJPP2016.4636
Article Number: 40840EB61828
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Development, characterization and assessment of botulinum toxin type A incorporated in nanocarriers

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Received 27 July, 2016; Accepted 16 November, 2016

Botulinum toxin type A is one of the most used products in mitigation of wrinkles and expression lines by promoting muscle paralysis. Because of their hydrophilic and high molecular mass, this active has low permeability through the skin. Thus, to exert its action as rejuvenation agent, botulinum toxin needs to be applied through intramuscular injections, causing pain and discomfort to the patient. An alternative to minimize this problem is encapsulation of the neuropeptide in nanocarriers that can easily cross the skin barrier. Thus, the aim of this work was to study the feasibility of encapsulation of botulinum toxin A in polymeric nanoparticles and liposomes. It was possible to demonstrate the formation of nanoparticles and liposomes loaded with botulinum toxin A. It was observed that liposomes containing botulinum toxin had an effect on neurotransmitters most effective in tests *in vitro*. The results demonstrate that it is possible to encapsulate botulinum toxin type A in liposome form without altering its biological action.

Key words: Botulinum toxin type A, liposomes, nanoparticles.

INTRODUCTION

Wrinkles are the most expressive signs of cutaneous aging, which is a complex biological phenomenon that depends on a combination of intrinsic and extrinsic factors. The intrinsic factors are responsible for chronological aging that is genetically determined and one of the main extrinsic factors is exposure to solar radiation that leads to photo-aging and causes alterations in the skin such as wrinkles, dry skin, loss of elasticity, irregular pigmentation, atrophy, collagen fragmentation and elastic fibers. The accumulation of elastin below the dermal-epidermal junction characterizes these effects and the elastosis that is typical of this aging (Khavkin and Ellis 2011). In addition, other factors must be considered, such as repetitive facial movements caused by a contraction of the facial muscles (Buck et al, 2009) that can lead to wrinkles or signs of expression. Since over a

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period of time, muscles become hypertrophied and acquire an increased tonicity. This natural phenomenon means that the furrows and wrinkles remain apparent even when the muscle is not contracted and the skin becomes flaccid and the wrinkles accentuated into deep grooves (Batistela et al, 2007).

Currently, the search for anti-aging products that can repair this phenomena, suggest the use of botulinum toxin type A, which is the regarded method for wrinkles. It is naturally produced from *Clostridium botulinum*, an anaerobic bacterium that produces seven serological types of toxin and type A is used clinically because it is the most powerful (Sposito 2004). Botulinum toxin acts by preventing the release of acetylcholine in the neuromuscular junction and causes paralysis (Blanes-Mira et al, 2002). In addition, this prevention is involved with the process of insulin release, that is, if there is no release of acetylcholine, there will be no release of insulin. Acetylcholine, released from nerve endings of the vagus nerve (parasympathetic nervous system), cholecystokinin, released by enteroendocrine cells from the intestinal mucosa, and the gastrointestinal inhibitory peptide are some substances that stimulate the release of insulin Kiba (2004). This neurotoxin was approved in 1989 for use in the treatment of squinting, eye-twitching and hemifacial spasms. In 2002, it was approved for cosmetic use to mitigate facial lines and in 2004 for the treatment of hyperhidrosis Mahajan and Brubaker (2007).

Botulinum toxin type A is a hydrophilic neuropeptide with high molar mass. It has a low permeability through the skin, mainly because of the lipophilic characteristics of the stratum corneum Gorouhi and Maibach (2009). Therefore, to carry out rejuvenation, botulinum toxin has to be applied by intramuscular injection; however, this procedure causes pain and discomfort to the patient.

Regarding this problem, technological development of new pharmaceutical methods has proved to be a more promising strategy to allow a free passage of medicines through the skin. There has been a great interest in the selective release of medication in the skin and hence there have been several studies of carrier systems with the aim of improving the selection and efficiency of the formulations Bruschi (2010).

Nanoparticles and liposomes are the most important of these carriers. Liposomes are spherical vesicles formed by lipid bilayers with a size that can vary from 20 nm to a number of micrometers. They can be used to encapsulate hydrophilic drugs in the aqueous cavity of the vesicle or lipophilic drugs in the lipid bilayer Lasic (1998) and Tyagi et al. (2006).

According to the process and composition of the nanoparticles, it is possible to obtain nanospheres or nanocapsules. Regarding the nanocapsules, the drug is found in a nucleus of the particle and nanospheres. The drug is encapsulated or adsorbed in the polymer mesh Brigger et al, (2002). The size of the nanoparticles varies from 10 to 1000 nm Pimentel et al. (2007).

In order to alleviating the discomfort of the patient, it is possible to encapsulate botulinum toxin into liposomes or even into nanoparticles. This can carry out their topical application and ensure their penetration and activity in the skin. There were many reports in the literature about the encapsulation of botulinum toxin Type A which underlines the innovative nature of this study.

MATERIALS AND METHODS

Reagents and solvents

Onabotulinumoxin A (Botox® 100 U, Allergan Pharmaceuticals, Westport, Irlanda); Poly(vinyl alcohol) (PVA) (*Mw* 13.000 a 23. 000 $g.mol^{-1}$, Sigma-Aldrich, St. Louis, USA); Cholesterol (Sigma-Aldrich, St. Louis, USA); Phosphatidylcholine (EMBRAFARMA, São Paulo, Brazil); Poly (*ε*-caprolactone) (PCL) (Mw70.000 a 90.000 $g.mol^{-1}$, Sigma-Aldrich, St. Louis, USA); and chemicals with analytical grade. Purified Milli-Q® water (Millipore, Bedford,USA) was used throughout the study.

Experimental procedure

Preparation of botulinum toxin A: Loaded nanoparticles

The nanoparticles containing botulinum toxin A were prepared by a double emulsion/solvent evaporation procedure Feczkó et al. (2008) and Jiao et al. (2002). The inner aqueous phase (A1) which consisted of 250 µL botulinum toxin A (20 $UI.mL^{-1}$) was added to an organic phase (O) containing 100 mg of poli(*ε*-caprolactona) in 20 mL diclorometh ane under vigorous mixing for 1 min at 22,000 $rev.min^{-1}$ (Ultra-turrax®, model T18 N, IKA®). The resulting water-in-oil (A_1/O) emulsion was further dispersed into 100 ml of PVA (Mw 13,000 – 26,000 $g.mol^{-1}$) aqueous solution 5%, under vigorous mixing (22.000 $rev.min^{-1}$) for 3 min resulting in a multiple emulsion (A_1/O/A_2). The double emulsion was kept under mechanical stirring (800 $rev.min^{-1}$) at room temperature for 4 h. After complete evaporation of the organic solvent, nanoparticles were separated by centrifugation (50.000 $rev.min^{-1}$, 20 min), washed with distilled water to remove the residual surfactant. After freeze drying, the samples were store with a desiccant under vacuum at room temperature. All formulations were obtained in triplicate. Unloaded nanoparticles were also prepared as negative controls.

Preparation of botulinum toxin A: Loaded liposomes

The multilamellar liposomes were prepared according to Esteves (2011). by the thin film hydration method. Briefly, L-*α*-Phosphatidylcholine (PC, 0.192 g) and cholesterol (CH, 0.060 g) were dissolved in chloroform. The lipid mixture was deposited as a thin film in a round-bottomed flask by rotary evaporator the chloroform under vacuum, at 40°C in a temperature-controlled water bath for a period of 2 h to ensure complete removal of traces of solvent. Hydration of the film was performed by adding 3 ml of saline solution (0.9%) and botulinum toxin A 250 µl to the flask. The dispersion was mechanically shaken for 30 min at 25°C. The liposomes remained refrigerated at 4°C overnight. Following this, the vesicles were lyophilized and stored in a desiccator under vacuum at room temperature. Unloaded liposomes were also prepared as negative controls.

Morphological characterization of nanoparticulated systems

Characterization of nanoparticulated systems

Optical microscopic evaluation: Optical microscopy involves passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample Schaffazick et al, (2003). The liposomes and nanoparticles were characterized by optical microscopy (Nikon Eclipse E200) at 400X magnification for analysis of the vesicle formation and morphology. Optical micrographs were acquired by a digital Nikon camera coupled to an optical microscope.

Scanning electron microscopy (SEM): Scanning electron microscopy have been very useful in obtaining information relative to the shape and size of the nanoparticles Schaffazick et al, (2003). Samples of nanoparticles were coated with a thin layer of gold by sputtering, using a Pelco 9100 sputter coater (Pelco, Clovis, CA, USA) and analyzed using scanning electron microscopy (Shimadzu Corporation, Kyoto, Japan) at an intensity of 10 kV, captured at various magnifications.

Atomic force microscopy (AFM): A sensitive and simple optical method for detecting the cantilever deflection in AFM Schaffazick et al, (2003). AFM analyses were recorded using a SPM9600 Scanning Probe (Shimadzu Corporation) with 20 nm radius of curvature and using silicon nitride (Si_3N_4) cantilever in contact mode. The scan size was 20 × 20 µm and the scan frequency of 2 Hz was selected.

Zeta potential analysis: The zeta potential reflects the surface potential of the particles, which is influenced by changes in the interface with the environment dispersant, due to the dissociation of functional groups on the surface particle or adsorption of ionic species present in the aqueous dispersion Schaffazick et al, (2003). The zeta potential of liposome and nanoparticles was determined by using a zetasizer Nano ZS (Malvern, UK). Prior to the analysis, the samples were diluted with pure water in the ratio 1:10 in pure water. The diluted samples were then analyzed at a voltage of 4 mV.

X-Ray diffraction analysis: In order to determine the association of the drug with nanoparticles, X-ray diffraction analysis indicates that the drug is molecularly dispersed in polymer matrix Schaffazick et al, (2003). The materials were analyzed with a Shimadzu X-ray diffractometer XRD-6000 (SHIMADZU CORPORATION), scan of 2° $min^{-1}$and 2θ from 5° to 55°, copper k-alpha radiation (λ = 1,5418 Å) 40 mA of current and 40 Kv voltage.

Spectroscopy evaluation in the infrared region with Fourier transform infrared spectroscopy (FTIR)

Infrared Spectroscopy is used to gather information about compound's structure, assess its purity, and sometimes to identify it Schaffazick et al, (2003).

Spectroscopy in the infrared region was carried out in tablets using 4 mg of each sample and 196 mg of spectroscopic grade KBr powder (2% mass), with IR Prestige-21 equipment (Shimdzu Co.), 64 scans.min^{-1}, and 4 cm^{-1} resolution.

Determination of the presence of botulinum toxin in the polymer nanoparticles and liposomes

The determination of the presence of botulinum toxin Type A was carried out after the release of nanoparticles and liposomes. To obtain released botulinum toxin, nanoparticles and liposomes containing botulinun toxin A were weighed to obtain the exact amount of 0.018 g and placed in 10 mL of phosphate buffer pH 6.8 (50 $mmol.L^{-1}$ for KH_2PO_4 and 22.4 $mmol.L^{-1}$ for NaOH). System was kept at a thermostatically controlled temperature of 37 ± 0.5°C and stirred at 75 rev. min^{-1} for 24 h in triplicate. The solution was filtered (0.22 nm poro size). Following this, an aliquot of the filtered sample was added to the KBr and dried in the oven at 37°C. With the use of this material, a tablet was prepared which was analyzed for FTIR in the analytical conditions described earlier.

Evaluation of the effects of botulinum toxin on the acetylcholine involved in the release of insulin, following the activation of the neurotransmitters

Animals

Male adult Wistar rats (weighing between 200 and 230 g) were kept in the Vivarium of the Biology Department in collective cells under controlled temperature (23 ± 2°C) and lighting (12h- light-dark cycle). Water and food rations were supplied at random.

Isolation of Langerhans islets

The isolation of the islets was conducted through the destruction of the exocrine portion of the pancreas through the collagenase digestion where, after an incision of the abdominal wall of the Wistar rats to expose the pancreas, an occlusion was performed of the duodenal extremity of the pancreatic duct. Following this, a Hank's solution containing collagenase Type V (1 $mg.mL^{-1}$) was injected in the hepatic portion of the same duct by means of a syringe connected to a cannula needle. After it was intumescent, the pancreas was removed and transferred to a plastic tube, with a stopper, containing 15 mL of Hank's solution.

After this, the tube was transferred to the water bath at 37°C for 20 min under oxygenation (5% CO_2/95% O_2). Later on, the tube was agitated manually for about 1 min to ensure the dissolution of the pancreatic tissue which was filtered and transferred to a beaker. The exudate was submitted to repeated cleaning with Hank's solution in an ice bath to block the collagenase activity and purify the pancreatic islets of the exocrine tissue. The islets were then collected and placed under a magnifying glass with the aid of a pipette. After being isolated, the islets were separated in groups and transferred to the plates of a Costar Petri dish containing 24 wells.

Static insulin secretion

The islets were initially incubated at 37°C for 1 h in Krebs-Ringer solution (1 ml) containing 5.6 mM of glucose. At the end of this period, the pre-incubation solution was replaced with 1 mL of Krebs with 16.7 mM solution of glucose. The samples that had to be analyzed were added to this solution: 100 µl of the solution of the nanoparticles containing the botulinum toxin type A, 100 µl of the solution of the liposomes containing the botulinum toxin type A and 100 µl of the botulinum toxin type A. The incubation lasted for 1 h at 37°C. When the incubation period was terminated, the plate was placed over the ice and with the aid of the magnifying glass. The supernatant of each well was transferred to Eppendorf pipettes and stored at -20°C until the measurements of the insulin dosages were performed.

Insulin quantification

The insulin that was secreted was quantified by means of a

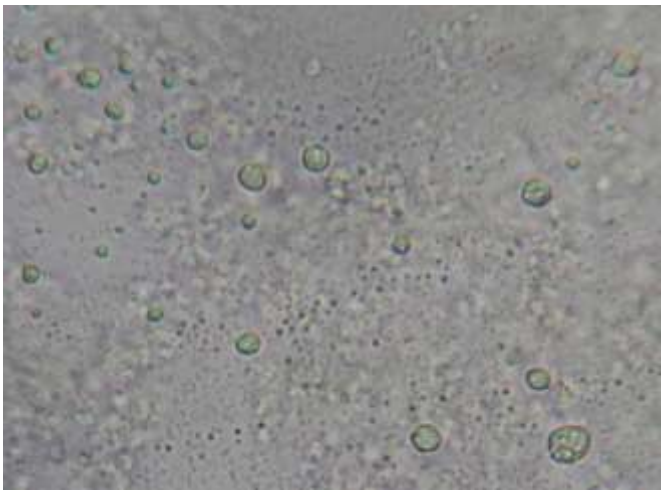

Figure 1. Formation of multiple emulsion in the preparatory process of the polymer nanoparticles.

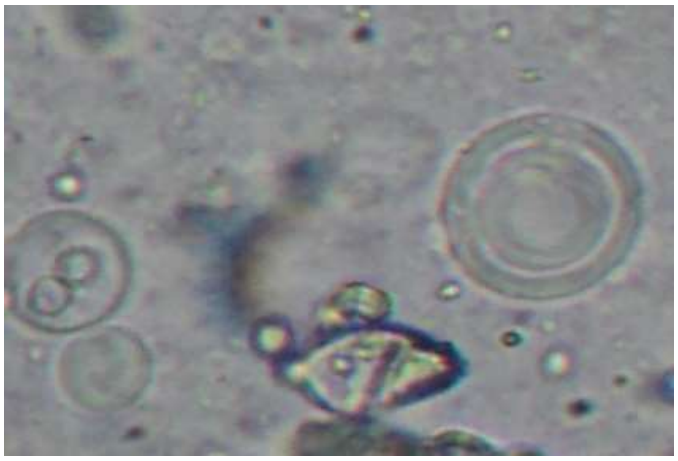

Figure 2. Multilamellar vesicles form.

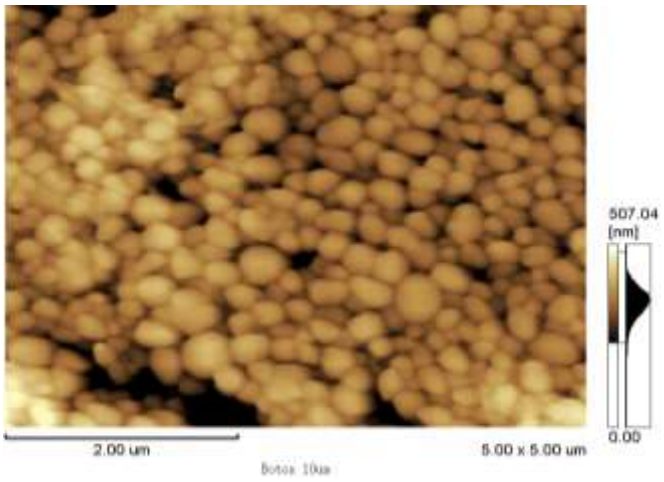

Figure 3. Nanoparticles containing botulinum toxin type A by AFM.

sandwich-type immunoassay carried out in places where there were direct chemoluminometric techniques which used constant amounts of antibodies. The first antibody is a mouse monoclonal anti-insulin marked with ester of acridine. The second, in the solid phase, is a mouse monoclonal anti-insulin antibody which is bonded by the covalently to paramagnetic particles.

Statistical analysis

All the data were expressed as an average standard deviation. The statistical comparisons were used to evaluate the dosing of the insulin. ANOVA statistical analysis of insulin dose tests (Tukey test).

RESULTS

Characterization of nanoparticulated materials containing Botulinum toxin A

Optical microscopy

In the first stage of the formation of the nanoparticles, it was observed that there was a multiple emulsion (Figure 1). The formation of this emulsion is indispensable for the incorporation of hydrophilic drugs like botulinum toxin type A Couvreur P et al, (2002), Soppimath et al, (2001) and Schaffazick et al. (2003).

The formation of liposomes can also be determined by optical microscopy (Figure 2). The liposomes that are formed appear as concentric and non-concentric multilamellar vesicles, which are the characteristics of liposomes obtained through the fine film technique Batista et al, (2007).

Morphological and surface analysis

The analysis carried out by AFM shows the nanoparticles containing toxin botulinum with a spherical shape and smooth and regular surface (Figure 3). Similar characteristics can be observed in the images obtained by MEV (Figure 4). The liposomes containing botulinum toxin type A evaluated in AFM are shown in Figure 5.

Determination of the average size, polydispersion and zeta potential

The nanoparticles loaded with botulinum toxin A had an average diameter of 578.4 nm. The graphical representation of the distribution of the sizes of the nanoparticles (Figure 6) shows a single population. The polydispersion index was 0.25, which suggests that there is a uniform distribution of the particles size and that this system is monodispersed.

Average size of 1363 nm liposomes was observed and a polydispersion index of 0.57. Figure 7 shows the distribution of the size containing botulinum toxin Type A.

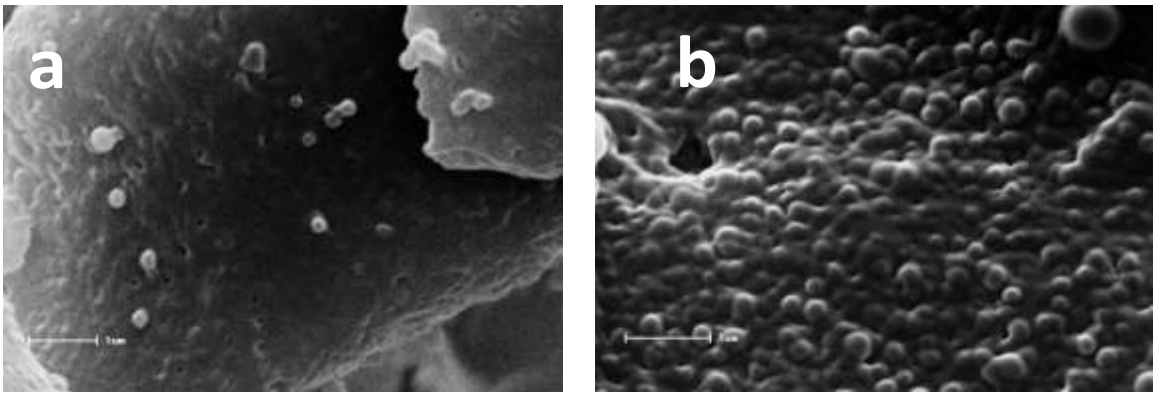

Figure 4. Morphological features of nanoparticles containing botulinum toxin type A obtained by SEM, 15K (a,b).

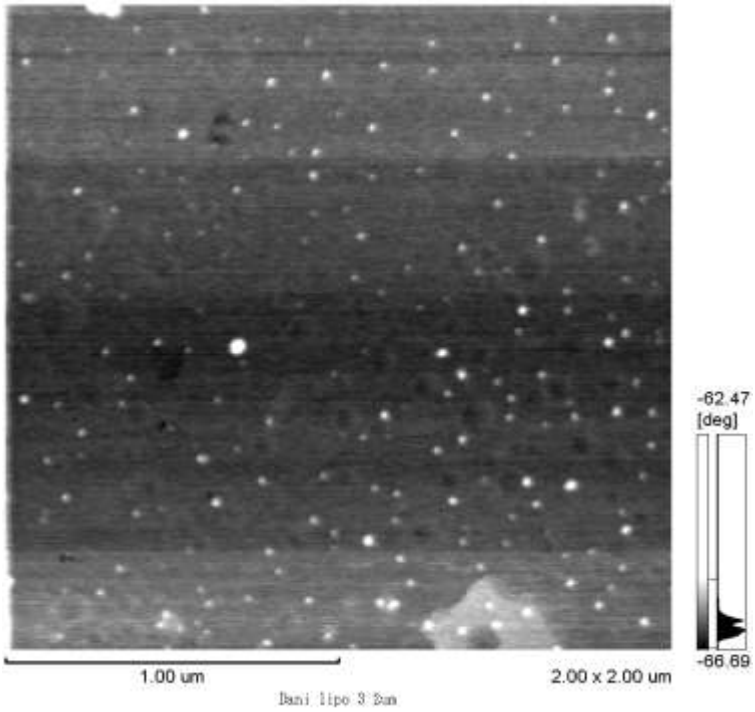

Figure 5. Liposomes, obtained by AFM.

Two sets of liposomes can be identified, which is also consistent with the optical microscopy images and the AFM (Figure 7). The zeta potential of the nanoparticles loaded with botulinum toxin A showed average values of -7.40 mV. The distribution of the zeta potential is represented in Figure 8. The liposomes containing botulinum toxin Type A showed average values of the zeta potential of -37.3 mV (Figure 9).

Characterization by X-ray diffraction

Figure 10 shows the results by X-ray diffraction for the PCL. The nanoparticles containing botulinum toxin A and the unloaded nanoparticles were used as negative control (N0).

Evaluation by spectroscopy in the FTIR region

The FTIR spectra of the nanoparticles that contain botulinum toxin A (NTB), empty nanoparticles (N0), botulinum toxin A (TB) and released botulinum toxin A (TBL) are represented in Figure 11.

The spectrum of the liposomes containing botulinum toxin A (LIPOTB), empty liposomes (L0), botulinum toxin

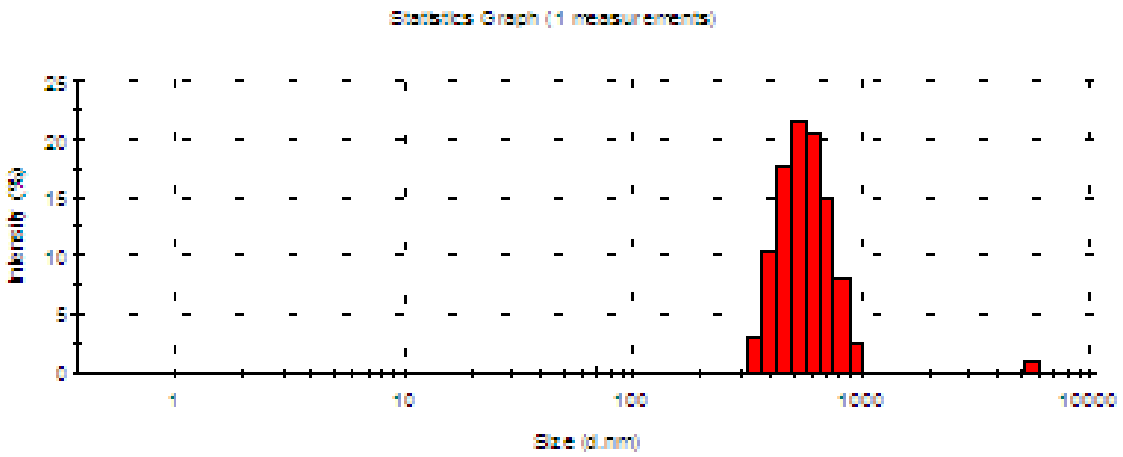

Figure 6. Nanoparticles containing botulinum toxin A.

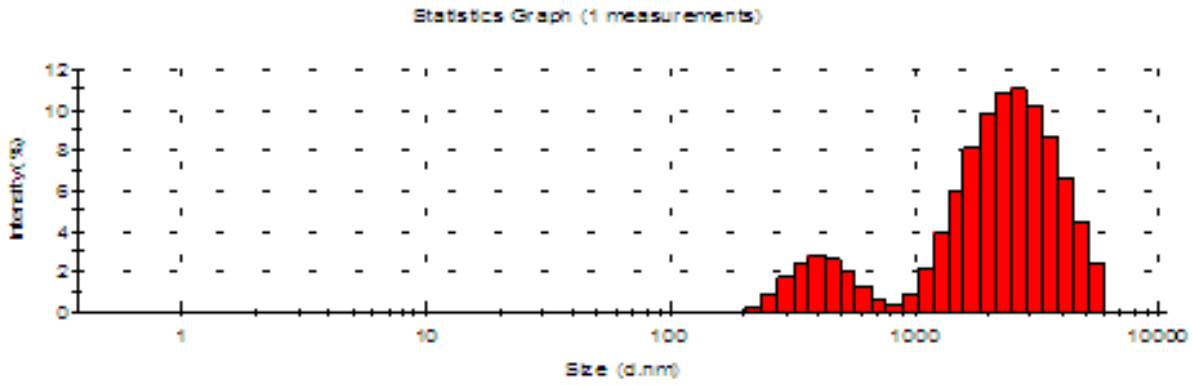

Figure 7. Size of liposomes loaded with botulinum toxin type A.

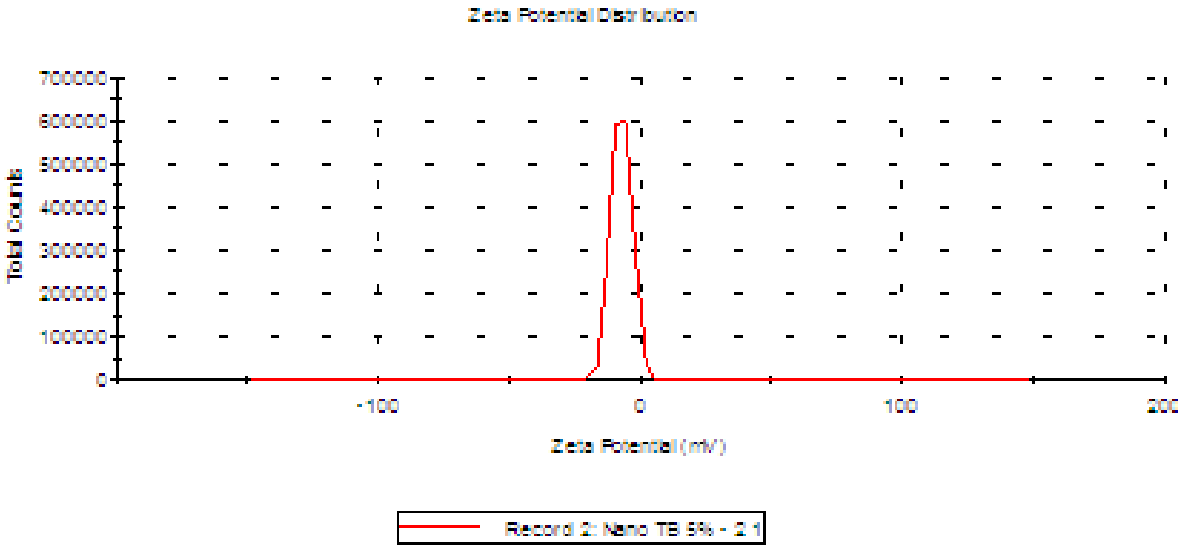

Figure 8. Zeta potential of nanoparticles loaded with botulinum toxin A.

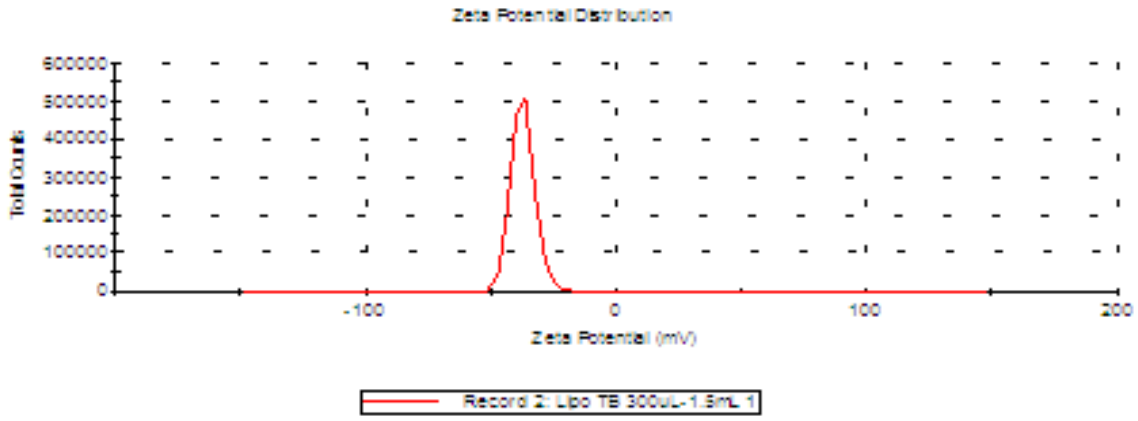

Figure 9. Zeta potential of liposomes loaded with botulinum toxin A.

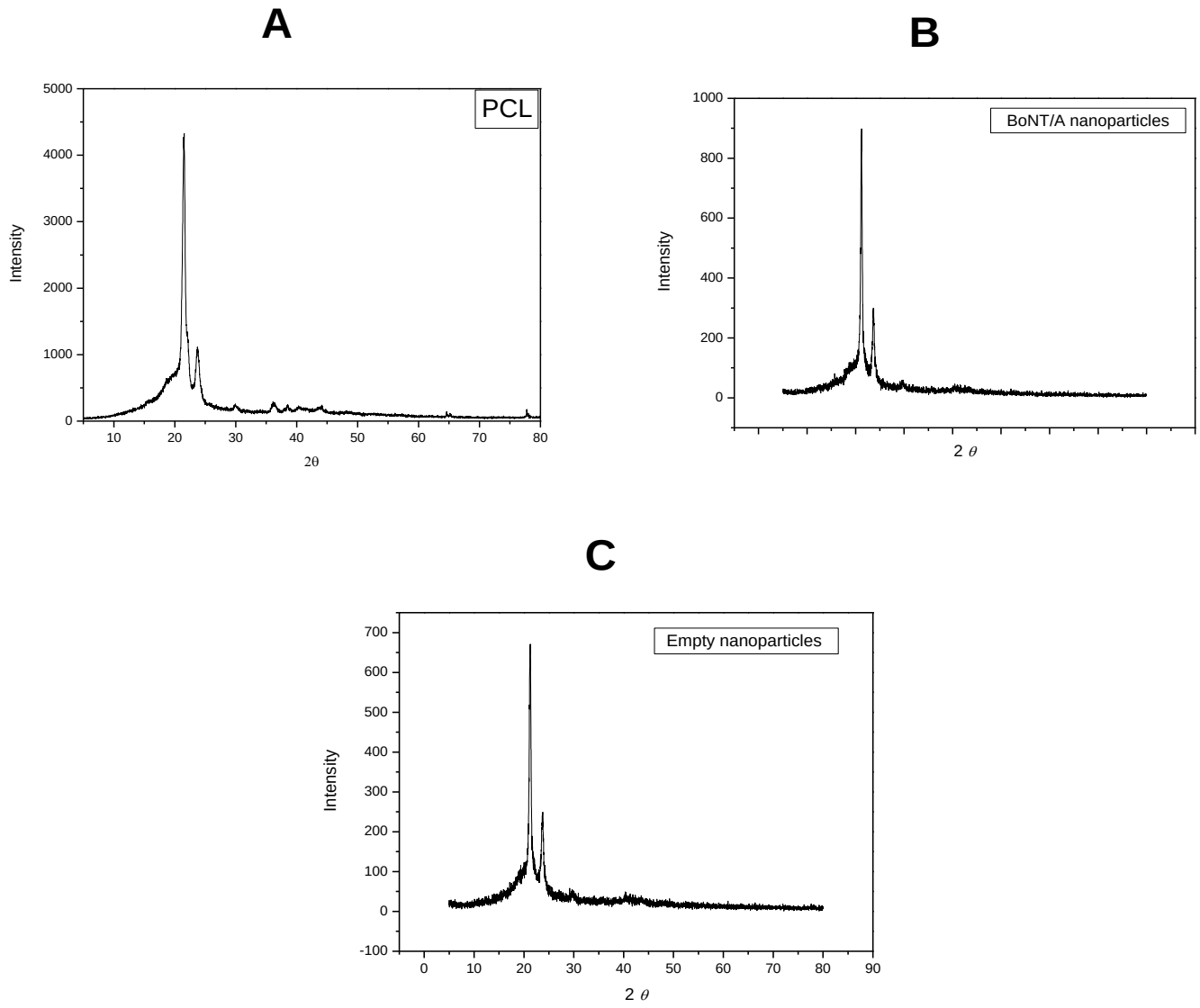

Figure 10. X-ray diffractograms of the PCL (a), nanoparticles containing botulinum toxin A (b), and empty nanoparticles (c).

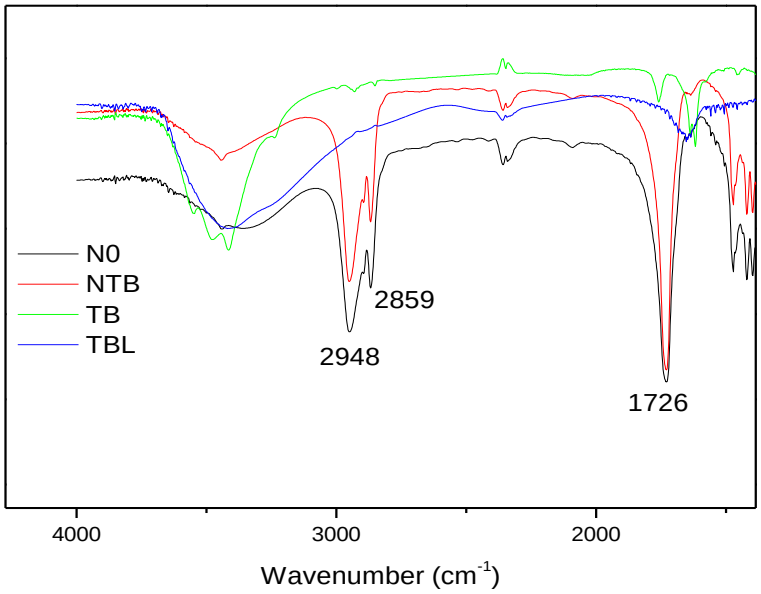

Figure 11. FTIR spectra of the PCL nanoparticles containing botulinum toxin A (NTB); empty nanoparticles (N0), botulinum toxin A (TB) and released botulinum toxin (TBL).

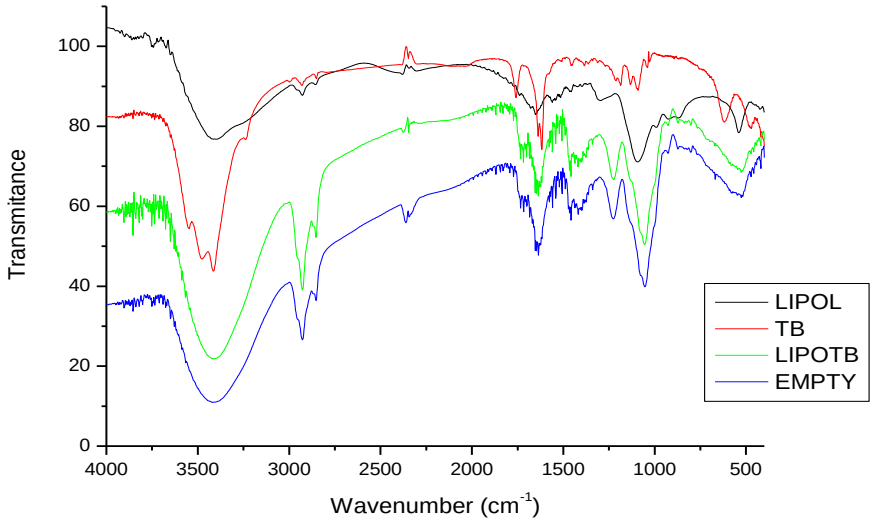

Figure 12. FTIR spectra of liposomes containing botulinum toxin A (LIPOTB), empty liposomes (L0), botulinum toxin Type A (TB), and released botulinum toxin (LIPOL).

Type A (TB) and botulinum toxin A released from the liposomes (LIPOL) are represented in Figure 12.

Evaluation of the effects of botulinum toxin on the release of insulin as a result of the activation of the neurotransmitters

The results of the samples analyzed on the release of insulin are shown in Table 1.

DISCUSSION

It can be seen that the vesicles that are formed have a spherical shape and regular surface, whereas the liposomes had heterogeneous sizes. This is probably due to the fact that in the formation of multilamellar vesicles both the number of lamellae and the shape can vary. The results were similar to those found by Paese (2008) Paese (2008). These values show a uniform pattern in the distribution of the size witch suggests a good level of

Table 1. Quantification of the insulin produced in islets of Langerhans after the incubation of the glucose solution produced by the botulinum toxin A and encapsulated in nanoparticles and liposomes.

Samples	Insulin (mU/mL)
Control (Glucose solution 16.7 mM)	15.9
Botulinum Toxin A encapsulated in nanoparticles	16.9
Botulinum Toxin A encapsulated in liposomes	381.2
Botulinum Toxin A	1078.2

suspension stability Gaumet et al, (2008). They confirm the observations made about the analysis of the images carried out by SEM and homogeneous particles were observed. According to Bouchemal et al. (2004), the size of the particle recommended for use in topical formulations (both pharmaceutical and cosmetic) is lower than 600 nm and as a result, the average diameter of the particles will be suitable for topical applications.

The negative values that are shown in zeta potential are caused by the presence of ester groups in the PCL Schaffazick et al. (2003) Bouchemal et al, (2004) and Müller et al, (2001). Lamprecht et al. (2001) obtained similar results with the PCL nanoparticles. Singh et al. (2006) used PVA in the preparation of the PCL nanoparticles and obtained a zeta potential of -2.6 ± 1.2 mV. Particles for cutaneous application with zeta potential (both negative and positive) are described in the literature Kulkamp et al, (2009).

An increased load found in the liposomes determines the degree of the repulsive force among the vesicles bringing about stability and prevents the agglomeration of the particles Sengodan et al, (2009). The X-ray of the empty nanoparticles (N0) as a negative control shows a diffraction pattern for crystalline material similar to that of pure PCL and indicates that the main peaks are at (2θ) in 21.36° and 23.57° depending on the semi crystalline property of the used polymer, as already noted by Verger et al. (1998) and Perez et al. (2000).

The profiles of the crystalline diffraction of the nanoparticles containing botulinum toxin Type A were similar to the diffractogram of pure PCL. However, owing to the low concentration of the active agent, it is not possible to establish whether or not there exists a molecular dispersion Lima and Andrade (2012).

The polycaprolactone spectrum showed a strong band of 1726 cm^{-1} corresponding to the vibration of the axial deformation of the C=O group and 2 bands at 2944 and 2865 cm^{-1} which can be attributed to the stretching of the C-H groups Lima and Andrade (2012) and Poletto et al, (2008). It can be noted further that the NTB and N0 spectra show the same bands as those of the PCL.

The TBL showed bands similar to those of TB and as well as this, did not show bands in the range of 2948 and 2859 cm^{-1} which are present in the PCL and in the empty nanoparticles (N0), which is a strong indication that the toxin was encapsulated in the nanoparticles.

The cholesterol spectrum shows a wide band at 3250 and 3500 cm^{-1} corresponding to the O-H vibration. The phosphatidylcholine shows 2 bands at 2850 and 2920 cm^{-1} corresponding to CH_2 and CH_3 bonds. The C=O is represented at 1730 cm^{-1} and the P=O bond is found to be 1088 cm^{-1}. It can be noted that within the liposomes spectra that contain the botulinum toxin A and the empty liposomes (Figure 12), they show bands that are characteristic of cholesterol and phosphatidylcholine as mentioned earlier.

The LIPOL shows bands similar to the botulinum toxin type A bands as well as not showing bands in the range of 2850 and 2920 cm^{-1} which are present in the liposomes either containing botulinum toxin A or empty.

The botulinum toxin type A (TB) led to a greater release of insulin, followed by botulinum toxin type A encapsulated in liposomes (LIPOTB), whereas lower accounts of insulin were observed in the samples that were incubated with the polymer nanoparticles that contained botulinum toxin Type A (NTB) and there was a negative control for the sample that consisted of glucose solution. The botulinum toxin A encapsulated in liposomes, showed a significant difference when compared with the level of control and the botulinum toxin A encapsulated in nanoparticles. However, there was no significant difference when compared with botulinum toxin A. In contrast, the botulinum toxin encapsulated in nanoparticles showed a significant difference when compared with botulinum toxin A, but did not show any difference when compared with the negative control. These results suggest that the activity of the botulinum toxin type A is maintained after its encapsulation in liposomes, but not when encapsulated in polymer nanoparticles. This fact can be attributed to the loss of botulinum toxin activity after the preparatory process.

Conclusion

The viability of producing polymer nanoparticles containing botulinum toxin Type A can be demonstrated by employing the multiple emulsion and solvent evaporation methods with a suitable size for application on the skin, although with a zeta potential that is below

the expected values. It was also possible to demonstrate that the formation of the multilamellar liposomes containing botulinum toxin Type A could be promising carriers of the botulinum toxin A. Among the nanosystems that were developed, it can be noted that the liposomes had a better effect with regard to the release of insulin than that shown by the nanoparticles of botulinum toxin Type A.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors thank the Postgraduate Program in Pharmaceutical Sciences of State University of Ponta Grossa for providing infrastructure, constant support, and encouragements.

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