

## Accepted Manuscript

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PII: S0144-8617(18)30410-7  
DOI: <https://doi.org/10.1016/j.carbpol.2018.04.032>  
Reference: CARP 13486

To appear in:

Received date: 19-1-2018  
Revised date: 6-4-2018  
Accepted date: 6-4-2018

Please cite this article as: Castro, Pedro M., Sousa, Flávia., Magalhães, Rui., Ruiz-Henestrosa, Victor Manuel Pizones., Pilosof, Ana MR., Madureira, Ana Raquel., Sarmento, Bruno., & Pintado, Manuela E., Incorporation of beads into oral films for buccal and oral delivery of bioactive molecules. *Carbohydrate Polymers* <https://doi.org/10.1016/j.carbpol.2018.04.032>

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## **Incorporation of beads into oral films for buccal and oral delivery of bioactive molecules**

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### **Highlights**

- Experimental design allowed to optimize a formulation of guar orodispersible films
- Alginate beads formulation was also optimized by experimental design
- Formulations were not cytotoxic to TR146 human buccal cells
- Combination of films and beads offers an effective buccal caffeine permeability
- Developed formulation offers a controlled release of carried molecules

**Abstract**

The association of alginate beads and guar-gum films in a single delivery system was idealized to promote a more effective buccal and oral delivery of bioactive molecules. A response surface method (experimental design approach) was performed to obtain optimal formulations of alginate beads to be incorporated into guar gum oral films as combined buccal and oral delivery systems for caffeine delivery. The combined formulation was further characterized regarding physicochemical properties, drug release, cell viability and buccal permeability. Beads average size, determined by dynamic light scattering (DLS), was of  $3.37 \pm 6.36 \mu\text{m}$ . Film thickness was set to  $62 \mu\text{m}$ . Scanning electron microscopy micrographs revealed that beads were evenly distributed onto the film matrix and beads size was in accordance to data obtained from DLS analysis. Evaluation of Fourier-transform infrared spectra did not indicate the formation of new covalent bonds between the matrix of guar-gum films, alginate beads and caffeine. *In vitro* release assays by dialysis membrane allowed understanding that the combination of guar-gum films and alginate beads assure a slower release of caffeine when compared with the delivery profile of free caffeine from alginate beads or guar-gum films alone. MTT assay, performed on human buccal carcinoma TR146 cell line, allowed concluding that neither guar-gum film, alginate beads nor guar-gum film incorporated into alginate beads significantly compromised cell viability after 12 h of exposure. As demonstrated by *in vitro* permeability assay using TR146 human buccal carcinoma cell lines, combination of guar-gum films and alginate beads also promoted a slower release and, thus, lower apparent permeability ( $1.15\text{E-}05 \pm 3.50\text{E-}06$ ) than for caffeine solution ( $2.68\text{E-}05 \pm 7.30\text{E-}06$ ), guar-gum film ( $3.12\text{E-}05 \pm 4.70\text{E-}06$ ) or alginate beads ( $2.01\text{E-}05 \pm 3.90\text{E-}06$ ). The conjugation of alginate beads within an orodispersible film matrix represents an effective oral/buccal delivery system that induces a controlled release along with an enhanced intimate contact with cell layers that may promote higher *in vivo* bioavailability of carried drugs.

**Keywords:**

Alginate Beads; Caffeine; Drug delivery; Experimental design; Oral films; Slow release.

## 1. Introduction

Buccal route is an attractive delivery route especially due to ease of administration and possibility to avoid first-pass metabolism [1]. Transport of drugs across buccal mucosa occurs exclusively by passive diffusion, an undifferentiated, unselective, transport mechanism [2]. Lipophilic bioactive molecules may permeate buccal mucosa by transcellular route whereas hydrophilic bioactive molecules are more prone to permeate buccal epithelia by intercellular route. Moreover, buccal epithelium presents a molecular weight cut-off that ranges from 500-1000 Da [3]. Hence, molecules with superior molecular weights may be hindered from transposing buccal epithelia and reach systemic circulation. Furthermore, saliva turnover limits the residence time of drugs within buccal mucosa, sometimes leading to premature swallowing [1].

Aiming to develop more effective buccal delivery systems, alginate beads containing caffeine as model drug were introduced within an orodispersible film matrix. Oral films are mucoadhesive oral delivery systems that disintegrate in the mouth in less than 30 s [3]. Besides from the inherent advantages shared with tablets or capsules (e.g. ease of administration and portability) administration of oral films does not require water. Moreover, oral films are especially useful for uncooperative patients since, once introduced into the mouth, and are very difficult to remove. Also, variables such as colour and taste are easily manipulated according to the preferences of the consumer/patient. Oral films are convenient delivery systems when buccal release is aimed [4].

Alginate is a natural anionic copolymer of 1,4-linked- $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid that is highly biocompatible and can be used to produce beads for buccal delivery of bioactive molecules [5]. Alginate beads can represent suitable delivery systems for the buccal mucosa, featuring mucoadhesion and sustained delivery of carried molecules. Production of alginate beads is cheap and does not imply using organic solvents, therefore being predictably safe for human consumption. For the entrapment of hydrophilic molecules, emulsification-internal gelation technique to produce alginate beads was used to provide high association efficiency for caffeine.

Alginate beads/microparticles have been used as delivery systems for buccal delivery of drugs but, to our knowledge, were not intended for buccal absorption, only aiming local activity [6-8]. Furthermore, alginate has been used for the production of buccal delivery systems (e.g. tablets) but not in the format of beads [9]. Moreover,

incorporation of alginate beads on film matrices represents an unconventional, conceptually new oral/buccal delivery system that conjugates the practicality and user-friendly characteristics of oral films and the slower release of carried bioactive molecules provided by alginate beads.

An experimental design based on response-surface method allows obtaining optimized formulations, assuring quality-by-design, thus avoiding excessive expending of time and resources and allowing to obtain robust and optimal results. In this study, both alginate beads and guar-gum films formulations were optimized using an experimental design approach and optimal formulations were prepared, based on characteristics (factors and responses) that are well-known for conditioning overall quality and performance of beads and oral films. Therefore, chosen variables for the optimization of guar-gum films were the relative amounts of guar-gum (matrix-forming polymer), sorbitol (plasticizer and sweetener) and citric acid (saliva-stimulating agent). Distance at burst and film burst strength were chosen as mechanical responses, being directly correlated with elasticity and rigidity, respectively. Film erosion (%) and the ratio of water-uptake over time ( $\text{mg}\cdot\text{s}^{-1}$ ) were set as representative responses of the capacity of the oral films to absorb water and disintegrate, releasing the content [10]. Film thickness was not controllable at the moment of the production of guar-gum films but is known as being an important factor for the variation of mechanical characteristics. Therefore, thickness was considered as a factor (independent variable) even though thickness values were not circumscribed to a pre-defined range at the moment of the experimental design [11]. Concerning the optimization of alginate beads, association efficiency (%), particle size ( $\mu\text{m}$ ), corresponding polydispersity index and  $\zeta$ -potential (mV) were set as responses for the variation of alginate and Tween® 80 concentrations.

Authors attempted to gather the controlled release and mucoadhesion offered by alginate beads with the convenience and mucoadhesion of guar-gum oral films to obtain a buccal and oral delivery system that enhances buccal absorption and promotes an increased bioavailability of carried bioactive molecule, when compared with alginate beads or orodispersible films alone.

## **2. Materials and Methods**

### **2.1. Materials**

Caffeine anhydrous (food chemicals codex, 99% purity), alginic acid (sodium salt from brown algae, molecular weight ranging from 80,000 to 120,000, 61% of mannuronic

acid and 39% of guluronic acid, as stated by supplier in safety and documentation section), guar-gum and D-sorbitol (assay purity  $\geq 98\%$ ) were purchased from Sigma-Aldrich (Steinheim, Germany). Citric acid monohydrate, calcium carbonate, potassium phosphate monobasic anhydrous, sodium phosphate dibasic were purchased from Merck (Darmstadt, Germany). Sodium chloride was purchased from Panreac (Barcelona, Spain). Methanol (HPLC gradient grade) was purchased from Fisher (Loughborough, United Kingdom). Deionized water was used to prepare all oral films formulations and ultrapure water was used to prepare caffeine standard solutions and eluents used in chromatography procedures. TR146 cell line (passage 9) was purchased from Sigma-Aldrich (Steinheim, Germany). Transwell® flasks (12 well) and inserts (collagen-coated, 1.12 cm<sup>2</sup> of culture area, 0.4  $\mu$ m 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 sulfoxide (DMSO) 99.7% was purchased from Fisher Bioreagents™ (EUA). For TR146 cell wash, pH of PBS was adjusted to 6.8, using a solution of hydrochloric acid 0.1 M.

## 2.2. Experimental design

Experimental design was performed recurring to SAS JMP® 9 software. Response surface method for the optimization of film formulation was defined using the amounts of guar-gum (polymer), sorbitol (plasticizer and sweetener) and citric acid (saliva production inducer) as factors (independent variables). Erosion, water-uptake/time ratio, distance at burst and film burst strength were set as responses (dependent variables).

Alginate beads formulation was optimized by setting the relative amounts of sodium alginate and Tween® 80 as factors and association efficiency,  $\zeta$ -potential, mean size and polydispersity index were set as responses.

### 2.2.1. Production and characterization of oral films

Preparation of oral films was performed using solvent casting technique [11]. Briefly, guar gum, citric acid and sorbitol were dissolved into 100 mL of ultra-pure water. Thereafter, resulting solution was spread onto a glass cast heated to 50 °C for 1 h. Resulting film was then maintained at room temperature for 12 h. Finally, individual films

(2 cm x 3 cm) were cut from the glass cast for further testing. Oral films were collected directly from the glass cast and packaged into thermo-sealed polyethylene sheets.

#### 2.2.1.1. Texture analysis

Texture analysis was performed on a texturometer equipment (TA.XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK). Force calibration for a 5 kg load cell was performed using a 2 kg weight and height calibration was performed for the film support rig and corresponding probe. Film burst strength (g) and distance at burst (mm) were considered as measures of rigidity and elasticity, respectively.

#### 2.2.1.2. Thickness measurement

Thickness of the oral films was measured using a calibrated vernier gauge caliper micrometer. Thickness was measured in five points of each oral film and the average value was determined [12].

#### 2.2.1.3. Water-uptake, erosion and disintegration time

Water-uptake (WU) was determined by placing Guar-gum films in contact with 1 mL of artificial saliva. Weight changes were registered at 30, 60, 90, and 120 s and WU was calculated according to Eq. (1) [10]. Afterwards, hydrated samples were introduced in an oven at 60 °C for 24 h and weight variation of oral films was recorded in order to determine erosion. Erosion (%) represents the amount of film that was lost during the contact with artificial saliva and was calculated according to Eq. (2).

$$\text{Water uptake (\%)} = \frac{(W_t - W_1)}{W_t} \times 100 \quad (1)$$

$$\text{Erosion (\%)} = \frac{(W_1 - W_3)}{W_1} \times 100 \quad (2)$$

where, W<sub>1</sub> is initial weight of tested oral films, W<sub>t</sub> is the weight of the oral films after contact with artificial saliva at determined periods of time and W<sub>3</sub> is the weight of dry oral films, after erosion.

Since, according to United States Food and Drug Administration (FDA), oral films, as oral delivery systems, must disintegrate within 30 s in the oral cavity, the water-uptake/time ratio was determined as an indicator of disintegration speed [13]. Water-uptake/time ratio was calculated according to Eq. (3).

$$\text{Water - uptake/time} = \frac{\text{Water - uptake}}{t(\text{max water - uptake})} \quad (3)$$

where, water-uptake (%) is an indicator of water taken up by the oral film (Eq. 1) and  $t(\text{max water-uptake})$  is the time (s) at which water-uptake (%) value was maximum.

## 2.2.2. Production and characterization of alginate beads

### 2.2.2.1. Production of alginate beads

Alginate beads were prepared by emulsification/internal gelation [14]. Briefly, calcium carbonate and caffeine were dissolved into an alginate solution. In a separate beaker, Tween<sup>®</sup> 80 was dispersed into 10 mL of liquid paraffin. Both dispersions were stirred for 30 min and then the alginate solution was added drop wise to the paraffin dispersion and the resulting emulsion was kept stirring (600 rpm) for 30 min. Then, glacial acetic acid was added drop wise to the emulsion to liberate calcium ions for gelation. Resulting emulsion was kept stirring (900 rpm) for 1 h followed by centrifugation (6,000 rpm, 15 °C) and the pellet was recovered and washed with PBS. Washing procedure was performed three times for each formulation of beads. Regarding the association of alginate beads with guar-gum films, beads were added to the film formulation and homogenized before being poured on a glass cast. Following, resulting formulation was heated to 50 °C for 1 h and then rested at room temperature for 12 h, as described for the production of guar-gum films in section 2.2.1.

### 2.2.2.2. Characterization of alginate beads

Caffeine association efficiency (AE), mean size,  $\zeta$ -potential, scanning electron microscopy (SEM) and delivery profile were the parameters used to characterize alginate beads.

#### 2.2.2.2.1. Particle size and $\zeta$ -potential analysis determination

All alginate bead formulations were diluted (1:100) with ultrapure 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).



#### 2.2.2.2.2. Caffeine association efficiency (AE)

Caffeine association efficiency was determined by dosing (HPLC-UV) the free caffeine content of the supernatant of each bead formulation after being centrifuged (6,000 rpm, 30 min, 16 °C). Caffeine concentration in the supernatant was determined by HPLC-UV on a Waters Alliance® instrument (Milford, MA, USA). Water and methanol mixture (60:40) was used as mobile phase, column temperature was defined as 35 °C and isocratic flow was set to 1 mL/min [15]. Samples were run through a Kromasil® C18 column, 5 µm (particle size) × 4.6 mm (internal diameter) × 250 mm (length) (AkzoNobel, Bohus, Sweden). UV detector wavelength was set to 270 nm. The injection volume was set to 50 µl. Finally, caffeine association efficiency was calculated according to the following Eq. (4):

$$\frac{W_{tc} - W_{sc}}{W_{tc}} \times 100 \quad (4)$$

where,  $W_{tc}$  stands for total weight of caffeine used in the alginate bead formulations and  $W_{sc}$  stands for caffeine collected from the supernatant after centrifugation.

### 2.3. Surface tension analysis

A pendant drop tensiometer (PAT-1, SINTERFACE Technologies, Berlin, Germany) was used to measure dynamic surface tension of developed formulations at the air/water interface. A drop of the delivery systems dissolved in artificial saliva solution is formed at the tip of a capillary (constant volume: 12 µl), which is into a cuvette with water saturated atmosphere to avoid droplet evaporation, covered by a compartment, which is maintained at constant temperature ( $20 \pm 0.2$  °C) by circulating water from a thermostat. It was allowed to stand for 30 min to reach constant temperature and humidity in the compartment. Then, the silhouette of this drop is cast onto a CCD camera and digitized. The digital images of the drop are recorded over time and fit to the Young-Laplace equation to accurately ( $\pm 0.1$  mN/m) determine surface tension using drop profile analysis tensiometry. All experiments were performed at 20 °C and at least two measurements have been done for each system. The computer controlled dosing system

allows to control a constant volume of the drop during the measurement and also to induce area deformations [16]. All the glass materials were properly cleaned using a mixture of ammonium persulfate and sulphuric acid and further rinsed with ultrapure water to eliminate all the possible surface active contaminants that could interfere in the measurements.

#### **2.4. Molecular Interactions analysis**

ATR-FTIR analysis was performed for guar-gum films and alginate beads (placebo and with caffeine) to assess eventual chemical interactions with caffeine. Analysis was conducted in a FTIR spectrometer, model ABB MB3000 (ABB, Switzerland), equipped with a deuterated triglycine sulphate detector and using a MIRacle™ single reflection horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate. Obtained spectra were baseline corrected using a 3-4 point adjustment. Area of obtained spectra was normalized to a 0-1 range. Spectra treatment was performed using the OriginPro® (version 9.0) software. Spectra of caffeinated guar-gum films, alginate beads, association of guar-gum films with alginate beads (GfB) and physical mixture of GfB formulation were subtracted from spectra of placebo guar-gum films, alginate beads and GfB, respectively [17]. Resulting spectra were compared with the spectra of pure caffeine anhydrous powder.

#### **2.5. Morphological analysis**

Morphological analysis was performed on a JEOL-5600 Lv Scanning Electron Microscope (Tokyo, Japan) equipped with SPRITE HR Four Axis Stagecontroller (Deben Research). Samples were placed on metallic stubs with carbon tape and coated with gold/palladium using a Sputter Coater (Polaron, Bad Schwalbach, Germany). Images were obtained for guar-gum films, alginate beads and GfB. using a spot size of 18-20 and a potential of 15-22 kV. All analyses were performed at room temperature (20 °C).

#### **2.6. Caffeine release profile**

*In vitro* release assays were performed in order to assess and compare release profiles of guar-gum films, alginate beads and GfB. Briefly, *in vitro* dialysis delivery assay was performed according to Wang, Liu, Sun, Wang, Wang and Zhu [18]. Briefly, the formulations (alginate beads, guar-gum films and GfB) and a caffeine control solution (2 mg/mL) were introduced into a 500 Da dialysis membrane. Dialysis membrane with a

pore size of 500 Da was chosen to mimic the pore size of buccal mucosa [3]. Immediately, the dialysis membrane was filled with the formulations and 35 mL of artificial saliva (pH= 6.8), clumped and dipped into 50 mL of phosphate buffered saline - PBS - solution (release media) pre-heated to 37 °C. The system was kept on continuous shaking (100 rpm). Aliquots of 5 mL were withdrawn from release media at 15, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660 and 720 min. Withdrawn volume was immediately replaced with 5 mL of PBS and pre-heated to 37 °C to preserve sink conditions.

## **2.7. Human buccal epithelium cell line**

Cell viability after contact with produced formulations and transepithelial permeability assay were performed using TR146 human buccal epithelium cell line. 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 [19]. 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 [20, 21].

TR146 cell line was purchased from Sigma-Aldrich (USA) and passage 9 was 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<sup>2</sup> T-flasks (T-75) and incubated in a 5% CO<sub>2</sub>/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).

### **2.7.1. 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 at density of 1 x 10<sup>4</sup> 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, to assess the cell viability after being in contact with developed formulations. Cell-

viability studies were performed after 24 h of culture, with previous supervision by light 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 drug delivery systems [22]. If TR146 cells were viable, succinic dehydrogenase was able to transform the tetrazolium salt into insoluble, purple-coloured, crystals of formazan [23]. 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 drug delivery formulations, 100  $\mu$ L 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 at 37 °C and in a 5% CO<sub>2</sub> atmosphere. After incubation time has passed, reagent was carefully removed, allowing the insoluble formazan crystals to remain in the bottom of the wells. 100  $\mu$ L 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=5 different, independent wells for the same experiment) was calculated according to Eq. (5):

$$\text{Cell viability (\%)} = \frac{\text{Experimental value} - \text{negative control}}{\text{Positive control} - \text{negative control}} \times 100 \quad (5)$$

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 [24].

### 2.7.2. Drug transepithelial diffusion study

Permeability assay also assessed in Corning® Transwell® inserts, using 12-well plates. TR146 buccal cells were seeded onto the inserts to mimic stratified epithelium of human buccal mucosa, as reported previously [20, 25]. 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 drug delivery formulations were introduced afterwards. Guar-gum films, alginate beads, GfB and free caffeine were tested for permeability (n= 5). Samples of 600  $\mu$ L were withdrawn from receptor part at 0, 15, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660 and 720 min. Withdrawn volume was immediately replaced with fresh PBS, pre-heated to 37  $^{\circ}$ C, to maintain sink conditions. The amount of permeated caffeine was quantified by HPLC-UV, as described in section 2.5.

The amount of caffeine that permeated Transwell<sup>®</sup> 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. (6) [26].

Papp ( $\text{cm}\cdot\text{s}^{-1}$ ) was calculated for free caffeine, guar-gum films, alginate beads and GfB, by normalizing the flux (J) over the concentration of caffeine in the donor compartment ( $C_0$ ) according to Eq. (7).

$$J = \frac{dQ}{A \times dt} \quad (6)$$

$$P_{app} = \frac{J}{C_0} \quad (7)$$

where,  $dQ/dt$  stands for the amount of permeated caffeine over time, A for the tissue surface area and  $C_0$  for the initial concentration of caffeine. All tested formulations presented the same initial concentration of caffeine (2 mg/mL) at the beginning of the drug trans-epithelial study.

Caffeine remaining entrapped by the TR146 cell monolayer was obtained by disruption of the monolayer with 1 mL of DMSO. Resulting solution was centrifuged (14,000 rpm, 15 min, 4  $^{\circ}$ C) and caffeine concentration in the supernatant was determined by HPLC-UV.

At the end of the drug trans-epithelial diffusion study, MTT mitochondrial viability assay was performed in order to verify the viability of TR146 cells [27]. Inserts incubated with culture medium only, during the caffeine trans-epithelial study (n= 5) were considered as positive control (i.e. total cell viability). Inserts incubated with Triton-X lysis solution (n= 3) were considered as negative control (i.e. reference for total cell death). MTT assay was performed as described in section 2.13.1.

## 2.8. 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 both alginate beads and guar-gum films. Mean values for each batch were compared with the values predicted in the model.

## 3. Results and discussion

An experimental design was performed to optimize two formulations as delivery systems that can be used alone or combined for an enhanced buccal delivery of bioactive molecules. A physico-chemical characterization (analysis of molecular interactions and morphological analysis), a release assay, TR146 cell viability test and permeability assay were performed for developed formulations.

### 3.1. Optimization of the formulation of guar-gum oral films and validation of results

Factorial design (response surface method) performed for optimization of the guar-gum films is briefly described in **Table 1**.

Prediction profiler (Figure S1, supplementary materials) obtained from the experimental design allowed elaborating an optimized guar-gum films formulation with desired values of erosion (%), distance at burst (mm), film burst (g) and water-uptake/time (% w/w.s<sup>-1</sup>) ratio.

Since very wide standard-deviation values were obtained for responses (dependent variables), three batches of the guar-gum films formula predicted by the statistical model as being optimal were prepared and erosion (%), distance at burst (mm), film burst strength (g) and water-uptake/time (mg/s) ratio were determined ( $n = 6$ ) and compared with theoretically predicted values. Oral films were prepared according to the prediction profiler, by dissolving 2.7 g of sorbitol, 2.0 g of guar gum and 0.380 g of citric acid in 100 mL of ultrapure water. Only oral films with 0.06 mm were selected for validation

purposes. Predicted and experimental values for the optimized formulation of guar-gum films are outlined in **Table 2**.

No statistical differences ( $p > 0.05$ ) were observed between mean predicted values and mean experimental values (Figure S2, supplementary materials).

Many other polymers have been previously reported as having film-forming properties at similar concentrations as observed for guar-gum films [11]. Indeed, chitosan and sodium carboxymethylcellulose oral films were previously optimised by our group but disintegration times were much higher and drug release was very distinct from developed guar-gum films [28]. Moreover, chitosan oral films are reported as being astringent and, therefore, potentially unpleasant, compromising patient/consumer compliance. On the other hand, guar-gum is commonly used in food products as thickener in a wide array of products and consumers already accept the flavour and used to find guar-gum referred on packaging labels [29].

Also, in comparison to mucoadhesive oral films recently developed, guar-gum films presented superior mechanical characteristics either regarding film burst strength (average of 1754.96 g for guar-gum films against 546.57 g for sodium carboxymethylcellulose films) or distance at burst (average of 5.77 mm for guar-gum films against 0.74 mm for sodium carboxymethylcellulose films) [30]. Distance at burst was similar to vaginal films (blend of 72% of hydroxypropylmethylcellulose, 18% polyvinyl alcohol and 10% glycerine) containing nanoparticles developed by our group (average of 5.77 mm for guar-gum films against 5.34 mm for vaginal films) indicating good elasticity characteristics [31].

Indeed, mechanical characteristics are very important either to assure stability during transport, accurate dosage of carried bioactive molecules or to avoid handling and administration issues [32].

Unfortunately, there is still a great lack of information regarding standardized technological characteristics (e.g. mechanical characterization, dissolution and disintegration assays, shelf-life assessment and appropriate storing and packaging conditions) making the optimization of oral films formulations a task requiring further studies [11, 33-35].

### **3.2. Optimization of the formulation of alginate beads and validation of results**

Alginate beads formulation was optimized with the purpose of obtaining high stability and enhancement of residence time of caffeine in contact with buccal mucosa.

The summary of the factorial design (response surface method) performed for the optimization of the formulation of alginate beads is outlined in **Table 3**. Relative amounts of sodium alginate (particle-forming polymer) and Tween® 80 (polysorbate, surfactant) were chosen as factors for the optimization of alginate beads. Association efficiency, mean particle size, polydispersity index and  $\zeta$ -potential were selected as responses, being considered of main importance for the characterization of beads. Caffeine anhydrous was the model drug carried by alginate beads, attending to achieve sustained release.

Prediction profiler (**Figure S3, supplementary materials**) allowed elaborating an optimized alginate beads formulation to achieve desired values of association efficiency (AE, %), mean particle size, polydispersity index (PdI) and  $\zeta$ -potential (mV).

As observed for guar-gum films, since very wide standard deviation values were obtained for responses (dependent variables), three batches of alginate beads were prepared and mean values of AE (%), size ( $\mu\text{m}$ ), PdI and  $\zeta$ -potential (mV) were determined and compared with the average values predicted by the statistical model. Alginate beads were prepared using 3.0% (% w/v) of alginate and 2.4% (% w/v) of Tween® 80. Predicted and experimental values for the optimized formulation of alginate beads films are outlined in **Table 4**.

No statistical differences ( $p > 0.05$ ) were observed between mean predicted values and mean experimental values, except for  $\zeta$ -potential. Indeed, experimental values for  $\zeta$ -potential were significantly inferior, i.e. more negative, than values predicted in the statistical model.

A graphical analysis of one sample T-tests performed for validation of the prediction profiler is provided in **Figure S4, supplementary materials**.

There is still a gap in the literature concerning microparticles as buccal delivery systems, albeit it has been reported by some researchers [36]. Nevertheless, most researchers are using nanoparticles aiming to increase apparent permeability of carried drugs and not to achieve a slower release of carried molecules. For instance, it has been reported that poly(lactide-co-glycolide) (PLGA) nanoparticles carrying acyclovir were incorporated into mucoadhesive buccal films and succeeded on increasing *ex vivo*



permeability of the drug across rabbit buccal mucosa [37]. Nevertheless, most works concerning the development of microparticles for buccal delivery are aiming local administration and not permeation across buccal mucosa to reach systemic circulation [38, 39]. Alginate was previously used to produce mucoadhesive microparticles by ionic gelation aiming to an increased residence time of drugs in the buccal mucosa. Effectively, alginate microparticles guaranteed an increased residence time of flurbiprofen and delmopinol when compared to reference solutions, decreasing the administration frequency [40].

### 3.3. Dynamic surface tension analysis

Analysis of water/air interfacial tension is outlined in **Figure 1**. The lower water/air surface tension was associated with higher possibility of the formulation to adhere to other solutions or surfaces (e.g. digestive juices or enteric tissues) [41]. Indeed, surface tension is a way of measuring the work of adhesion. The smaller the work of internal adhesion within the drop, the greater the possibility of interaction with external environment.

The interfacial behaviour of developed formulations along with individualized components of the beads (i.e. Sodium alginate and Tween 80) reflected significant differences that can be translated into distinct mucoadhesion profiles. Indeed, it was observed that caffeine did not present interfacial activity.

Artificial saliva solution presented a small variation of superficial tension along time as well, even though slightly more pronounced when compared to caffeine.

On the other hand, sodium alginate presented tensioactive activity with a variation of 24 mN/m during the 7,000 seg of the assay, indicating a potentially favourable interaction with gastrointestinal mucosa. Tween 80 (polysorbate) induced the most extensive reduction of surface tension with time [42]. Effectively, Tween 80 is well-known to present very expressive tensioactive activity and a high drop stability was expected.

GfB and caffeine-loaded alginate beads presented the higher stabilization (i.e. the lower the surface tension, the higher the possibility of interacting with external media as the buccal mucosa or absorptive epithelia along the gastrointestinal tract) capacity of all formulations, indicating that the association of caffeine with alginate beads is expected to

offer a greater interaction with gastrointestinal tract mucosa when compared with caffeine or excipients alone.

Moreover, the incorporation of caffeine-loaded alginate beads within the guar-gum film matrix led to the greatest decrease in the surface tension of the drop (tween 80 is not being considered due to intrinsic tensioactive activity) indicating that the guar-gum film-alginate beads synergism may potentially offer a higher mucoadhesion along gastrointestinal tract when compared to the remaining formulations [41].

### 3.4. Molecular interaction analysis

Attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) analysis was performed to perceive the onset of new bonds between caffeine and developed formulations during production steps.

Resulting infrared spectra achieved from the subtraction of alginate beads, guar-gum films, GfB and for the physical mixture of all excipients of GfB and caffeine from corresponding placebo formulations is outlined in **Figure 2**. Spectra of caffeine anhydrous powder was used for comparison with obtained subtracted spectra.

Typical caffeine bands that appear at  $1700\text{-}1600\text{ cm}^{-1}$  are representative of the C=O stretch of amide I [43]. C-N stretch coupled with N-H bending of the amide II present in the caffeine molecule are represented by the bands at  $1600\text{-}1500\text{ cm}^{-1}$ . The bands showed in the range of  $1666\text{-}1550\text{ cm}^{-1}$  are addressed to the stretches of C=C, C=O and C=N bonds of caffeine molecules [44, 45]. Subtracted spectra for alginate beads, guar-gum films, GfB and physical mixture of all excipients of GfB represented in **Figure 2**, showed the same characteristic infrared bands as caffeine anhydrous infrared spectra. Thus, there is no indication of the formation of new chemical bonds between caffeine and the excipients of alginate beads or guar-gum oral films during formulations processes. Also, there are no significant differences between caffeine spectra and the subtracted spectra for the physical mixture, revealing that the excipients do not induce alterations in the chemical structure of caffeine. Spectral analysis indicates that the formation of new chemical bonds that may hinder caffeine release from developed delivery systems is not observed. Instead, caffeine release from guar-gum films, alginate beads or GfB may be affected mainly by physical shielding and/or electrostatic bonds [28].

### 3.5. Morphology analysis

Scanning electron microscopy (SEM) was used to perform a visual recognition of the matrix structure of oral films and alginate beads, in order to obtain further information regarding caffeine delivery profile.

Morphology analysis of alginate beads, guar-gum films and alginate beads dispersed on oral films was performed by SEM (Figure S5, **Supplementary materials**).

Alginate beads were added to guar-gum films formulation before solvent casting for the blend to be as homogeneous as possible. The fact that alginate beads are hydrophilic could represent a stability problem since it would be very likely that alginate would be dissolved in the guar-gum films matrix during solvent casting procedure. However, it is noticeable that alginate beads are homogeneously spread on the film matrix, indicating good stability of alginate beads. Also, bead size and morphology observed in the microphotographs are coherent with the size predicted by the statistical model and corroborated by DLS analysis that indicated that mean particle size is around 3.58  $\mu\text{m}$ . Both size and shape of alginate beads were homogeneous. Also, some particle agglomeration seems to occur. Nevertheless, homogeneous dispersion of alginate beads into/onto the guar-gum films matrix indicates that caffeine dosage in each film is very similar and, thus, caffeine release profile is likely to be similar when testing different batches. Therefore, the chances of a successful future scale-up are promising.

### **3.6. *In vitro* caffeine release assay**

The release assay using dialysis membranes was performed to characterize the delivery profile of caffeine from alginate beads, guar-gum films and the combination of both delivery systems (GfB) aiming to test a conceptually new oral delivery system. Pore size of dialysis membrane (500 Da) was chosen according to the intercellular space between epithelial cells of buccal mucosa [3, 46].

For the assessment of release profile of caffeine from developed delivery systems, guar-gum films and alginate beads alone and in combination were tested. **Figure 3** represents delivery profile from guar-gum films, alginate beads and GfB compared with a control solution of free caffeine (2 mg/mL).

The higher flux of caffeine from control solution crossed the dialysis membrane occurred in the first 15 min, indicating that caffeine flux easily occurred from the inside of the dialysis membrane to the outside, following Fick's first law. Caffeine is a small ( $194.194 \text{ g}\cdot\text{mol}^{-1}$ ), highly hydrophilic ( $2.16\text{E}04 \text{ mg/L}$ ) molecule and a fast efflux from the

dialysis membrane was predictable [47, 48]. Release profile of caffeine content in control solution was almost linear in the first 60 min, approaching a zero-order release kinetics and reaching a plateau from 60 min till the end of the release study. Indeed, caffeine is expected to permeate buccal mucosa according to the simplified version of Fick's first law (Eq. (5)) by paracellular path [49, 50].

$$J = P(C_{donor} - C_{receiver}) \quad (5)$$

where,  $P$  is the permeability coefficient,  $C_{donor}$  and  $C_{receiver}$  are the concentrations of caffeine inside the dialysis membrane and on the receiver compartment, respectively, and  $J$  is the flux from the dialysis membrane (donor compartment) to the receiver compartment [50]. Caffeine release from guar-gum films was significantly slower than the control caffeine solution but fast disintegration of the film led, yet, to a significantly fast caffeine release to the outside of the dialysis membrane. Effectively, the same noticeable burst release of bioactive molecules loaded in a thin film was observed in other studies, indicating a clear trend regarding delivery of bioactive molecules from thin films [51, 52]. The differences observed between guar-gum films release profile and control may be due to physical and/or electrostatic hindrance of caffeine release from guar-gum films matrix. Caffeine release from alginate beads across dialysis membrane was significantly slower when compared with guar-gum films or control. The fact that caffeine molecules are associated (either inside or at the surface) to the alginate beads may delay the release of caffeine, therefore preventing a sooner passage across dialysis membrane pores. Indeed, alginate beads, may have to disintegrate for the entrapped caffeine to be free and able to cross the membrane. The combination of guar-gum films and alginate beads to form one delivery system offers the highest impedance of caffeine release over time. Indeed, the conjugation of guar-gum film and alginate matrices implies that caffeine must be released from a double barrier before contacting with the absorptive epithelium. Both film and bead matrices must disintegrate and dissolve to allow the complete release of caffeine. Nevertheless, caffeine release in the first 60 min was very similar for GfB and alginate beads. The initial burst release may be due to some premature release of caffeine from alginate beads shortly after the production of the formulations, as verified in other studies [31, 51].

### 3.7. Cell viability studies

TR146 cells were used to evaluate potential toxicity caused by different concentrations of caffeine-loaded alginate beads, guar-gum films and GfB. Placebo formulations with the same mass as caffeine-loaded delivery systems were used as controls. Cell viability was assessed by MTT reduction assay.

Results of cell viability *in vitro* assay for placebo and caffeine-loaded alginate beads, guar-gum films and alginate beads incorporated into guar-gum films are shown in **Figure 4**.

None of the tested concentrations of caffeine alone or incorporated into alginate beads, guar-gum films or GfB did significantly compromise cellular viability of TR146 cells, after 12 h of exposure. Total TR146 cell viability indicates that, when administered *per os* there was no evidence that suggests that some of the drug delivery systems (either alone or combined) are hazardous for the buccal epithelium.

### **3.8. Permeability assay on TR146 monolayers**

TR146 human buccal carcinoma cells were used to determine buccal permeability of caffeine from developed formulations.

The permeation profiles through TR146 cells grown on Transwell® inserts were distinct according to the formulation (**Figure 5**).

Values of the apparent permeability ( $\text{cm}\cdot\text{s}^{-1}$ ) for all the formulations are outlined in **Table S1, Supplementary contents**.

Results obtained for caffeine control solution (2 mg/mL) are in accordance with previously reported caffeine buccal apparent permeability [53, 54]. Also, caffeine control solution presented a permeability profile that strongly correlates with diffusion from dialysis membrane (**Figure 2**). Indeed, caffeine permeation also occurred according to Fick's first law following a zero-order kinetics. Caffeine released from guar-gum films permeated TR146 cell layer faster than the control caffeine solution for 100 min. After being inserted into the Transwell® inserts, guar-gum films began disintegrating. Then, fragments resulting from disintegration of guar-gum films deposited and adhered to the apical layer of TR146 cells. Immediate adhesion of guar-gum films fragments offered a higher contact surface area with the cell layer and thus, more effective caffeine permeation. Effectively, mucoadhesive delivery systems were reported, by peer researchers, to increase permeation of carried molecules across TR146 cell layer [55].

Both alginate beads and GfB offered slower caffeine permeation when compared with guar-gum films or caffeine control solution. Lower permeation of caffeine across TR146 cell layer may be at least partially due to the slower caffeine release, as observed in the caffeine release assay (**Figure 2**). In fact, as previously observed for the release assay using dialysis membranes, GfB promoted a slower release of caffeine when compared with control, alginate beads or guar-gum films alone. The cross-analysis of dialysis release assay and *in vitro* permeability assay on TR146 buccal mucosa cells indicated a strong correlation between caffeine release profile and permeability effectiveness. The fact that, when caffeine-loaded alginate beads is combined with guar-gum films, caffeine is doubly hindered from being released since both alginate and guar-gum matrices must be disintegrated and dissolved before caffeine contacts with buccal cells. Moreover, since alginate beads present high mean particle size, permeation of the whole beads is not likely to happen unless the integrity of buccal epithelium is somehow compromised. Also, since transepithelial permeation study was performed using a static model, swallowing, salivation, masticatory movements or even phonation were not considered. Indeed, it is predictable that a caffeine solution is almost immediately swallowed after being introduced in the oral cavity in *in vivo* conditions.

Moreover, after cellular disruption of the TR146 monolayer, it was possible to observe (**Figure 6**) that most caffeine was present either within or retained by the cell layer. The fact that guar-gum films, alginate beads and GfB were capable to maintain caffeine in intimate contact with the monolayer may enhance permeability of caffeine over time, especially in *in vivo* conditions.

#### 4. Conclusions

Two new oral/buccal delivery formulations, consisting on a guar gum based film and caffeine-loaded alginate beads, were optimized by experimental design. Robustness of developed predictive profilers was successfully validated, except for  $\zeta$ -potential of alginate beads (experimental values for  $\zeta$ -potential of alginate beads were more negative than predicted values). Nevertheless, more extreme  $\zeta$ -potential values are beneficial to the stability of alginate beads and less prone to induce toxicity due to disruption of cellular membranes. Thus, attempting to achieve a conceptually new delivery system aiming buccal and oral delivery of bioactive compounds, alginate beads were dispersed in the matrix of guar-gum films. Surface tension analysis allowed to conclude that the

synergism between alginate beads and guar-gum films offered a higher water-air stability when compared with isolated formulations. Therefore, it is predicted that GfB present good capacity to intimately interact with buccal mucosa. ATR-FTIR analysis did not indicate the occurrence of new chemical bonds between caffeine and guar-gum films or alginate beads. Indeed, subtracted spectra (placebo formulations subtracted from caffeinated formulations) for guar-gum films, alginate beads and GfB present the same characteristic bands as caffeine anhydrous, demonstrating that chemical structure of caffeine was not altered during or after inclusion into guar-gum films, alginate beads or in the combination, GfB. Morphological characterization by SEM demonstrated a homogeneous dispersion of alginate beads on the guar-gum films matrix, indicating that caffeine content is very likely to be homogeneous in each film unit, a good indicator if scale-up production is intended. Alginate beads, guar-gum films and GfB did not compromise cell viability when tested on TR146 cells, by MTT assay. *In vitro* release profile of caffeine (dialysis membrane) and drug trans-epithelial assay (TR146 buccal cells grown on Transwells®) allowed concluding that the combination of guar-gum films with alginate beads represent a suitable delivery system when a slower release of carried bioactive molecule into oral cavity is intended, when compared with guar-gum films and alginate beads alone and with a control (caffeine solution). Also, the highest concentration of caffeine was found absorbed/adsorbed to TR146 cell layer when compared with alginate beads, guar-gum films or caffeine alone. Results indicate that there is a synergistic effect between alginate beads and guar-gum films that promotes an intimate contact with buccal epithelia, leading to a more extensive absorption over time, corroborating the hypothesis stated by the authors.

There are not yet similar delivery systems combining beads and films for oral and buccal delivery of bioactive molecules reported in the literature even though nanoparticles were already incorporated into films for delivery of anti-HIV microbicides drugs in previous works performed by our group [31, 51]. GfB may represent an innovative approach on the buccal delivery of hydrophilic bioactive molecules such as caffeine to assure controlled effects, being also especially suitable for paediatric or psychiatric patients that may be uncooperative to therapy.

### **Acknowledgments**

This article is a result of the project NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the

PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Pedro M. Castro would like to thank Comissão de Coordenação e Desenvolvimento Regional do Norte (CCDR-N), Portugal, for his PhD grant (NORTE-08-5369-FSE-000007).

Flávia Sousa would like to thank Fundação para a Ciência e a Tecnologia (FCT), Portugal for her PhD grant (Grant SFRH/BD/112201/2015).

The authors acknowledge the support granted by national funds from FCT through project PTDC/BBB-NAN/3249/2014.

This work was also financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274)



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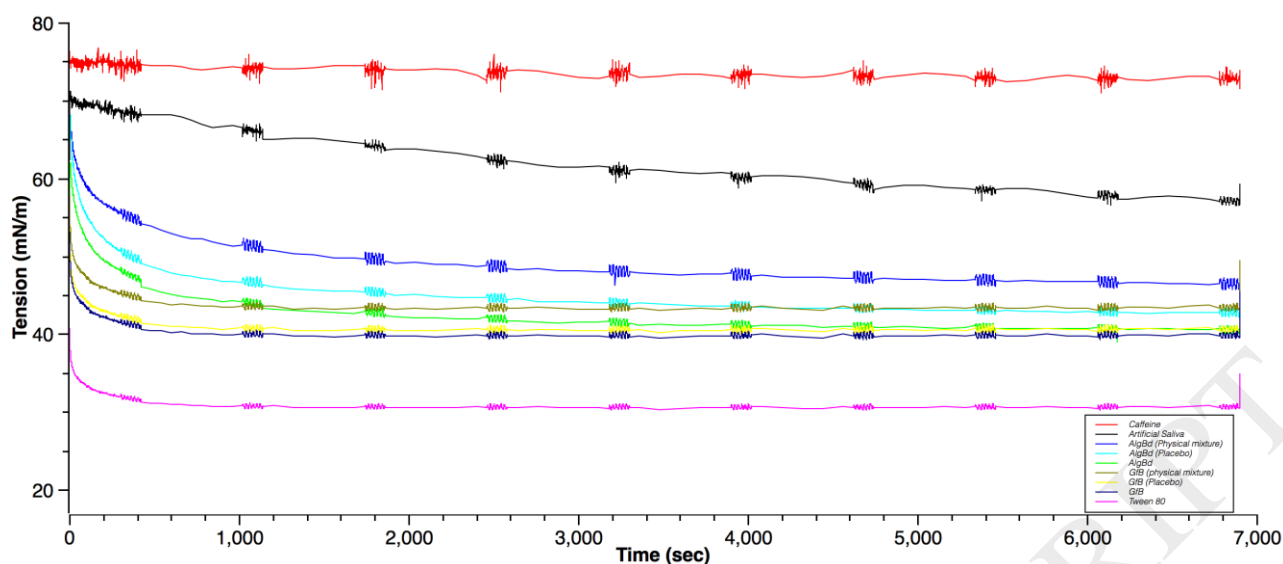
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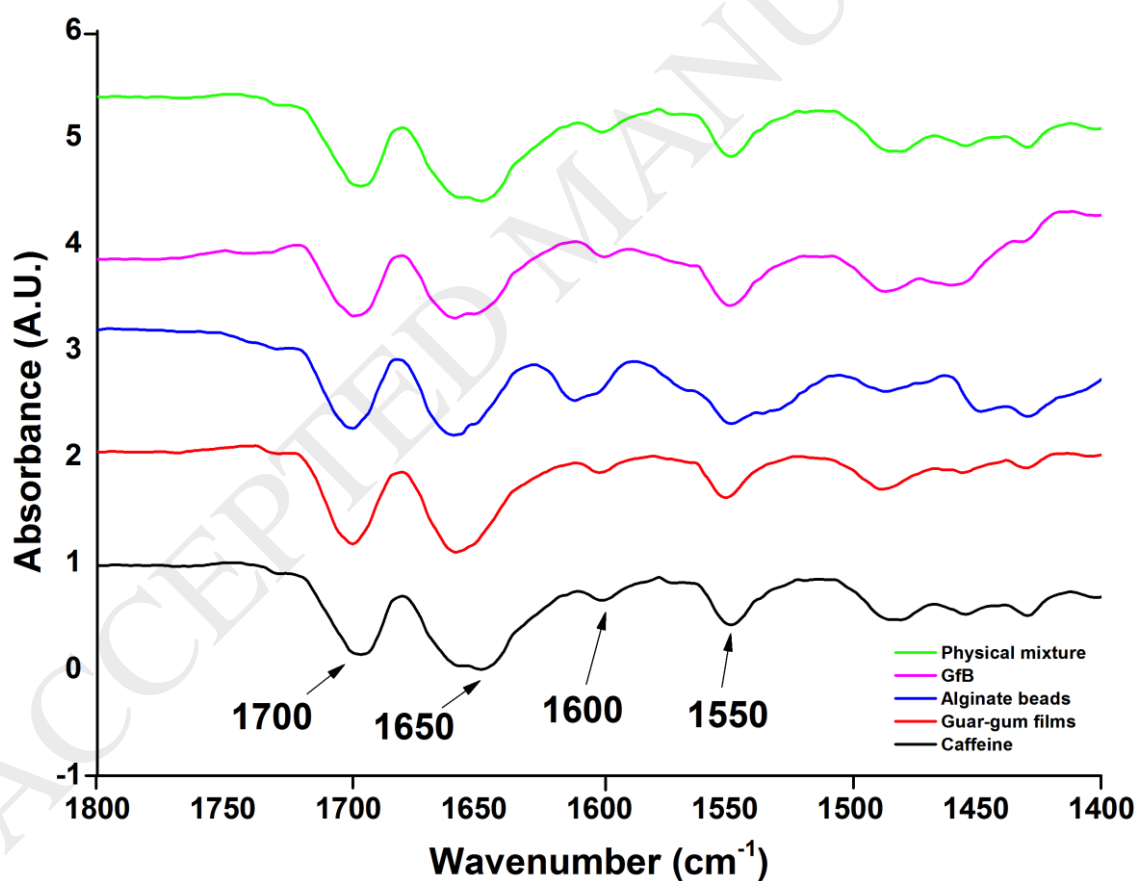
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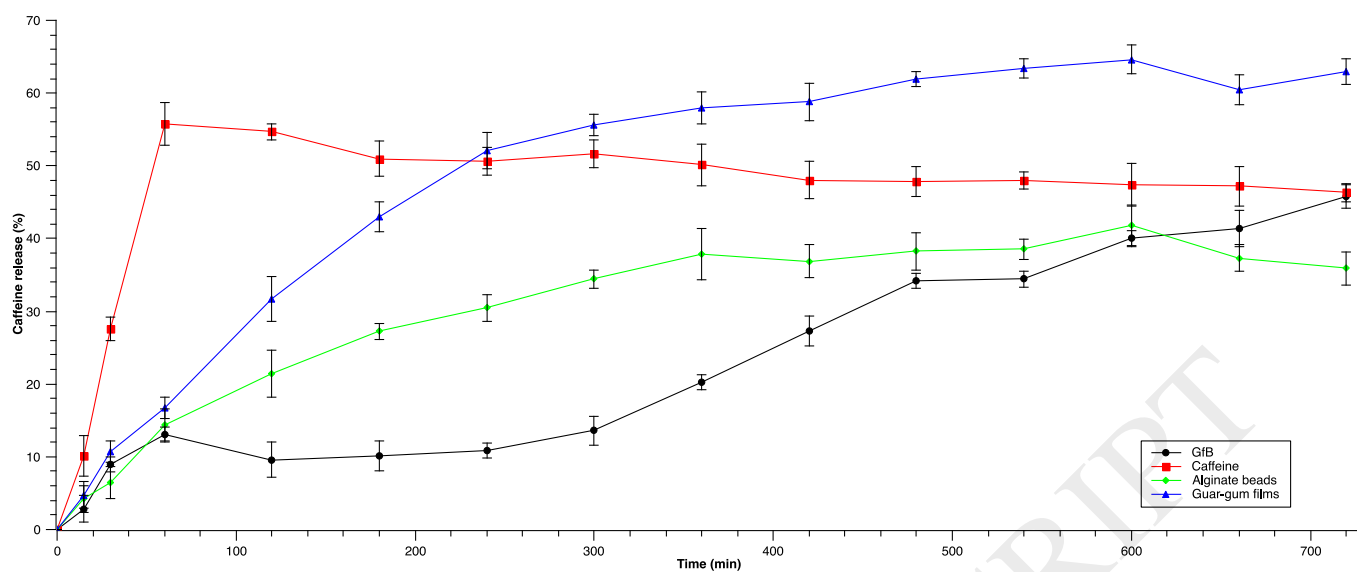
ACCEPTED MANUSCRIPT



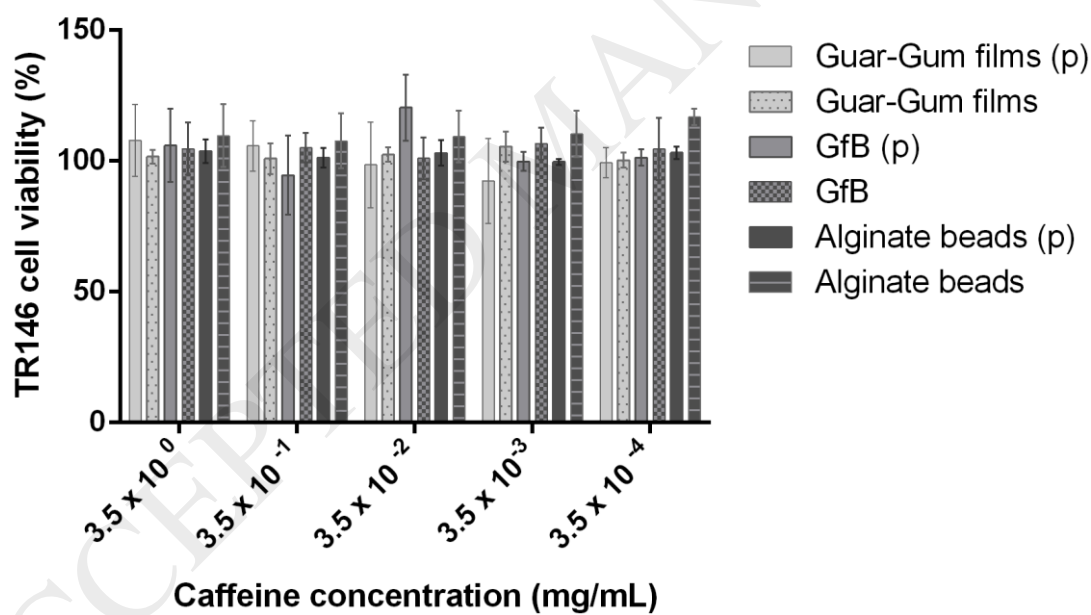
**Figure 1:** Time evolution of water/air superficial tension for the alginate beads containing caffeine along with respective , physical mixture and isolated formulation components.



**Figure 2:** Subtracted FTIR spectra corresponding to the caffeine present on alginate beads, GfB, guar-gum films caffeine anhydrous powder and the physical mixture of all excipients of GfB.



**Figure 3:** Caffeine cumulative release (mean  $\pm$  standard deviation, n=5) across a 500 Da dialysis membrane.



**Figure 4:** Cytotoxicity assessment of different concentrations of caffeinated (alginate beads, guar-gum films and GfB), placebo (alginate beads(p), guar-gum films(p) and GfB(p)) formulations.

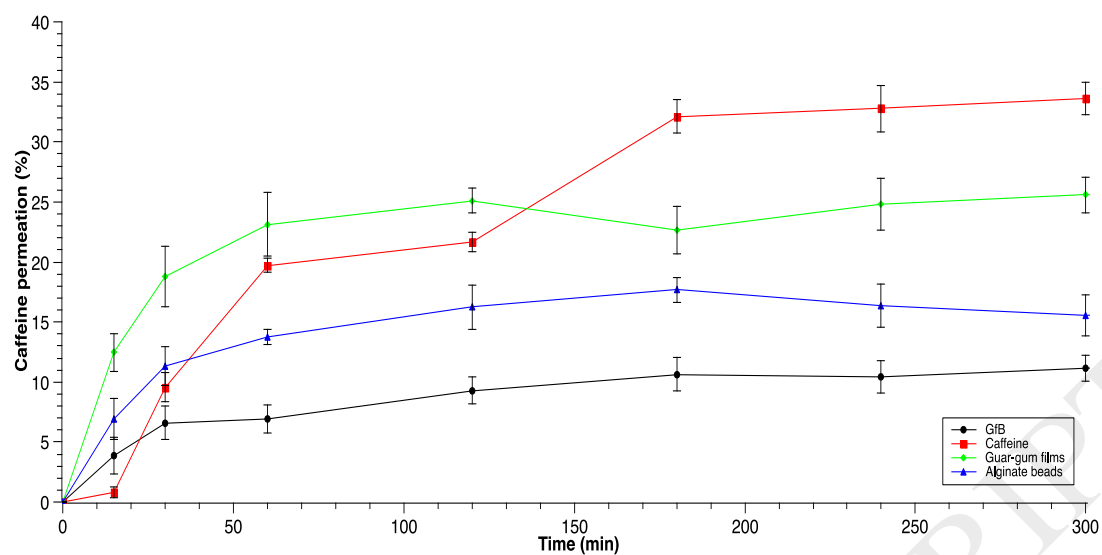


Figure 5: Cumulative permeability of caffeine (mean  $\pm$  standard deviation, n=3) across TR146 cells seeded in Transwell®.

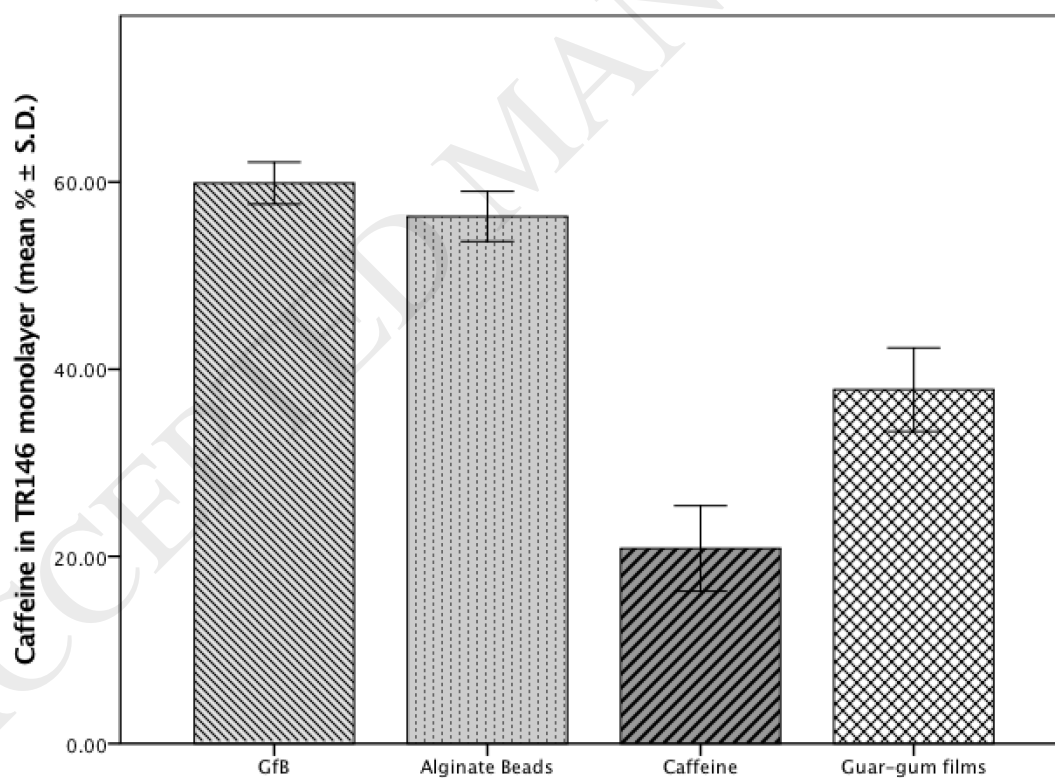


Figure 6: Caffeine (mean  $\pm$  S.D.) collected from TR146 cell monolayer, by delivery system

**Table 1:** Factorial design parameters established for the optimization of the formulation of guar gum oral films

<b>Factorial design parameters (Guar-gum films)</b>				
<b>Level (code unit)</b>	<b>Guar gum concentration (% w/v)</b>	<b>Sorbitol concentration (% w/v)</b>	<b>Citric acid concentration (% w/v)</b>	<b>Caffeine concentration (mg/mL)</b>
-1	2.5	3.75	0.75	16.7
0	3.75	5.69	1.0	16.7
1	5.0	7.50	1.25	16.7

**Table 2:** Predicted and experimental values obtained for the optimized formulation of guar-gum film

	<b>Predicted value (mean <math>\pm</math> SD)</b>	<b>Experimental value (mean <math>\pm</math> SD; n=6)</b>
Erosion (%)	39.33 $\pm$ 24.61	41.78 $\pm$ 3.97
WU/time(max WU) (mg/s)	4.46 $\pm$ 1.48	4.28 $\pm$ 0.33
Distance at burst (mm)	5.77 $\pm$ 3.07	5.39 $\pm$ 0.65
Film burst strength (g)	1754.96 $\pm$ 2567.36	1595.42 $\pm$ 300.13

**Table 3:** Factorial design parameters established for the optimization of the formulation of alginate beads

<b>Factorial design parameters (Alginate beads)</b>			
<b>Level (code unit)</b>	<b>Alginate concentration (% w/v)</b>	<b>Tween 80 concentration (% w/v)</b>	<b>Caffeine concentration (mg/mL)</b>
-1	2	2	16.7
0	3	3	16.7
1	4	4	16.7



**Table 4:** Predicted and experimental values obtained for the optimized formulation of alginate beads.

	Predicted value (mean $\pm$ SD)	Experimental value (mean $\pm$ SD; n=6)
Association efficiency (%)	80.25 $\pm$ 4.35	81.57 $\pm$ 6.59
Particle size ( $\mu\text{m}$ )	3.57 $\pm$ 2.25	3.37 $\pm$ 0.64
Polydispersity index	0.300 $\pm$ 0.192	0.348 $\pm$ 0.060
$\zeta$ -potential (mV)	-19.93 $\pm$ 7.18	-25.27 $\pm$ 1.50