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Development of nanoparticles coated with cassava bagasse pectin (*Manihot esculenta* Crantz) containing β-carotene for mucoadhesive applications

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Abstract: Pectin (PC) extracted from a solid residue from cassava roots (*Manihot esculenta* Crantz) was used to coat nanoparticles (NP) containing β-carotene (BC) aiming at the gastrointestinal administration of this lipophilic nutraceutical. The NP were prepared by spontaneous emulsification method using food grade components. Pectin-coated NP have been successfully prepared as confirmed by the increased particle size and negative surface charges due to the pectin's anionic nature. NP showed spherical shape and monodisperse distribution, with a mean size of 21.3 nm (polydispersity index (PDI) 0.29) for BC PC T80-NP (nanoparticle with β-carotene, pectin and Tween 80) and 261.4 nm (PDI 0.1) for BC PC T20-NP (nanoparticle with β-carotene, pectin and Tween 20). BC was encapsulated at amounts of 530 and 324 μg/ml for BC PC T80-NP and BC PC T20-NP, respectively, with high encapsulation efficiency (> 95%), increasing its antioxidant capacity *in vitro*, besides no cytotoxic effect. However, only BC PC T20-NP was stable over a 90 days storage period (4°C) and revealed a strong interaction between pectin and mucin. These results suggest that pectin-coated BC PC T20-NP is a promising strategy to improve the bioavailability and permeation of BC for administration through mucosal surfaces.

Key words: β-carotene, cassava bagasse, *Manihot esculenta* Crantz, mucoadhesion, nanoparticles, pectin.

INTRODUCTION

β-carotene (BC) has several biological activities, being the main carotenoid with provitamin A activity in human diet (Saini et al. 2015). BC also shows strong antioxidant activity, eliminating hydroxyl and superoxide radicals and, thus, avoiding lipid peroxidation of low density lipoproteins (LDL) (Böhm et al. 2002). However, despite its importance to human health, this compound has some constraints for its nutraceutical use; for example, its sensibility to the chemical degradation by radical oxidation triggered by the singlet oxygen produced when it is exposed to light, high temperatures or heavy metals (Achir et al. 2010). In addition, BC is a hydrophobic molecule with high melting point and hence its solubility in water, as well as oral bioavailability, are low. Therefore, these characteristics present obstacles to its incorporation in food (Rao et al. 2013) and/or to the development of new aqueous based formulations.

Polysaccharide-coated nanoparticles (NP) with mucoadhesive properties might be a strategy to increase the time of contact of bioactive compounds with biological membranes (e.g. mucosal surfaces), optimizing their permeability and bioavailability (Rose & Voynow 2006). Biopolymers, such as pectins (PC) has become attractive for the formulation of drug carriers and/or nutraceuticals due to their non-toxic traits, resistance to degradation by proteases and amylases, and mucoadhesive properties (Marras-Marquez et al. 2015). PC are abundant in nature, present in the plant cell wall, but with their commercial sources limited mostly to the apple pomace and citrus peels [14]. In addition, pectin's chemical composition, structure (degree of esterification, e.g.) and physicochemical properties are distinct according to the plant species donor (Marras-Marquez et al. 2015, Chan et al. 2017).

In this context, the research and development of NP coated with PC from alternative botanical sources is justified because this biopolymer presents a great variation in its chemical composition and, therefore, can offer different functional characteristics (Sila et al. 2009). Such differences can occur, for example, with respect to their resistance to enzymatic degradation or mucoadhesive properties that might be interesting for increasing the retention period of active compounds in the absorption site, eventually improving its therapeutic efficacy. Thus, cassava (*Manihot esculenta* Crantz) bagasse, a by-product from the processing of roots for the production of starch, has virtually no economic value and is a source of PC with properties not yet investigated regarding the development of delivery systems for hydrophobic nutraceuticals.

Previous studies on nano-based products containing PC extracted from the cassava bagasse have not been reported in the scientific literature so far. Thus, the development of PC coated-NP containing BC provides subsidies both regarding the mucoadhesive interactions of this polymer and the performance of new strategies in optimizing the encapsulation of BC, a nutraceutical with low bioavailability and easily degradable. The main objective of this study was to develop and characterize NP coated with cassava bagasse PC (*Manihot esculenta* Crantz) loaded with BC for mucoadhesive applications.

MATERIALS AND METHODS

Materials

The cassava bagasse was obtained from the agroindustry Fecularia Machado (Sombrio county, Santa Catarina state, southern Brazil) from roots harvested in 2014/2015. Bagasse was produced as a solid by-product during the extraction of cassava starch. Polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20) were purchased from Vetec (Rio de Janeiro, Brazil). PIC Química (São Paulo, Brazil) kindly donated caprylic/capric triglyceride (Miglyol 812 N), a medium chain triglyceride (MCT), and corn oil was bought from a local supermarket. β-carotene (BC, ≥ 97% purity grade) and 2, 2-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO) and methanol, acetonitrile, and acetone (HPLC grade) from Tedia Company (Fairfield, OH) All other chemicals were analytical grade reagents.

Pectin extraction

As adapted from Maraschin et al. (2000), 50 g (dry weight) cassava bagasse were weighed, added of 3V 90% (v/v) dimethylsulfoxide (DMSO) solution, and incubated at 25ºC for 26 h, with constant stirring. Further, the biomass was incubated (water bath, 40ºC, 35 min) and centrifuged (4,000 rpm, 10 min, 25ºC). The supernatant, i.e., fraction S1 containing starch, was discarded and the residue (R1) collected, oven dried at 50ºC

until achieve constant weight. Three volumes 0.5% (w/v) ammonium oxalate solution, were added to R1, following incubation for 2 h, at 60°C. The biomass was centrifuged (4,000 rpm, 10 min, 25°C) and to the supernatant, i.e., pectic fraction, 3V ethanol (92.8° GL) were added, followed by incubation for 3 days at 4°C to obtain a flocculated PC. This PC fraction was recovered by centrifuging (4,000 rpm, 10 min, 25°C), freeze-dried for 24 h and stored at -20°C.

Preparation of pectin-coated β-carotene nanoparticles

BC-loaded NP were prepared by the spontaneous emulsification method as described previously (Anton & Vandamme 2009), with modifications. In order to optimize the preparation conditions, NP were prepared using different types and concentrations of surfactant and oil. Briefly, the oil phase composed by 0.1% BC, surfactant (Tween 20 or Tween 80), and oil (MCT or corn oil) was heated at ~ 80°C, under magnetic stirring, until complete solubilization of the carotenoid (< 5 min). Then, the oil phase was poured into 10 ml of aqueous phase containing water at 80°C, under moderate magnetic stirring. Table I shows the preparation conditions for the NP developed.

The preparation of pectin-coated NP was carried out from previous dispersion of PC in the aqueous phase, following filtration through 14 µm pore size filter paper. The PC concentration varied from 0.01 to 0.5% (w/v). Unloaded pectin-coated NP, prepared with different surfactants (Tween 80 or Tween 20, named Blank PC T80-NP and Blank PC T20-NP, respectively), were also obtained under the same experimental conditions for comparison purposes. The development stages of the NPs are represented in the scheme of figure 1.

Table I. Composition of the formulations studied for obtaining nanoparticles with β-carotene by spontaneous emulsification.

Composition	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
β-carotene (%, w/v)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Corn oil (%, w/v)			10	10			10	10						
MCT (%, w/v)	10	10			10	10			10	10	5	1	5	1
Tween 20 (%, w/v)	20		10		10			10	5				5	5
Tween 80 (%, w/v)		20		20		10	10			5	5	5		
Water (ml)	10	10	10	10	10	10	10	10	10	10	10	10	10	10

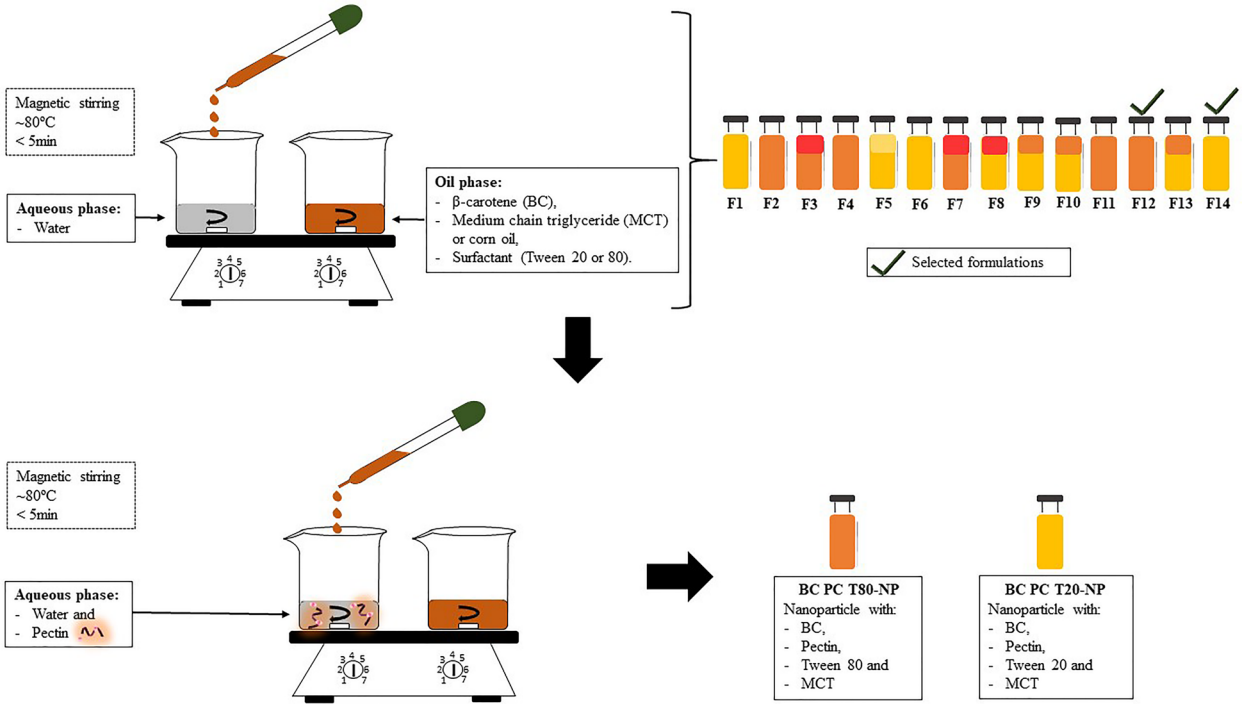

Figure 1. Schematic representation of the stages of development of nanoparticles.

Measurements of particle size and zeta potential

By means of the dynamic scattering of light (DLS) and laser Doppler anemometry the average particle size and the zeta potential were determined. The samples were diluted in ultra-pure water and the measurements performed at 25°C. The size analyzes were carried out at a fixed spreading angle (90°), while to measure the zeta potential (ζ) the samples were placed in electrophoretic cells with an electrical potential established at ±150 mV. Thus, using the Smoluchowski's equation, the zeta potential values were calculated as mean values of the electrophoretic mobility. These parameters were obtained using the Zetasizer Nano ZS90 equipment (Malvern Instruments, Worcestershire, UK).

Morphology of nanoparticles

The morphology of the NP was performed using a Jeol JEM-1011 transmission electron microscope (TEM, Jeol Ltd., Tokyo, Japan). Uncoated and pectin-coated NP were previously diluted (1: 10) in ultra-pure water, deposited on carbon-coated copper grids, and stained with 2% (w/v) uranyl acetate solution for 30 min.

Stability study

The evaluation of the physical stability of pectin-coated NP loaded with BC was performed after a storage of samples for 90 days, in amber glass bottles at -4°C to prevent possible degradation from exposure to light and high temperature. After 0, 7, 30, and 90 days the average particle size, polydispersity index, zeta potential, and pH were measured.

HPLC analysis

Chromatographic conditions

HPLC analyses were performed on a UHPLC Thermo Scientific UltiMate 3000 RS Dual System

(Thermo Fisher Scientific, San Jose, CA) equipped with a C18 reverse phase column (Supelco 15 cm x 4.6 mm, 5 µm) coupled to a pre-column (Sigma-Aldrich 2 cm x 4.0 mm, 5 µm) and UV-visible spectrophotometric detector operating at 450 nm. Elution utilized methanol: acetonitrile (90: 10, v/v) as the mobile phase at 1 ml/min flow. Quantification of analytical grade BC (Sigma-Aldrich, MO, USA) was done by diluting it with chloroform: acetone (1: 1, v/v) solution, followed by building a standard curve (1.6 to 100 µg/ml - $y = 1.259x$, $r^2 = 0.99$) considering the analyte's peak area from three consecutive injections per sample for concentration calculations.

Determination of BC content

To quantify BC, 100 ml nanoparticle were added to 900 ml acetone and stirred until complete solubilization. Subsequently, this dispersion was filtered for the removal of PC through a funnel of regenerated cellulose (RC-Vliesverstärkt - 0.2 µm). NP prepared without BC (blank nanoparticles) were prepared under the same conditions to check if any compound of the formulation interfered in the analysis. After preparation, solutions were pipetted into amber vials and injected three times into a UHPLC apparatus. BC content was expressed as mg BC/ml nanoparticle. The real BC concentration was estimated by comparing the total amount of the compound found in the suspension and that added to the formulation.

Determination of encapsulation efficiency

The NP suspensions were placed in Amicon Centrifugal Filter Devices (100 kDa, Millipore Corp., Billerica, MA) and centrifuged (6,200 rpm, 30 min) to separate the encapsulated BC (supernatant) from the free one (filtered). The filtered contents were transferred to amber vials and further analyzed by UHPLC as previous described. Thus, the encapsulation efficiency (EE) (%) was determined from the difference between the total concentration of BC found in the NP suspensions after their complete dissolution in acetone and the concentration of the analyte recovered after the ultra-filtration/centrifugation procedure.

In vitro antioxidant activity

The antioxidant activity of the free and NP forms of BC was determined by the 2, 2-diphenyl-2-picrylhydrazyl (DPPH) method (Kim et al. 2002). From a DPPH stock solution (7.9 mg DPPH in 2.5 ml methanol) diluted to a concentration of 1: 100 (v/v), an 80% methanol solution was prepared. The samples were diluted to different concentrations (mg/ml) for BC PC T80-NP, BC PC T20-NP (in water), and BC (in chloroform/acetone 1:1, v/v). Thus, a sample (10 µl) was added to 290 µl diluted DPPH solution, shaken, and held out of the light for 30 min. After incubation, the absorbance of samples was measured on a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA) at 530 nm. The DPPH solution was used as a control. The percentual inhibition of the DPPH radical was calculated from the following equation:

$$DPPH\ Inhibition\ (\%) = \left((abs.\ DPPH - abs.\ Sample/abs.\ DPPH)\right)100$$

Where, abs. DPPH is the absorbance of the DPPH solution and abs. Sample is the absorbance of the sample.

Interaction between mucin and pectin-coated nanoparticles

The evaluation of the interaction between mucin and pectin-coated with NP was made according to the method previously described by Mazzarino et al. (2012). The bovine submaxillary gland mucin (BSM) was prepared at the concentration of 250 µg/ml in a sodium phosphate buffer (0.2 M), pH 8. NP suspensions at different

concentrations (1 to 40%, v/v) were added to 2 ml mucin dispersion and allowed to stir for 15 min, at room temperature. Subsequently, the mean particle size of the mucin-nanoparticle aggregates was measured using a Zetasizer Nano ZS90.

Cytotoxicity assay with Caco-2 cells

Caco-2 cells (#HTB-37), obtained from the American Type Culture Collection (ATCC), were cultured and maintained in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% L-glutamine. Cells were grown in a humidity atmosphere and 5% CO_2, at 37°C until reaching 80-90% confluence and seeded in 96 well plates (2.5 x 10^4 cells/well) for 48h incubation. After, Caco-2 cells were exposed to different concentrations of free BC, T80-NP, and T20-NP nanoparticles for 6h. After incubation, 100 µl 10% trichloroacetic acid (TCA) were added to each well to fix the cells for one hour (at 4°C). The plates were then washed with water to remove TCA and stained with sulforhodamine B (SRB) for 30 min. Subsequently, the plate was washed with 1% acetic acid to remove unbound SRB (Vichai & Kirtikara 2006). The protein-bound dye was then dissolved in 10 mM Tris-Base [tris (hydroxymethyl) aminomethane] (5 min) solution and the optical densities (OD) were read at 510 nm on a Spectra Max M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). This methodology was also used to determine the maximum concentration of tetrahydrofuran (THF) that was used to solubilize BC with the culture medium.

Statistical analysis

Descriptive statistics were presented as mean and standard deviation. The one-way ANOVA followed by the Dunnett's *post hoc* test ($p < 0.05$) was applied to Gaussian distribution data. Statistical analyses were performed with the support of Excel (Microsoft Office, 2016) and GraphPad Prism (v. 7.0) softwares.

RESULTS AND DISCUSSION

Development of nanoparticles

In this work, NP containing BC were obtained by the spontaneous emulsification method without the addition of organic solvents and using food grade ingredients. The development of NP is a promising strategy for delivery of lipophilic molecules due to the small size that confers to these systems physical stability and high bioavailability, important traits required for numerous products in the food and pharmaceutical industries (Komaiko & Mcclements 2016). The production of NP can be made from high-demanding energy techniques, such as high pressure homogenization that breaks the oil phase into small droplets (Solans & Isabel 2012). Alternatively, because they are simpler, cheaper and more energy efficient as simple stirring is required, low-energy emulsification methods, such as spontaneous emulsification, have gain considerable attention in recent years (Solans & Isabel 2012, Komaiko & Mcclements 2016).

Spontaneous emulsification occurs when two immiscible liquids emulsify without any external aid, such as electrical or mechanical energy (Komaiko & Mcclements 2016). In food systems due to the concern for safety, taste and cost, it is desirable avoid the use of organosolvents. Thus, an organic phase, containing oil and a hydrophilic surfactant, and an aqueous phase composed by water and possibly a co-surfactant, are commonly used (Anton & Vandamme 2009). This process can be affected by a number of factors including the preparation conditions such as composition, type, and concentration of surfactant and oil,

among others (Komaiko & Mcclements 2016). In order to optimize the NP preparation, four parameters were evaluated: the type of oil (MCT or corn oil), concentration thereof (1, 5, and 10% w/v), type of surfactant (Tween 20 or 80), and its concentration (5, 10, and 20% w/v). All NP suspensions showed yellow to orange colors, ranging from translucent to milky aspect.

The mean particle size, polydispersity index (PDI), and zeta potential of developed NP are shown in Table II. All formulations prepared with corn oil showed phase separation. In this study, formulations with BC precipitates or inhomogeneous ones after visual inspection (e.g., presenting phase separation) were considered unstable and not analyzed. Based on this result, NP prepared with MCT oil in different concentrations were chosen for further evaluation. Chang et al. (2013) reported that MCT substitution by long chain triglyceride (LCT) carriers, such as in corn and canola oils, did not form stable NP for reasons not yet well understood. According to these authors, possibly the molecular characteristics between MCT and LCT, such as chain size and saturation, may have affected the physicochemical properties determining the formation of spontaneous emulsions, such as viscosity, interfacial tension, phase behavior or optimum curvature.

The mean particle size, polydispersity index (PDI), and zeta potential of developed NP are shown in Table II. Some formulations formed phase separation or BC precipitates shortly after preparation, as others (F6) were initially homogeneous, but became unstable after a few days of storage. However, those ones that remained homogeneous were evaluated and all showed a PDI < 0.26, confirming a monomodal distribution of particles.

The results revealed the particle size as a dependent variable of the NP composition. The average particle size and PDI decreased when the Tween 80 concentration was greater than MCT one. Thus, in F12 formulation the particle size and PDI were the smallest among all formulations, i.e., 16.1 nm and 0.142, where the surfactant/oil concentration was 5% and 1% (w/v), respectively, i.e., the amount of Tween 80 was 5 times greater than that of MCT. It is known that the hydrophobic portion of the surfactant molecule adsorbs around oil droplets, at the oil/water interface, while the hydrophilic portion is solvated by water molecules from the external aqueous medium (Tadros 2017). Thus, it is possible to notice that upon a higher

Table II. Mean particle size, polydispersity index and zeta potential of the BC nanoparticles developed by spontaneous emulsification method.

Parameters	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
Particle size (nm)	ND*	54.2 ± 0.56	ND*	ND*	ND*	180.5 ± 6.45	ND*	ND*	ND*	ND*	88.5 ± 1.25	16.1 ± 0.31	ND*	132.0 ± 3.77
Polydispersity index	ND*	0.164 ± 0.018	ND*	ND*	ND*	0.259 ± 0.017	ND*	ND*	ND*	ND*	0.224 ± 0.030	0.142 ± 0.022	ND*	0.164 ± 0.004
Zeta potential (mV)	ND*	-12.0 ± 1.04	ND*	ND*	ND*	-15.2 ± 0.44	ND*	ND*	ND*	ND*	-15.8 ± 1.45	-7.3 ± 0.84	ND*	-18.0 ± 2.54

Data are represented as mean ± standard deviation (n = 3). *ND= not defined (these formulations were not measured because after preparation they did not remain homogeneous).

concentration of Tween 80 in relation to MCT, there will be enough surfactant in the oil-water interfacial layer to completely cover the surface of BC-MCT droplets, thus avoiding coalescence and decreasing the size of NPs, as well as maintaining a desired monomodal distribution, with the reduction of the PDI (Saberi et al. 2013). In addition, there is a reduction in the oil-water interfacial tension with the largest amount of surfactant molecules diffusing from the organic phase to the aqueous one, leading to the formation of thicker oil droplets in this interfacial zone (Anton & Vandamme 2009). This explains why F2 formulation presented an average particle size 54.2 nm and PDI 0.164, values slightly superior to those observed for the F12 formulation. Importantly, this occurred because F2 formulation presented a 2: 1 surfactant/oil ratio, that is, the amount of Tween 80 was only twice that of the MCT.

On the other hand, the average particle size and the PDI also decreased following the reduction of the MCT and Tween 80 contents, but when in equal proportion for both (1: 1). Thus, particle size and PDI values of 180.5 nm and 0.259, respectively, were found in F6 formulation with oil and surfactant concentrations of 10% (w/v). Besides, by reducing the contents of these constituents to 5% (w/v - F11 formulation), particle size and PDI values were still lower, i.e., 88.5 nm and 0.224. However, the particle size and PDI of these formulations were larger than F2 and F12 ones, probably because this proportional equity (MCT/Tween 80 1: 1) decreased the efficiency of Tween 80 to completely cover the surface of BC-MCT droplets. In addition, it could increase the instability of the system as demonstrated by the higher PDI and even by the phase separation detected in F6 formulation after a few days of storage. This result is in line with those obtained by Saberi et al. (2013) who found that when a specific surfactant concentration is exceeded, measurements of particle size distribution show bimodal distributions. In addition, above a certain level of surfactant an increase in the drop size is expected due to the formation of a highly viscous liquid crystalline phase that makes it difficult to spontaneously break the oil-water interface (Wang et al. 2009).

Another factor in the composition of the system that can influence the formation of spontaneous emulsions is the surface charge of the particles. High potential zeta values (> 30 mV) are good indicators of stability in the systems, since droplets with higher surface charge increase the electrostatic repulsion between the oil droplets of the NP, thus preventing aggregation and destabilization (Vallar et al. 1999, Jin et al. 2018). The zeta potential values for all formulations were low in module, i.e., -7.3 to -18.0 mV, probably resulting from the stabilization of MCT droplets with BC by nonionic surfactants which are characteristic of Tween 20 and 80 (Chanamai et al. 2002, Strickley 2004). Interestingly, despite this nonionic nature, these slightly negative values can be attributed to anionic impurities present in surfactants, e.g., fatty acids that result in particles with negative surface charges (Wu et al. 2017). In this case, the physical stabilization promoted by these nonionic surfactants occurred by the steric and non-electrostatic mechanism through the adsorption of the oil-water interface, which reduces interfacial tension and improves the protection of the aggregation drop (Krstonošić et al. 2009, Bai et al. 2016).

Among the formulations obtained, F12 and F14 have the same concentrations of all components, differing only in the surfactants (i.e., Tween) used. Formulation F12 showed a micellar particle size (16.1 nm), demonstrating that Tween 80 had a direct influence on this variable when compared to the formulation prepared with Tween 20 (F14, particle size = 132.0

nm). Tween 80 has been reported to form smaller droplets than Tween 20 due to differences in its molecular geometry. This can be attributed to the fact that Tween 80 has a single unsaturated hydrophobic tail that is folded, while Tween 20 has a single linear saturated hydrophobic tail (Chang et al. 2013, Guttoff et al. 2015). Based on these physicochemical properties and taking into account the lowest concentrations of oil and surfactant required, formulations containing 1% MCT and 5% Tween 80 (F12) or Tween 20 (F14) were selected for further preparation of pectin-coated NP.

The NP was chosen based on the results of particle size and polydispersity analysis. Formulations F12 and F14 gathered relevant characteristics to be compared each other. Both formulations presented suitable average particle size for the purposes of this investigation and low rates of polydispersity. F12 exhibited micellar dimensions at 16.1 nm, while F14 largely differed from it at 132 nm, produced with different surfactants. Given this scenario, they were chosen to further assess the variables of interest in the continuation of the study.

Coating of nanoparticles with pectin

PC concentrations from 0.01 to 0.5% (w/v) were tested maintaining, however, all other preparation conditions (Table I) used for F12 and F14 formulations. The results showed that the particle size and zeta potential of NP suspensions are dependent on the PC concentration, as can be seen in Figs. 2a and b. By increasing PC concentration an increase in zeta potential values was detected, what is also evident when compared to the low surface charge values noted for NP not coated with the biopolymer (Table II), confirming the coating of the NP with PC in the developed formulations. NP prepared with Tween 80 and different concentrations of PC obtained zeta potential values ranged from -29.3 mV to -42.4 mV, while lower values in module (-20.7 mV to -27.3 mV) were found when using Tween 20. Thus, it is possible to infer the existence of discrepancies between the PC-Tween 80 and PC-Tween 20 interactions, since a stronger adsorption of the biopolymer to the first surfactant seems to occur. This increase in the surface charge occurs due to the anionic nature of PC adsorbed at the oil-water interface, contributing with electrostatic forces and steric

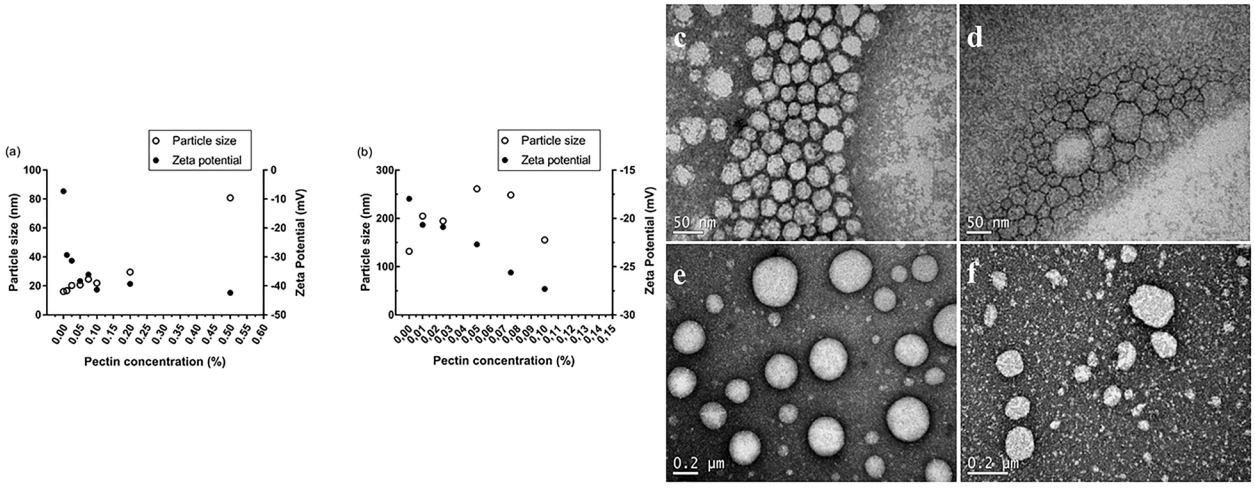

Figure 2. Particle size and zeta potential of the nanoparticles prepared with Tween 80 (a) and Tween 20 (b) according to the concentrations of pectin. Transmission electron microscopy of BC PC T80-NP (c) and BC PC T20-NP (e) with pectin and BC T80-NP (d) and BC T20-NP (f) without pectin.

repulsion that increase the stability of the NP (Chen et al. 2006).

Another relevant feature of PC in terms of its stability is its chemical structure. Some studies have associated the presence of acetyl and methyl groups with the interfacial activity of the PC in NP, leading to a consequent stabilization of the system. In previous studies (unpublished data), the PC extracted and used in the present investigation had its degree of methyl esterification (DM) determined by nuclear magnetic resonance of hydrogen (^{1}H-NMR) presenting a value of 32.39% and characterizing it as a low methoxylation (LM) biopolymer. Schmidt et al. (2015) detected a direct relationship between DM and the citrus PC emulsion capacity by increasing DM from ~ 70% to ~ 80%. However, other authors have investigated citrus PC with DM ranging from 22 to 73% and inferred that the content of methyl esters had little importance in the emulsification capacity of that polysaccharide (Akhtar et al. 2002).

However, despite the amphiphilic character, LM pectins are more hydrophilic than high methoxylated ones due to the greater availability of hydroxyl groups in its structure, which interact with the polar portions (e.g., OH^-) of the surfactant molecules by means of hydrogen bonds. Thus, it is suggested that such interactions may be responsible for increasing the particle size as PC concentration increases in formulations. In Fig. 2a it is possible to observe that the system containing PC-Tween 80 showed a discrete increase in particle size for the first concentration of that biopolymer and a greater variation in the concentrations of 0.2 to 0.5%. In its turn, the results found for the PC-Tween 20 NP system (Fig. 2b) reveal that, in general, an increase in the particle size was noted up to the concentration at 0.075%. For higher concentrations, an imbalance occurred with a drastic reduction in 0.1%, as the systems containing PC at 0.2 and 0.5% could not be measured because they did not remain homogeneous after preparation. This is probably due to a weaker adsorption between PC-Tween 20 as compared to PC-Tween 80, as noted by the zeta potential values discussed above. Therefore, there are optimum concentrations of PC to stabilize the nano-based system and values above that boundary caused phase separation or precipitation for the formulation with Tween 20.

Additionally, it is worth mentioning that in addition to the differences in molecular geometry, the surfactants also present a distinct hydrophilic-lipophilic balance (HLB). This system classifies hydrophilic surfactants as having high HLB values (> 10), while lipophilic surfactants show values below 10 (Macedo et al. 2006). The HLB of Tween 80 and 20 are 15.0 and 16.7, respectively, and although both are classified as hydrophilic these differences may also influence the interactions with PC molecules (Chang & McClements 2014). These HLB values are relevant because they are high enough for these surfactants to be hydrophilic, but not high enough to be insoluble in the organic phase.[19].

Several studies have pointed to PC as an excellent stabilizer for food hydrocolloids, such as nanoemulsions (Dutta & Sahu 2012). One of the interesting characteristics of this biopolymer is its amphiphilic character, which helps to reduce the interfacial tension between the oil and the aqueous phase, improving NP stability (Guerra-Rosas et al. 2016). Another relevant feature of using PC in the development of NP is its viscosity. The increase of this polymer augment the viscosity of the aqueous phase and, therefore, the stabilization of the system, since it diminishes the gravitational movement of the oil droplets retarding or avoiding the coalescence (Guerra-Rosas et al. 2016).

The PDI of the formulation containing Tween 80 ranged from 0.26 to 0.60. The NP PC concentrations under 0.1% showed a monodisperse system (<0.3), except 0.025% (0.32). Meanwhile, NP prepared with Tween 20 showed PDI values between 0.09 and 0.14, confirming the monodisperse distribution of particles for all concentrations of PC. Based on the physicochemical properties, the formulations with Tween 80 and Tween 20, both with 0.05% PC, were selected for the next evaluations and characterization, and hereafter they will be named BC PC T80-NP (nanoparticle with BC, pectin, and Tween 80) and BC PC T20-NP (nanoparticle with BC, pectin, and Tween 20).

Transmission electron microscopy (TEM) images (Fig. 2c, d, e, f) showed the spherical and more regular shape of the PC-coated NP (BC PC T80-NP and BC PC T20-NP) in contrast to those without the biopolymer (BC T80-NP and BC T20-NP). In addition, TEM exhibited an average particle size for NP with PC around 21 and 261 nm, respectively for BC PC T80-NP and BC PC T20-NP. In their turn, NPs without PC averaged around 16 nm (BC T80-NP) and 132 nm (BC T20-NP), similar to those obtained by DLS studies.

Stability study

Stability studies of PC-coated NP were performed after storage of samples in amber glass bottles at 4°C, during 90 days. BC is known to be sensitive to chemical degradation when exposed to high temperatures, heavy metals and oxidation by the singlet oxygen produced when exposed to light (Achir et al. 2010). It is worth mentioning that its storage at 4ºC improves the stability of encapsulated BC comparatively to higher temperatures (Luo et al. 2017). All NP maintained homogeneous appearance over the period of study. However, gradual loss of color (orange and yellow to almost colorless) was observed for both BC PC T80-NP and BC PC T20-NP, indicating a partial degradation of BC. Similar results were reported by Luo et al. (2017) investigating the stability of BC nanoemulsions stored at 4ºC, 25ºC, and 55ºC for 14 days. In their study, the authors affirm that the gradual loss of color is common to BC during storage and is related to their chemical instability.

The partial degradation of BC herein reported is in line with the results obtained by Tan & Nakajima (2005) under the same temperature and storage time used in this study. According to those authors, one of the hypotheses that explains this phenomenon is the influence of the BC's surface area, which in the nanometer range is significantly greater, as compared to bulk crystalline β-carotene. Thus, there may be a reduction in the stability of NP, providing more contact surface between BC particles and the aqueous environment.

As shown in Fig. 3, BC PC T20-NP formulation maintained the monodisperse distribution of the particles with no significant changes in particle size and zeta potential values. However, BC PC T80-NP formulation showed a significant decrease in the zeta potential values in module (-37.4 to -17.9 mV), thereby reducing the repulsive forces between the oil droplets, culminating with their coalescence and generating an increase in particle size, which ranged from 21.3 to 38.5 nm, indicating low stability of the colloidal system. In addition, both formulations exhibited significant reductions between the initial and final pH values, e.g., from 5.8 to 4.7 and from 6.2 to 4.5 for BC PC T80-NP and BC PC T20-NP, respectively.

According to Bai et al. (2017), the magnitude of the negative value of the NP charges, that is, the zeta potential, decreased with the reduction in pH due to the protonation of carboxylic functional groups in the PC chains under acidic conditions. This effect was noted in a very pronounced way in BC PC T80-NP, probably due

to the stronger adsorption of the biopolymer to Tween 80, as previously discussed. In fact, the results showed that the addition of PC provided an meaningful increase in zeta potential and, with acidification, an inverse response was detected. On the other hand, BC PC T20-NP, despite the reduction in pH, maintained its zeta potential without significant changes (-22.7 to -23.4 mV), showing that its PC chains did not suffer relevant protonation effects. It is suggested that these differences in behavior between the two formulations can be explained by the degree of interaction of PC with the type of surfactant and also due to the dimensions of NP, where BC PC T80-NP, with a micellar particle size, has greater specific surface area, which makes it more reactive.

Despite the reduction of pH, the stability study demonstrated that both formulations maintained relevant physicochemical properties in relation to their particle sizes and, in particular to the PDI, maintaining the required single-mode distribution.

Concentration of β-carotene and encapsulation

Table III shows the total concentration of BC in the nanoformulations which varied from 324 to 530 µg/ml for BC PC T20-NP and BC PC T80-NP, respectively. A reduction of the total amount of BC in relation to the initial one added (1 mg/ml) may result of its degradation during the heating for solubilization. This is because the development of NP capable of encapsulating and protecting BC is relevant due to its diverse

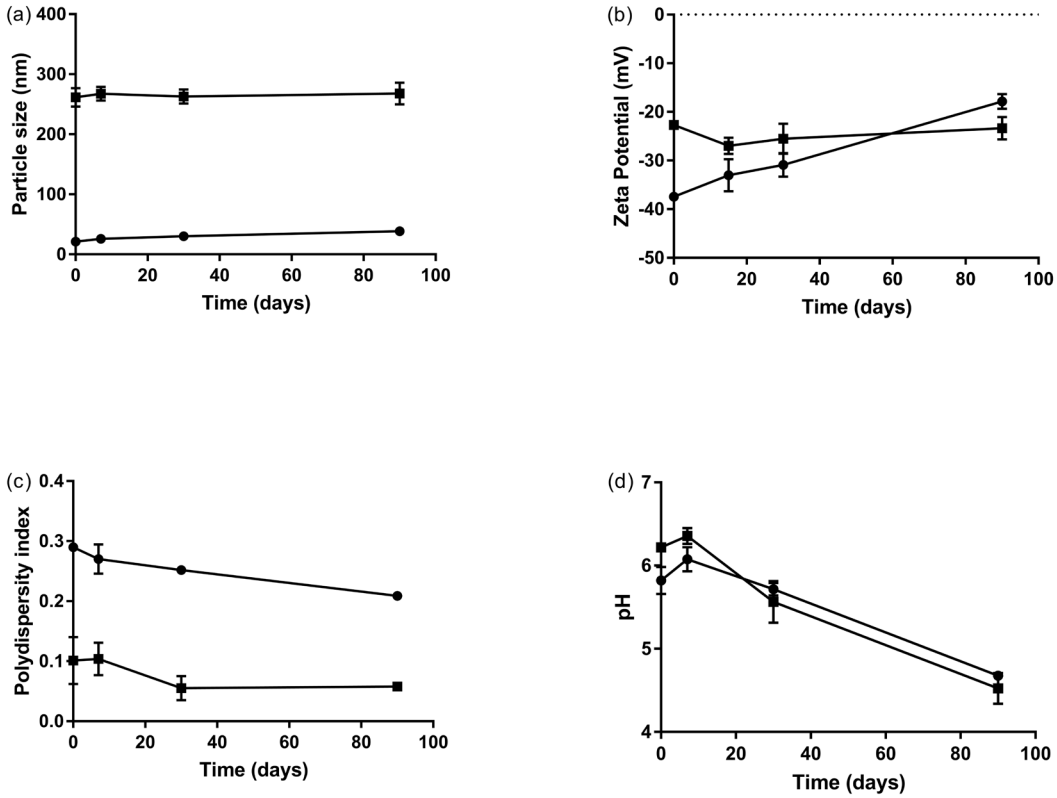

Figure 3. Stability study of the BC PC T80-NP (●) and BC PC T20-NP (■) nanoparticles. Particle size (a), zeta potential (b), polydispersity index (c), and pH (d) of nanoparticles stored for 90 days at 4°C. Data are represented by mean ± standard deviation (n= 3).

biological activities important to the promotion of human health. However, at the same time it is a challenge due to the BC's hydrophobic nature and the difficulties in maintaining its stability during processing (Colle et al. 2016). It is well known that some nonpolar solvents promote the solubilization of BC, however, in order to develop NP without the addition of organic solvents and using food grade ingredients, alternatives were sought to solubilize it with edible oils and, if possible, with absence or minimal heating due to its thermal sensitivity. However, the warming of the reaction medium for the solubilization of BC during the preparation of NP is widely described in the literature using oils at 50-60°C for 5 or 10 min (Qian et al. 2012, Luo et al. 2017), or even higher temperatures such as 140°C (Jo & Kwon 2014). In this study, nonetheless, those methodologies were not reproducible and it was necessary to add a surfactant ingredient and a minimum heating (70 to 80°C) in order to completely solubilize BC. Thus, the temperature and the heating time were standardized and controlled. However, the quantification of BC by UHPLC revealed that even the short heating period herein used during the preparation of the NP caused the loss of that pigment. The BC losses during the preparation of NP, to a greater or lesser extent, are common and recurrent in most scientific works (Tan & Nakajima 2005).

The challenge of working with BC is widely discussed in the scientific literature. Indeed, studies have attempted to develop optimized protocols in order to preserve at most BC during the processing and preservation of foods (Colle et al. 2016). This context also extends to the development of NP. Thus, the present study started using a high BC concentration, predicting future losses of that pigment over the process of developing NP. However, despite the losses found, the results demonstrated that the nanoencapsulated bioactive performed better than its free form. In fact, it is worth mentioning that the use of NP has been an effective strategy to improve the bioavailability of nutraceutical compounds (Yao et al. 2014). In addition, considering the mucoadhesive property of PC in this process, there is an extension of the nutraceutical retention period at the absorption site, thus increasing its therapeutic efficacy. In addition to this, the mucosal surface has an abundant blood supply and a sufficient blood flow rate that accelerates the adsorption process of the mucoadhesive system. Thus, in principle, the dose of the bioactive to be administered orally can be reduced substantially (Alexander et al. 2011, Asija 2014) and low concentrations of BC, in a nanoderivative form for instance, may be sufficient for achieving the biological effect of interest.

The difference in the total content of BC for the PC-coated NP possibly occurred due to the traits of the surfactants ingredients in the formulations that directly affected the particle size and their interactions with the biopolymer, thus suggesting a higher or lower efficiency in

Table III. β-carotene content (µg/ml) and encapsulation efficiency (EE%) of pectin-coated nanoparticles.

Formulation	Content (µg/ml)	EE (%)
BC PC T80-NP	530 ± 0.04	95.7 ± 0.22
BC PC T20-NP	324 ± 0.01	98.6 ± 0.02

Data represented in mean ± standard deviation (n = 3).

the protection of the bioactive investigated. As discussed above, PC has a stronger adsorption to the surface of the micelles containing Tween 80, in addition, the HLB value of that surfactant is lower than Tween 20, directly influencing the formation of emulsions and explaining the higher amount of BC detected in BC PC T80-NP.

The EE for both formulations showed to be higher than 95% (Table III), probably due to the low solubility of the bioactive compound in the outer aqueous phase of the NP suspensions. This demonstrates that the physicochemical properties and the design of the developed delivery system were adequate in the encapsulation of the BC.

Based on the results discussed above, a schematic representation of NP coated with PC is proposed in Fig. 4. In this illustration the PC molecules are anchored to the surface of NP stabilized by nonionic surfactants (Tween 20 or 80).

Antioxidant activity in vitro

The antioxidant activity of free and nanoencapsulated BC was measured by the DPPH radical scavenging potential. This method is based on the donor ability of hydrogen atoms or an electron transfer from the antioxidant compound to the DPPH radical forming a stable compound (Karadag et al. 2009). The results show that the encapsulation of BC into PC-coated NPs increases their antioxidant potential when compared to the free form of the carotene. The value of the effective concentration required to reduce the initial concentration of DPPH radicals by 50% (EC50) was also determined for the samples (Brand-Williams et al. 1995, Lu and Foo 2000). As shown in Table IV, the EC50 values of the PC-coated NP were significantly lower

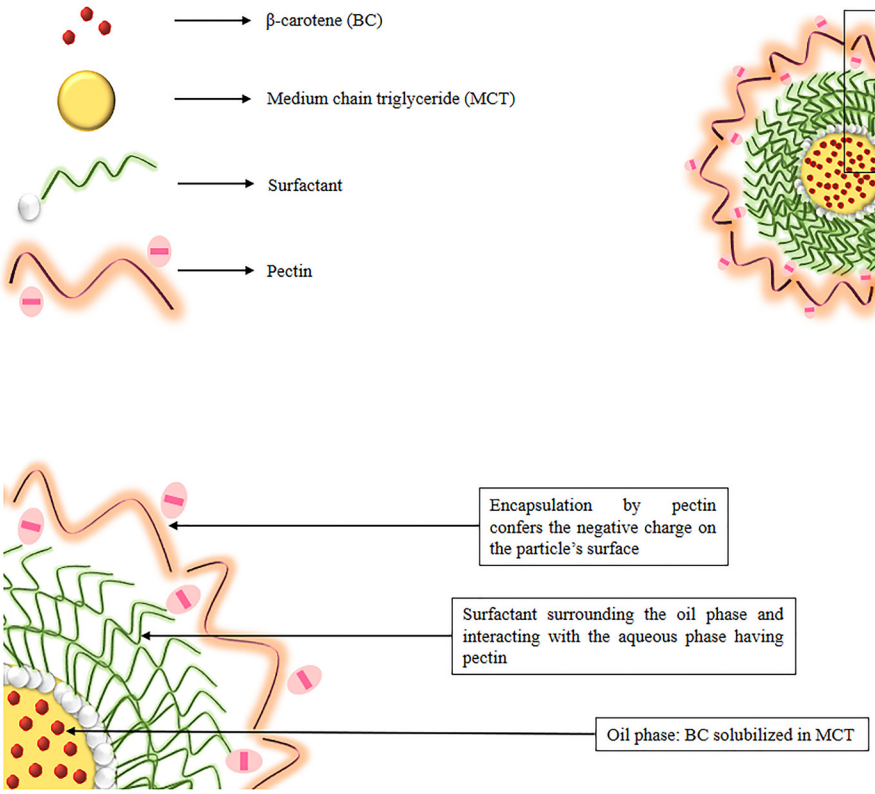

Figure 4. Schematic representation of nanoparticles coated with pectin containing β-carotene.

than those for free BC, indicating the improved antioxidant efficiency of the compound when nanoencapsulated. There are few articles about antioxidant properties of encapsulated carotenoids, but these results are in agreement with studies performed by Yi et al. (2015) where NP coated with dietary proteins, at 10 ug/ml, presented higher antioxidant potential (between 66.8 and 72.8%) than free BC (16.7%) as determined by the DPPH assay.

Interactions between mucin and coated nanoparticles with pectin

Experiments were carried out to determine the interaction between the PC-coated NP and mucin (BSM). BSM dispersion showed a particle size of 294.5 nm and high polydispersity (0.608). However, when the BC PC T20-NP nanoformulation was added to the BSM dispersion, the average particle size decreased. This finding may result from the compaction generated by the interaction between the mucin and the PC constituent of the NP. Indeed, the compaction of the NP is easily observed by its PDI value, which increases as the concentration of NPs increases. Likewise, by increasing BC PC T20-NP concentrations from 1.0 to 40% (v/v), the particle size augmented from 231.7 to 294.6 nm (Figure 5a). In addition, NP concentrations between 20 to 40% (v/v) in BSM dispersion showed a difference of particle size of 26.8 nm. Thus, it is evident that the particle size directly depends on the concentration of the PC-coated NP, suggesting the formation of aggregates resulting of a strong interaction between PC and mucin.

For the BC PC T80-NP nanoparticle and the BSM dispersions, due to the reduced micelle particle size (20.7 nm) we were not able in detecting changes in particle size. Eventually, by using more sensitive techniques such as quartz crystal microbalance or surface plasmon resonance (Mazzarino et al. 2014a, b) it would be possible to measure particle size.

Table V shows the particle size values of dispersion of mucin and uncoated and PC-coated NP loaded with BC. The addition of PC-coated BC PC T80-NP nanoparticle to the mucin dispersions resulted in an increase of 9.2 nm compared to PC-free NP, while for BC PC T20-NP this increase was higher (24 nm), demonstrating the excellent ability of PC to interact with the mucin by the mechanism of adhesion. PC has a large number of hydrogen bonding groups, such as the carboxylic groups (-COOH), which make possible the hydrogen bond forming with the mucin's functional groups. Thus, it has been proposed that the molecular interactions in the NP system occurs by adsorption mechanisms of the PC in the mucin molecules (Lee et al. 2000). In addition, the DM of PC may also influence the mucoadhesion process. Schmidgall & Hensel (2002) reported a more significant interaction against the mucous membranes of the colon by LM PC comparatively to the high methoxylation HM PC that were ineffective. This may occur because LM PC has fewer methyl groups and therefore a greater number of carboxylic groups available to participate in the mucoadhesion process. As the PC of the present study is a LM one, this trait evidences the mucoadhesive properties of the PC-coated NP, which exhibited exceptional interaction with the mucins.

Thirawong et al. (2008) investigating PC-mucin interactions reported this polysaccharide as a promising mucoadhesive polymer in the development of gastrointestinal drug delivery systems. Consequently, the ability of NP to interact with the mucosal surface can prolong the contact time of the bioactive incorporated into the delivery system and thereby improve the permeation and bioavailability of BC and/or other lipophilic nutraceuticals, for instance.

Table IV. Antioxidant activity of β-carotene and nanoparticles determined by the DPPH method.

Nanoparticles	EC_{50} (mg/ml)
β-carotene	0.552 ± 0.07[a]
BC PC T80-NP	0.080 ± 0.01[b]
BC PC T20-NP	0.074 ± 0.01[b]

Data are represented by mean ± standard deviation (n = 3).
EC_{50}: Effective concentration to reduce 50% of DPPH radicals.
Different letters indicate statistical differences between treatments (p <0.0001).

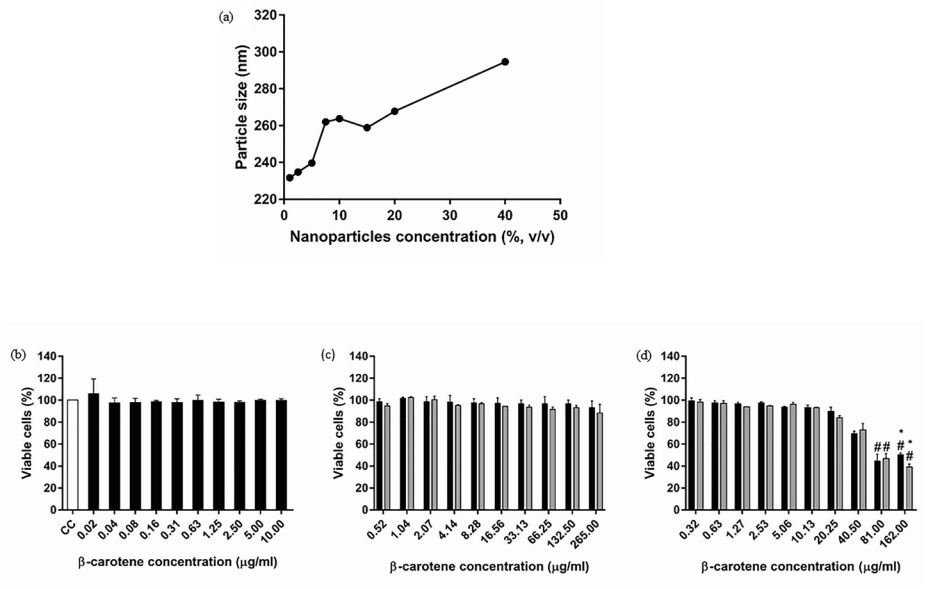

Figure 5. Variation of the particle size (main peak) of the colloidal mucin system (250 μg/ml) as a function of the addition of different concentrations of pectin-coated nanoparticles (BC PC T20-NP) (a). Cytotoxic effect of free β-carotene (CC= cell control) (b), BC PC T80-NP (black bars) and Blank PC T80-NP (gray bars) (c), and BC PC T20-NP (black bars) and Blank PC T20-NP (gray bars) (d) on Caco-2 cell lines after 6h incubation. Data are represented by mean ± standard deviation (n = 3). Distinct symbols (* and #) indicate statistical differences between treatments and the control

Table V. Particle size of dispersions containing mucin and uncoated (BC PC T80-NP and BC T80-NP 2.5%, v/v) and pectin-coated (BC PC T20-NP and BC T20-NP 20%, v/v) nanoparticles loaded with β-carotene.

Samples	Particle size (nm)
BC PC T80-NP	37.1
BC T80-NP	27.9
BC PC T20-NP	267.8
BC T20-NP	243.8

Citotoxicity in Caco-2 cells

In vitro cytotoxicity studies of free BC, BC loaded, and unloaded PC-coated NP were performed on Caco-2 cell lines. This cell culture is commonly used since it is derived from human intestinal adenocarcinoma and has been used to provide a useful model of cell culture of the small intestinal epithelium. In addition, it is in the small intestine that most of the digestion and absorption of bioactive compounds takes place (Natoli et al. 2012).

Due to the low solubility of BC in aqueous medium, 0.125% THF (v/v) was the maximum concentration used for BC solubilization in culture medium harmless to the cell viability. Thus, BC at 10 µg/ml was the maximum content possible within these parameters.

The *in vitro* cytotoxicity data were obtained for the free BC, unloaded, and BC loaded-NP (BC PC T80-NP and BC PC T20-NP) on Caco-2 cells after 6h incubation are shown in Fig. 5b, c, d. When compared to control, no significant reductions in cell viability were detected after treatment with free BC and BC PC T80-NP. Interestingly, the two highest concentrations assayed (81 and 162 µg/ml) of BC PC T20-NP NP and Blank PC T20-NP showed the highest cytotoxicity effect, since approximately 50-60% of the cells were dead at the end of the incubation time. As this effect was also observed in the NP without the bioactive, it is suggested that the reduction in cell viability occurred by the cytotoxic effect of Tween 20. Yi et al. (2014) also found no cytotoxic effect on Caco-2 cell lines after exposure to free BC. These results are interesting, since none of the constituents of the nanoformulations were toxic at the maximum concentrations of BC.

CONCLUSIONS

Two nanoparticle systems coated with PC extracted from a residual biomass of cassava roots after starch extraction were developed. These nanoformulations underwent an optimization study and were prepared effectively. Despite the degradation of BC, probably associated with the heating required for its solubilization in the preparation of the formulations, PC-coated NP efficiently encapsulated BC, presenting an excellent EE (> 95%) and a much better ability regarding the free radical scavenging (DPPH assay) when compared to the free bioactive compound. In addition, BC PC T80-NP was not cytotoxic to Caco-2 cell lines at any of the concentrations investigated, as BC PC T20-NP was cytotoxic only at the highest concentrations (81 and 162 µg/ml). Taking into consideration the two PC-coated NP developed, the BC PC T20-NP formulation showed to be more promising due to the high stability over the 90 day-storage period. In addition, the mucoadhesive properties of BC PC T20-NP were demonstrated by the excellent ability of mucin interaction through the adhesion mechanism.

Given this scenario, it is worth mentioning that the data herein shown are unprecedented results regarding the evaluation of NP coated with PC extracted from a cassava residue. This botanical source is unusual for the development of nanoformulations as reported in the scientific literature where citrus, apple pomace, and beet are commonly used. Thus, these NP are thought to be promising for the oral administration of BC, since the mucoadhesive properties of the cassava PC facilitate their interaction in the gastrointestinal tract by improving its absorption. In addition, this model can also be used to deliver other lipophilic nutraceuticals improving their therapeutic efficacy for administration to mucosal surfaces.

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Letícia Mazzarino and Marcelo Maraschin reviewed and supervised the work. Bianca Coelho carried out the experiments and wrote the manuscript. Clarissa Feltrin, Naira F.Z. Schneider, Heloísa S. Pitz and Cláudia M.O. Simões contributed to cytotoxicity assays. Ana Paula L. Voytena and Daniela S. Coelho collaborated with HPLC trials. Enilto O. Neubert contributed with the research and acquisition of samples. All authors discussed the results and contributed to the final manuscript.