Film-nanoparticle composite for enhanced oral delivery of alpha-casozepine

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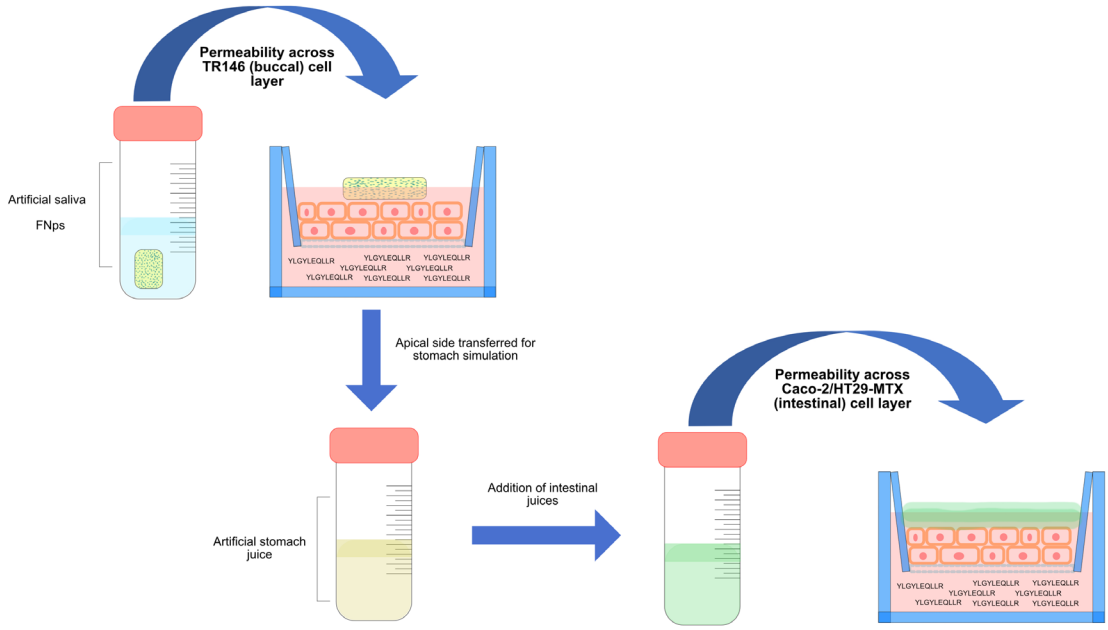
Abstract

Whey-derived α-casozepine (YLGYLEQLLR) was associated with previously optimized guar-gum film-PLGA nanoparticles (FNp), aiming to increase both permeability across absorptive epithelia but also stability across gastrointestinal tract. Developed formulations did not compromise the viability of TR146 buccal cell line nor Caco-2 and HT29 intestinal cell lines. Permeability across both buccal and intestinal cell barriers was enhanced when α-casozepine was carried by FNp system, when compared with film and nanoparticles alone or with control solution. None of developed formulations induced significant loss of cell viability as determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. It was verified that FNp delivery system promoted a significantly slower release of relaxing peptide when compared with PLGA nanoparticles and guar-gum films alone and with peptide control solution. Additionally, apparent permeability of α-casozepine across TR146 human buccal carcinoma cells carried by FNp (6.074E-06 $cm.s^{-1}$) was indeed superior when compared with peptide loaded in PLGA nanoparticles (3.084E-06 $cm.s^{-1}$) and in films (3.252E-06 $cm.s^{-1}$) alone or with free peptide control solution (7.594E-07 $cm.s^{-1}$). Both FNp and PLGA nanoparticles alone enhanced the permeability of relaxing peptide (1.433E-08 and 2.748E-08 $cm.s^{-1}$, respectively) compared with guar-gum films alone (9.045E-08 $cm.s^{-1}$) or peptide control solution (9.007E-09 $cm.s^{-1}$).



1. Introduction

Bioactive proteins and peptides present several advantages over conventional bioactive molecules as high specificity to target-receptors and superior biologic mimicking of physiological processes occurring in the organisms. Higher specificity leads to lower occurrence of unwanted side effects. Indeed, recombinant insulin production for the management of diabetes *mellitus* was of paramount importance for the exponential growth on research and development of biologics (Castro et al., 2015).

Whey protein is thoroughly studied as an important source of bioactive proteins and peptides. Indeed, peptides with antihypertensive, neuroactive, relaxing, immune and regulatory of the gastrointestinal tract have been obtained from the hydrolysis of whey protein (Dullius et al., 2018). α-casozepine is a decapeptide obtained from the hydrolysis of whey protein that presents anxiolytic activity and was reported to exert benzodiazepine-like activity, presenting homology with the diazepam binding inhibitor (Yoshikawa, 2015). Nevertheless, oral administration of α-casozepine leads to a lower bioavailability of about four times when compared with intraperitoneal administration (Benoit et al., 2017). Limited activity following oral administration of α-casozepine may be due to pH conditions and enzymatic activity in the gastrointestinal tract (Batista et al., 2018). Moreover, low permeability across intestinal mucosa and hepatic first-pass (or pre-systemic) effect also lead to a decrease in the bioavailability of bioactive molecules administered *per os*.

Buccal administration route represents a suitable alternative to conventional oral administration, being equally convenient but avoiding crossing gastrointestinal tract and hepatic first-pass effect (Morales and Brayden, 2017). Nevertheless, buccal mucosa is not as absorptive as intestinal and epithelia does not present specific molecule transporting systems. Also, saliva production and stimulation of the receptors in the palate and pharynx inevitably lead to swallowing.

Aiming bioavailability enhancement, α-casozepine was loaded in a buccal/oral delivery system comprised of previously optimized poly(lactic-co-glycolic acid) – PLGA - nanoparticles and guar-gum films (FNp). Guar-gum films were previously developed aiming a burst disintegration within 30 s, thus releasing the PLGA nanoparticles within the buccal mucosa and promoting buccal absorption of carried peptide. Due to limited absorptivity of buccal epithelia, PLGA nanoparticles were previously optimized by the group to present a

small size (~160 nm) aiming to enhance permeability of carried bioactive molecules across buccal epithelia (Batista et al., 2018; Castro et al., 2015). When swallowing of the formulation occurs, PLGA nanoparticles are reported to provide protection of carried bioactive molecules across stomach and intestinal extreme pH and enzymatic activity. It has also been reported that PLGA nanoparticles enhance intestinal uptake of carried molecules, with special affinity to M-cells (Fievez et al., 2009; Garinot et al., 2007).

2. Materials and methods

2.1. Materials

The relaxing peptide with the sequence YLGYLEQLLR (99.7%) was purchased from GenScript (China). Poly (lactic-co-glycolic acid) (50:50) was kindly offered by Purac. Ethyl acetate 99.5%, poly(vinyl alcohol) and D-sorbitol (assay purity ≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Citric acid monohydrate, potassium phosphate monobasic anhydrous and sodium phosphate dibasic were purchased from Merck (Darmstadt, Germany). Sodium chloride was purchased from Panreac (Barcelona, Spain). Deionized water was used to prepare all oral films formulations and ultrapure water was used to prepare peptide standard solutions and eluents used in chromatography procedures. TR146 cell line (passage 9) was purchased from Sigma-Aldrich (Stenheim, Germany). Transwell® flasks (12 well) and inserts (collagen-coated, 1.12 cm^2 of culture area, 0.4 μm pore size and 12 mm membrane diameter) were purchased from Corning (New York, USA). 96-well plates were purchased from Thermo Scientific (Denmark). Fetal Bovine Serum (FBS), HAMS-F12 culture medium and Pen-Strep (10 000 U Penicillin, 10 000 U Streptomycin) were purchased from Lonza® (Verviers, Belgium). TrypLE™ express was purchased from Gibco® (Denmark). Thiazolyl Blue Tetrazolium Bromide (MTT) Ultra-Pure was purchased from VWR (Solon, USA). Dimethyl sulphoxide (DMSO) 99.7% was purchased from Fisher Bioreagents™ (EUA). Phosphate buffer saline (PBS) solution was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of monobasic sodium phosphate and 0.24 g of dibasic potassium phosphate to 1 L of ultrapure water and by adjusting pH to 7.4 using a solution of sodium hydroxide 0.1M. For TR146 cell wash, pH of PBS was adjusted to 6.8, using a solution of hydrochloric acid 0.1 M. Artificial saliva was prepared using 8.0 g/L of sodium chloride, 0.19 g/L of monobasic potassium phosphate and 2.38 g/L of dibasic sodium phosphate and pH was set to 6.8 using phosphoric acid. Finally, α-amylase (Sigma) was added to obtain a solution of 100 U/mL.

2.2. Production and characterization of PLGA nanoparticles

The formulation of PLGA nanoparticles was previously optimized by the group. PLGA nanoparticles were prepared by double emulsion technique, as described by Araújo et al. with slight modifications (Araujo et al., 2014). Briefly, a solution of the bioactive peptides was added dropwise to a solution of PLGA dissolved in 2 mL of ethyl acetate and sonicated for 30 s at 70% amplitude with a ultrasonicator (SONICS VibraCell EUA), resulting in a W/O emulsion. A solution of PVA was simultaneously prepared in separate and added to the sonicated PLGA solution. Immediately, the resulting simple emulsion was sonicated for 30 s and 70% amplitude, resulting in a w/o/w double emulsion. The double emulsion was kept stirring (600 rpm) for 3 h for the complete evaporation of ethyl acetate to occur, leading to the hardening of the nanoparticles.

2.2.1. Characterization of PLGA nanoparticles

PLGA nanoparticles were diluted (1:50) with Milli-Q water before particle size and zeta-potential analysis. Particle size and polydispersity index were determined by dynamic light scattering (DLS). Zeta-potential was determined by phase analysis light scattering. All measurements were performed in triplicate in a Zetasizer Nano ZSP equipment (Malvern Instruments Ltd, Worcestershire, UK).

2.2.1.1. Peptide association efficiency (AE)

The LC-ESI-UHR-QqTOF-MS analysis was performed on a UltiMate 3000 Dionex UHPLC (Thermo Scientific), coupled to a Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer with 50,000 Full-Sensitivity Resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany).

Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100 mm x 2.1 mm, 2.2 um) (Dionex) a 60 ºC. Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B). The gradient started with 5% during increased to 95% in 7 min, which was kept constant for 2 min and; returned to 5 % B in 1 min and maintained at 5% B for an additional 5 min at a flow rate of 0.25 mL/min. The injection volume was 3 µL. Parameters for MS analysis were set using positive ionization mode with spectra acquired over a mass range m/z 150 to 2500. The parameters were as follow: capillary voltage, 4.5 kV; drying gas temperature, 180 ºC, drying gas flow, 8.0 L/min; nebulizing gas pressure, 1.6 bar, collision RF, 1500 Vpp; transfer time, 100 us and prepulse storage, 10 us. Post-acquisition internal mass calibration used ESI-L Low/concentration tuning mix solution delivered by a syringe pump at the start of each chromatographic analysis.

Relaxing peptide association efficiency was calculated according to the following Eq. (6):

$$\frac{Wtc - Wsc}{Wtc} \times 100 \qquad (6)$$

where, Wtc stands for total weight of relaxing peptide used in the formulations and Wsc stands for relaxing peptide collected from the supernatant after centrifugation.

2.3. Morphological characterization and stability of PLGA nanoparticles

The analysis of morphological characteristics and respective alterations of peptide-loaded PLGA nanoparticles over time was performed by atomic force microscopy (Nanoscope 8 Multimode AFM equipped with an E-type scanner – Bruker, U.S.A.). Regarding the morphological characterization of peptide-loaded PLGA nanoparticles, freshly prepared according to the method reported in section 5.1.3.2., the sample was diluted (1:1000 proportion), spread on a mica discand dried by spinning for 5 min. Imaging was performed with ScanAsyst air probes.

To assess the stability of PLGA nanoparticles, samples were analysed in liquid, aiming to understand if the peptide release is related with erosion of the carrier particle. Time-lapse imaging of hydrated specimens was obtained by adsorbing the nanoparticles on freshly-cleaved mica and imaging with the fluidic cell filled with the desired buffer. Imaging was performed with ScanAsyst fluid+ probes. The extension of erosion extension was evaluated by determining particle height along time. The morphology of the borders of the particle was also observed. Briefly, sequential imaging of the same PLGA nanoparticle was performed every 10 min, for 490 min, using a pH 7.4 PBS buffer as dispersant. After 490 min, the buffer in the

AFM fluidic cell was totally replaced with a pH 2.0 HCl solution and height and morphology of the nanoparticle were also determined every 10 min.

2.4. Preparation of guar-gum films and assembling process with PLGA nanoparticles

Guar-gum films were previously optimized by our group and were prepared by the solvent casting technique (Castro et al., 2017; Castro et al., 2018b). Briefly, 54 mg of sorbitol, 40 mg of guar-gum and 7.6 mg of citric acid were added to 2 mL of distilled water and stirred until complete dissolution. Resulting solution was poured in a Petri dish and heated to 37 ºC for 1 h and kept at room temperature for the following 12 h. For the incorporation of PLGA nanoparticles within the film matrix, sorbitol, guar-gum and citric acid were dissolved in 2 mL of a previously prepared nanoparticle dispersion instead of the distilled water.

2.5. Peptide integrity analysis

Chemical and structural integrity of α-casozepine was determined by attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy. Analysis was performed on the peptide alone and incorporated in PLGA nanoparticles, guar-gum films and FNps. ATR-FTIR analysis were conducted in a FTIR spectrometer, model ABB MB3000 (ABB, Switzerland), equipped with a deuterated triglycine sulphate detector and using a MIRacle^{TM} single reflection horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate. All spectra were acquired with 256 scans and 4 cm^{-1} resolution, in the region of 4000-500 cm^{-1}.

2.6. Tongue adhesion of developed formulations

Mucoadhesion to tongue assessment was performed for α-casozepine-loaded FNp and guar-gum films on a texturometer (TA.XT plus Texture Analyser, Stable Micro Systems, United Kingdom). Mounted load cell presented a 5 kg capacity and force was calibrated with a 2 kg weight. Briefly, the formulations were adhered to the testing probe (squared shape, 6.25 cm^2) and a cow tongue (obtained fresh from a local slaughterhouse) was mounted on the texturometer support platform. Following, 3.5 mL of artificial saliva (pre-heated to 37 ºC) were poured dropwise on the top of the tongue. The probe was set to descend until contact between the formulation and the tongue occurred. Contact force was set to 5 g and contact time was set to 30 s. Thereafter, the probe ascended at a speed rate of 0.1 mm/s and debonding force was registered, allowing to determine adhesiveness (N), work of adhesion (N.s) and debonding distance (mm).

2.7. Human buccal epithelium cell line culture

Trans-epithelial permeability assay and cell viability after contact with produced formulations were performed using TR146 human buccal epithelium cell line culture. TR146 cell line was chosen due to great resemblance of normal human buccal mucosa, namely regarding undifferentiated, non-keratinized stratified epithelium, morphological and functional characteristics as activity of carboxypeptidase, esterase and aminopeptidase (Morck Nielsen and Romer Rassing, 2000). Also, expression of K4, K10, K13, K16 and K19 keratins, membrane-associated receptors for involucrin and epidermal growth factors also reflect other common characteristics to normal human buccal epithelium cells (Jacobsen et al., 1999; Jacobsen et al., 1995).

TR146 cell line was purchased from Sigma-Aldrich (USA) and passages 9 to 14 were used. The culture medium consisted of HAMS F-12 medium enriched with 2 mM Glutamine (Lonza), 10% (V/V) fetal bovine serum (FBS) and 1% (V/V) of penicillin-streptomycin antibiotic blend. TR146 cells were seeded and maintained in 75 cm^2 T-flasks (T-75) and incubated in a 5% CO_2/95% air and 98% relative humidity atmosphere. The culture medium was replaced every two days. When 70-80% of cell confluence was reached, cells were detached from T-75 flasks using 2 mL of TrypLE™ Express. Detached cells were then prepared and seeded either in other T-75 flasks, 96-well culture plates (Nunc®) or in Transwell® inserts 12-well culture plates purchased from Corning® (Germany).

2.8. Co-culture of Caco-2/HT29-MTX cell lines

Caco-2 (Caucasian colon adenocarcinoma) cell line is widely used for the study of intestinal permeation of bioactive molecules (Araujo and Sarmento, 2013). Nevertheless, Caco-2 monoculture was not significantly representative of duodenum epithelia due to the formation of tight junctions that typically occur in the colon but not in the small intestine, leading to a hindrance of the absorption of hydrophilic molecules. Also, Caco-2 monoculture is exclusively composed of enterocytes and overexpress efflux transporters, typical of an excretory rather than absorptive epithelia. Therefore, HT29-MTX (Caucasian colon adenocarcinoma grade II) cell line is used in co-culture with Caco-2 cells. HT29-MTX were chosen due to mucus producing ability, as occurs in the duodenum mucosa.

Thus Caco-2/HT29-MTX co-culture was used to better mimic the *in vivo* conditions that occur in the duodenum. HT29-MTX and Caco-2 cell lines were grown separately in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin and streptomycin and 1% (v/v) of non-essential aminoacids, at 37 ºC under a 5% CO_2 water-saturated atmosphere. Upon 70-80% confluence, cells were detached as described in section 2.4. Co-culture seeding in Transwells® was performed in a 9:1 ratio of Caco-2 (3 x 10^5 cells/well) and HT29 (3 x 10^5 cells/well) cells, respectively (Antunes et al., 2013).

2.9. Cell mitochondrial activity assessment

Cell-viability studies were carried out on proliferating cells, chosen when TR146 cells were 70-80% confluent in T-75 flasks and properly detached as described above. After detachment, cells were re-suspended in medium and seeded in 96-well plates with a concentration of 1 x 10^4 cells/mL, 200 µL per well. The same cell concentration was adopted in the 12-well plates but using 500 µL of cell suspension, after *in vitro* permeability assay. Cell-viability studies were performed after 24 h of culture, with previous supervision by optical microscopy of the morphology and confluence of the cells in the plate wells. MTT assay allows to assess mitochondrial viability and, therefore, cell viability after 12 h contact with prepared delivery systems (da Silva et al., 2016). If TR146 cells were viable, succinic dehydrogenase was able to transform the tetrazolium salt into insoluble, purple-coloured, crystals of formazan (Mosmann, 1983). Medium with 1% (V/V) Triton X-100 solution was added as lysis buffer and served as positive control. Negative control consisted of cells in contact with medium only. After treatment with produced formulations, 100 µL of the MTT reagent (0.5 mg/mL prepared in culture medium) was added to each well and the plates were incubated for 4 h. After incubation time has passed, reagent was carefully removed, allowing the insoluble formazan crystals to remain in the bottom of the wells. 100 µL of DMSO per well was used to solubilize the formazan crystals in a dark room and, after 15 min of agitation on an orbital shaker, the absorbance at 570 nm and 630 nm was read on a FLUOstar OPTIMA microplate reader (United

Kingdom), in triplicate. Absorbance values for all readings at 630 nm were subtracted from the absorbance values read at 570 nm. Cell viability (%, n=6 different, independent wells for the same experiment) was calculated according to Eq. (7):

$$\textbf{\textit{Cell viability}}\ (\%) = \frac{\textbf{\textit{Experimental value}} - \textbf{\textit{negative control}}}{\textbf{\textit{Positive control}} - \textbf{\textit{negative control}}} \times \mathbf{100} \qquad (7)$$

Concentration of the formulations tested for potential commitment of TR146 cell viability were chosen according to the average amount of saliva produced in the human mouth when in contact with food products (Watanabe and Dawes, 1988). The same concentration was tested for the Caco-2/HT29-MTX co-culture.

2.10. Peptide trans-epithelial diffusion study

Permeability assay was assessed in Corning® Transwell inserts, using 12-well plates. TR146 buccal cells were seeded into the inserts to mimic stratified epithelium of human buccal mucosa, as reported previously (Jacobsen et al., 1995; Zeng et al., 2015). Briefly, TR146 cell line was used due to the reported similarities between keratinization profile and metabolic activity with physiologic human buccal mucosa cells. TR146 cells were seeded on the inserts and the medium was changed every two days for 21 days. For medium replacement, medium was removed from the wells and 0.5 and 1.5 mL of fresh culture medium was added to the apical and basolateral sides, respectively. On the day of the study, culture medium was totally removed. Medium in the basolateral side (receptor part) was replaced with 1.5 mL of PBS, pH 6.8. Medium in the apical side (donor part) was replaced with fresh medium and peptide delivery formulations were introduced afterwards. Guar-gum films, PLGA nanoparticles, FNp and free relaxing peptide (n= 5) were tested. Samples of 600 µL were withdrawn from receptor part at 0, 15, 30, 60, 120, 180 and 240 min. Withdrawn volume was immediately replaced with fresh PBS, pre-heated to 37 ºC, to maintain sink conditions. The permeation experiments were carried out in 5 wells per peptide delivery system sample. After the final samples were drawn from the basolateral side, apical side content was fully aspirated and 1.0 mL of DMSO was used to destroy the cells in each well for the quantification of relaxing peptide adsorbed to the cell surface or present within the cells or in the transepithelial space. The concentration of relaxing peptide was determined by LC-MS using the same method as reported in section 5.1.3.2.2.

The fractional amount of relaxing peptide that permeated Transwell® inserts with the stratified epithelium formed by confluent TR146 cells (dQ) was determined over the time intervals (dt) and the flux (J) was determined by calculating the slope of the resulting plots, according to Eq. (8) (di Cagno et al., 2015).

$$J = \frac{dQ}{A \times dt} \qquad (8)$$

Papp ($cm.s^{-1}$) was calculated for free relaxing peptide, guar-gum films, PLGA nanoparticles and FNp, by normalizing the flux (J) over the concentration of relaxing peptide in the donor compartment (C_0) according to Eq. (9).

$$Papp = \frac{J}{C0} \qquad (9)$$

where, dQ/dt stands for the amount of permeated relaxing peptide over time, A for the tissue surface area and C_0 for the initial concentration of permeated relaxing peptide. All tested

formulations presented the same initial concentration of relaxing peptide (500 μg/mL) at the beginning of the peptide trans-epithelial study.

2.11. Cell monolayer integrity

Transepithelial electrical resistance (TEER) of TR146 monoculture and Caco-2/HT29 co-culture was measured every two days along cell growth and after every sample collection when the permeability assay was performed, to assess the cell growth rate and cell viability after contacting with tested formulations, using a Millicell® ERS-2 Voltohmmeter (Merck, Germany) (Araujo and Sarmento, 2013; Sander et al., 2013). During permeability experiments, TEER values for TR146 cell culture and for Caco-2/HT29-MTX were always above 130 $\Omega.cm^2$ and 250 $\Omega.cm^2$, respectively, indicating that cell cultures were viable along the assay (Jacobsen et al., 1995; Pan et al., 2015).

2.12. Statistical analysis

Statistical analysis regarding dissolution profile data was performed using IBM® SPSS® Statistics version 22.

Shapiro-Wilk ($n< 50$) test was used to verify if the values obtained for the responses in the experimental design were normally distributed. One sample T test was used to verify the existence of statistically significant differences between predictive models and experimental values. Experimental values were obtained from three samples selected from three new batches, for PLGA nanoparticles. Mean values for each batch were compared with the values predicted in the model.

3. Results and discussion

3.1. Characterization of α-casozepine-loaded PLGA nanoparticles

Developed nanoparticles were characterized regarding mean size, polydispersity index, zeta-pontential and peptide association efficiency and results are outlined in **Table 1**.

Table 1: Characteristics of placebo and peptide-loaded PLGA nanoparticles

Formulation	Association Efficiency (%)	Mean size (nm)	Polydispersity Index	Zeta-potential (mV)
PLGA nanoparticles (Placebo)	**N/A**	**20.39 ± 18.21**	**0.201 ± 0.034**	**-9.83 ± 1.57**
Peptide-loaded PLGA nanoparticles	**82.02 ± 2.26**	**163.31 ± 11.21**	**0.254 ± 0.032**	**-11.02 ± 1.90**

A high association efficiency of the peptide to the PLGA nanoparticles was obtained, indicating that a high portion of α-casozepine added to the formulation is either adsorbed to the surface or incorporated within the polymeric matrix of the nanoparticles. Moreover, the inclusion of the peptide in the PLGA nanoparticle formulation induced a statistically significant ($P< 0.05$) increase of mean size of the particles. Indeed, an increase in size of loaded particles, compared to unloaded particles, has been reported in the literature (Lozoya-Agullo et al., 2018). Also, an increase on polydispersity index was also reported for peptide-loaded PLGA

nanoparticles when compared with placebo nanoparticles. On the other hand, no statistically differences between formulations regarding zeta-potential were observed.

3.2. Morphology and stability characterization of PLGA nanoparticles

The images obtained from the AFM analysis of PLGA nanoparticles, outlined in **Figure 1**, allowed to conclude that, despite the observable polydispersity, the observed size of the particles is in accordance to the previously reported data, obtained by dynamic light scattering (Castro et al., 2018a).

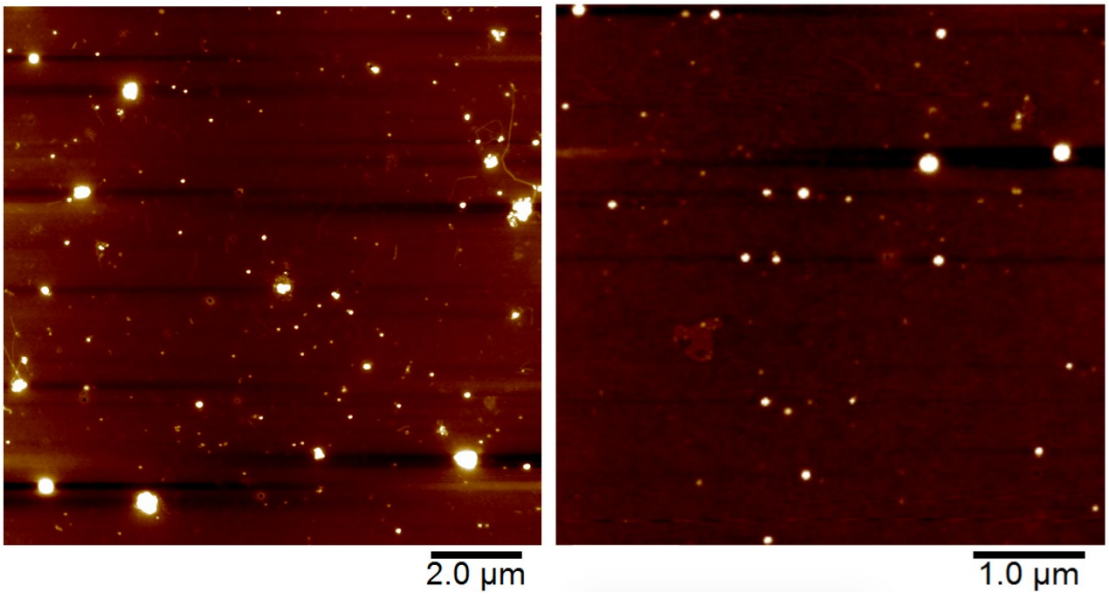


Figure 1: Atomic Force Microscopy images of peptide-loaded PLGA nanoparticles.

Besides the observed reproducible size, PLGA nanoparticles are widely reported as promoting long-lasting release of the carried bioactive molecule mainly due to slow erosion along time (Dinarvand et al., 2011; Hines and Kaplan, 2013). While sequentially in contact with a pH 7.4 PBS buffer (480 min) and a pH 2.0 HCl solution (480 min), the height of one PLGA nanoparticle tracked by the AFM decreased as outlined in **Figure 2**.

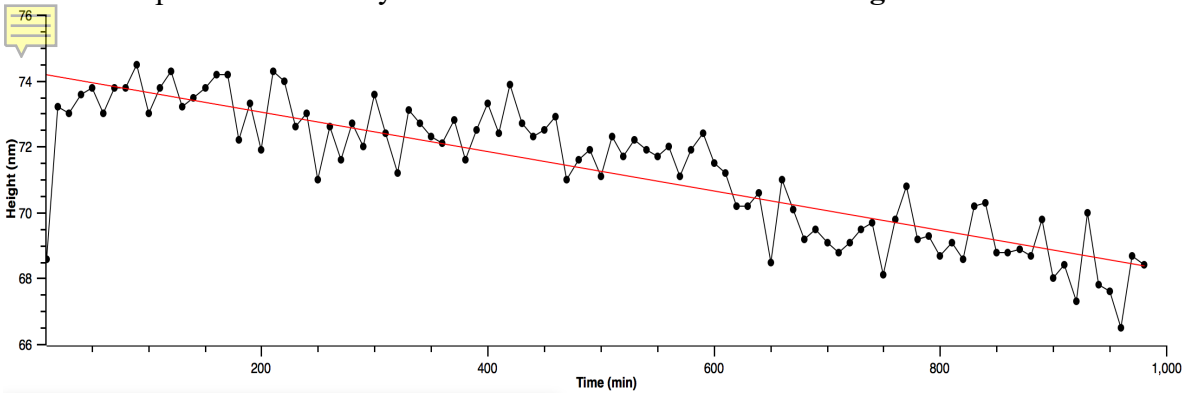


Figure 2: Height variation of PLGA nanoparticles in pH 7.4 PBS buffer (first 490 min) and in pH 2.0 HCl solution (from 490 to 980 min).

Even though a decrease on the height of the particle was observed, the erosion of the particle was slow over the 980 min. Indeed, the decrease of the height of the particle was of ~5 nm in 980 min, representing only 6.7% of the initial height of the particle or about 19% of its volume (assuming a spherical shape). Moreover, the height decrease tendency was not

significantly altered by changing the pH of the external solution (no changes in the slope value were observed from 0-490 min and 490-980 min). Results may indicate that the release of the peptide from the PLGA nanoparticle may be more extensively due to de-adsorption from the carrier that from erosion of the particle.

1.1.1.1. Peptide integrity analysis

The infrared spectra of α-casozepine alone or loaded in developed formulations was obtained by ATR-FTIR, aiming to understand if any alteration of the chemical structure of the peptide occurred after inclusion in guar-gum films, PLGA nanoparticles or FNps. The spectra are outlined in **Figure 3**.

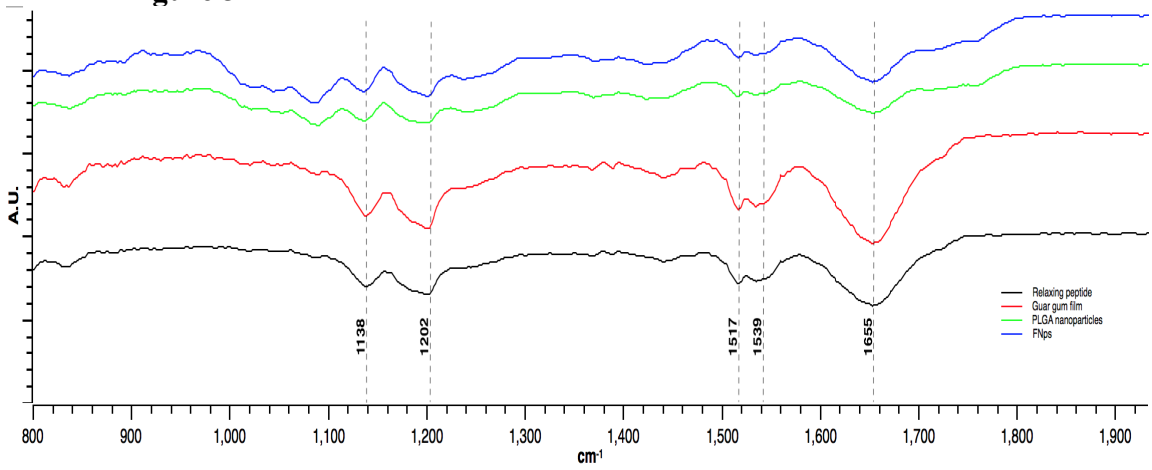


Figure 3: Infrared spectra of a-casozepine and α-casozepine-loaded guar-gum films, PLGA nanoparticles and FNps.

Bands between 1100 cm-1 and 1250 cm-1 (within fingerprint region) indicated the presence of C-C-O and C-OH bonds that are present either in the peptide molecules and in the chemical composition of developed formulations. Peptide bond characteristic bands resulting from the bending motions of the N-H groups and from the stretching of the C-N bonds were observed between 1490 cm-1 and 1580 cm-1 (amide II band) (Nixon, 2014). Moreover, a band between 1740 cm-1 and 1580 cm-1, corresponding to amide I band was observed and indicated the occurrence of C=O stretching vibrations and of C-N stretching.

The fact that the regions of both amide I and amide II of alpha-casozepine and alpha-casozepine-loaded formulations are overlapping is an indicator that there was no modification of the chemical structure of carried peptide during incorporation or production of delivery systems (Castro et al., 2018b).

3.3. Tongue adhesion of developed formulations

Tongue adhesion was performed for FNp formulation, aiming to assess if the addition of peptide-loaded PLGA nanoparticles to the film formulation would change the tongue adhesion properties. As outlined in **Table 2**, the inclusion of PLGA nanoparticles in the guar-gum film matrix significantly increased adhesiveness and work of adhesion of the formulation when compared with guar-gum film alone. Indeed, it has been reported that polymers with a high number of available polar groups (i.e. –COOH or –OH groups) are strongly mucoadhesive, therefore justifying the increased tongue adhesion when nanoparticles were added to the guar-gum matrix (Longer et al., 1985; Mathiowitz, 1999; Peppas and Sahlin, 1996; Ponchel and Irache, 1998; Reineke et al., 2013).

Table 2: Tongue adhesion properties of guar-gum films and peptide-loaded FNp

Formulation	Adhesiveness (N)	Work of adhesion (N.sec)
Guar-gum film*	0.026 ± 0.003	0.118 ± 0.062
FNps	0.041 ± 0.002	1.715 ± 0.084

3.4. TR146 and Caco-2/HT29 cell viability

Cell viability was determined by MTT for both for TR146 human buccal cell line and co-culture of Caco-2/HT29-MTX intestinal cells. Tested concentrations of formulations did not significantly compromise cell viability of both buccal and intestinal cell layers, as outlined in **Figure 4** and **Figure 5.**

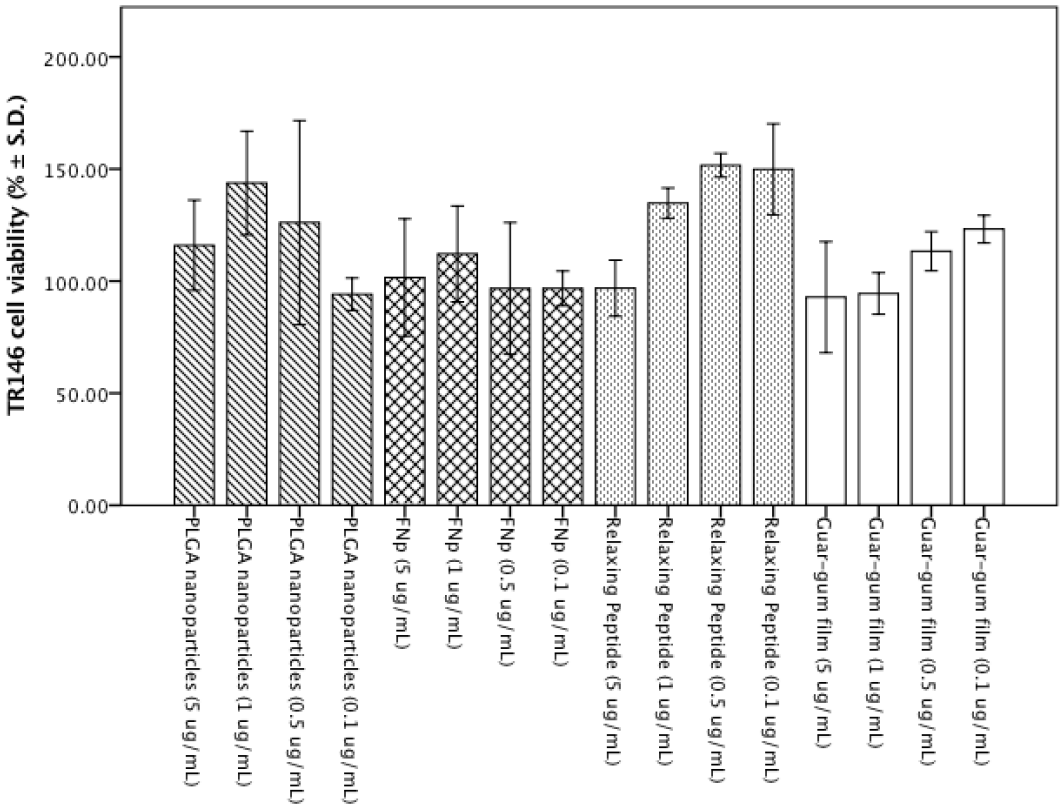


Figure 4: Mitochondrial viability of TR146 cells after contact with developed formulations for 12 h.

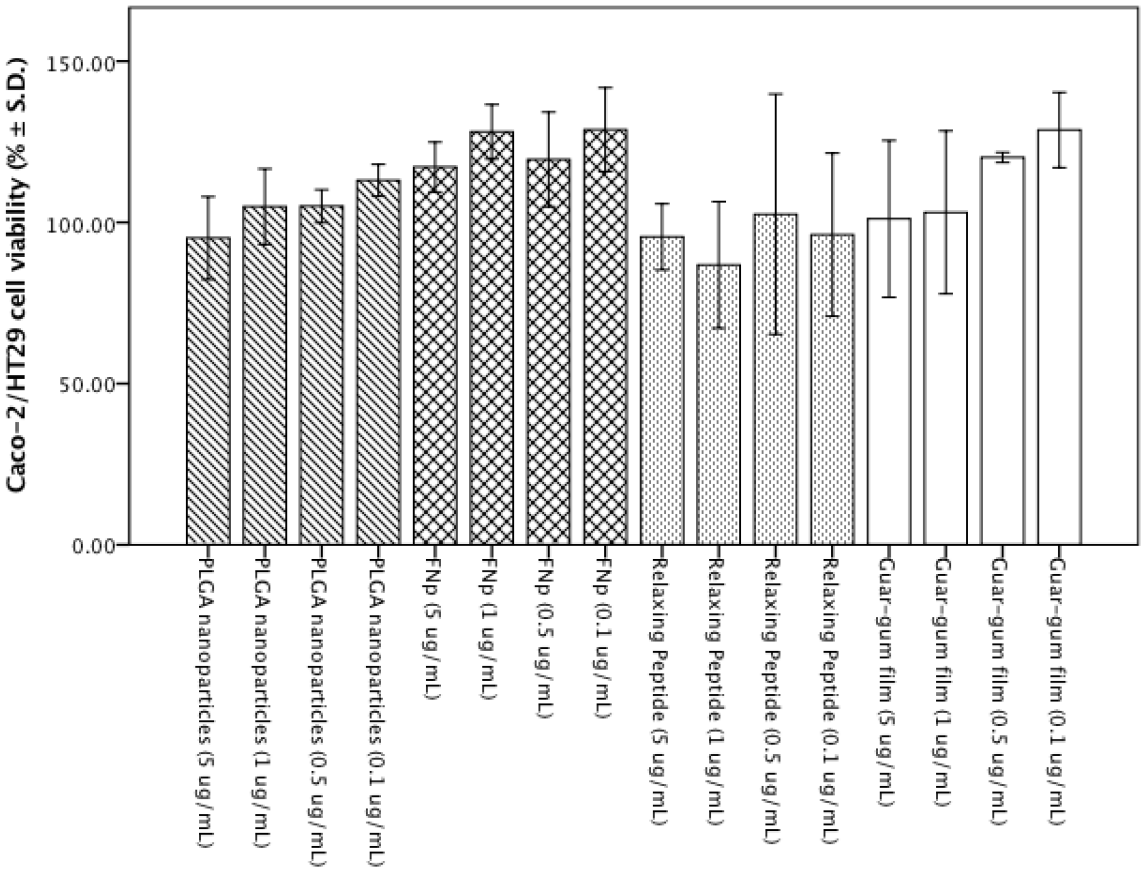


Figure 5: Mitochondrial viability of Caco-2/HT29-MTX cells co-culture after contact with developed formulations for 12 h.

Loss of cell viability was not expected since all materials used are generally recognised as safe (GRAS) at tested concentrations ((FDA), 2017a, b, c). Moreover, α-casozepine originates from whey protein hydrolysis and was not, therefore, expected to compromise cell viability. Results also indicated that *in vivo* toxicity should not be expected after oral administration of developed formulations, at tested concentrations.

3.5. Peptide transepithelial diffusion and protection across gastrointestinal tract

Developed formulations were intended to enhance permeation of carried peptide across absorptive membranes but also to offer protection along the gastrointestinal tract. It was verified that FNp system guaranteed higher peptide permeation across buccal mucosa when compared to PLGA nanoparticles and guar-gum films alone or peptide solution (control) as outlined in **Figure 6**. Moreover, the non-occurrence of molecules with different molecular weight observable in the mass spectrum indicated that degradation of the α-casozepine (either resulting in smaller peptides or in aggregation) did not occur after simulation of mouth digestion. Conversely, after simulation of stomach and intestinal simulation, new peptides were formed, likely resulting from the degradation of α-casozepine.

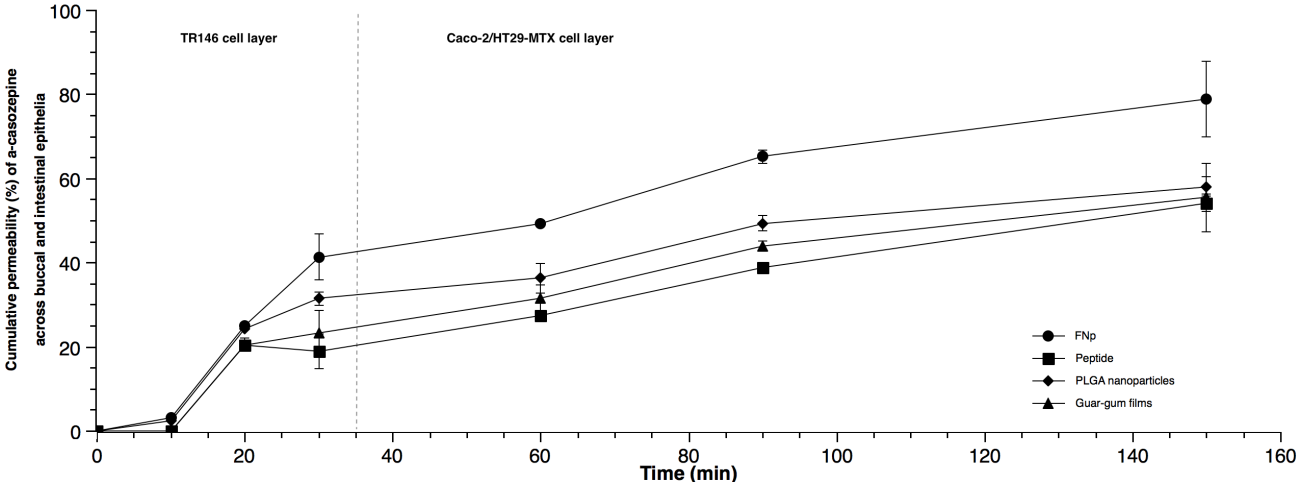


Figure 6: Cumulative permeation of α-casozepine across TR146 (buccal) and Caco-2/HT29 (intestinal) cell layers

Apparent permeability of relaxing peptide carried by FNp was significantly higher ($P < 0.05$) across TR146 cell layers when compared with remaining formulations and control solution as outlined in **Table 3**. Results obtained for Caco-2/HT29-MTX cell layers suggest that the steps of gastrointestinal tract simulation that followed contributed to the complete disintegration of tested formulations, therefore leading to the establishment of a *plateau* stage. Indeed, differences in apparent permeability across Caco-2/HT29-MTX cell layers for tested formulations were very modest, indicating that the greatest portion of relaxing peptide was already released to the digest by the time of intestinal section. Therefore, peptide permeation from 60 min to 150 min approached an equilibrium stage between the apical and basolateral sides of the cell layers.

Table 3: Apparent permeability (Papp) of α-casozepine across TR146 and Caco-2/HT29 cell layers

Formulation	Papp ($cm.s^{-1}$) across TR146 cell layers	Papp ($cm.s^{-1}$) across Caco-2/HT29 cell layers
FNp	1.77E-04 ± 2.48E-05	1.37E-04 ± 4.27E-05
PLGA nanoparticles	1.34E-04 ± 6.98E-06	1.01E-04 ± 1.03E-05
Guar-gum films	1.07E-04 ± 2.47E-05	1.11E-04 ± 4.83E-05
Peptide solution	8.79E-05 ± 1.88E-05	1.24E-04 ± 8.96E-06

4. Conclusions

Research regarding oral delivery of bioactive molecules as proteins and peptides has been focusing thoroughly in the permeability enhancement across intestinal mucosa. Nonetheless, buccal route presents several advantages as bypassing hepatic first-pass effect and avoiding contact with extreme pH and enzymes either in stomach and in the intestine. A conceptually new formulation was successfully developed from the association of guar-gum films and PLGA nanoparticles as carriers for α-casozepine. Peptide chemical profile was not altered while carried by developed formulations, as determined by the analysis of the spectra obtained

by ATR-FTIR. Tongue adhesion properties were significantly increased after adding PLGA nanoparticles to guar-gum film formulation. Moreover, the delivery system developed from the combination of PLGA nanoparticles and guar-gum orodispersible films was not cytotoxic for TR146 buccal carcinoma cells or Caco-2/HT29-MTX co-culture and demonstrated effectiveness regarding the enhancement of both buccal and intestinal permeation of carried relaxing peptide along with protection of the molecule across gastrointestinal tract. Results indicated that developed delivery system are good candidates to be tested *in vivo* for effectiveness.

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