

# Alginate-based Films for Investigative Delivery of Flavonoids

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November 2019

## Abstract

The aim of this work was to develop alginate-based films for the controlled release of flavonoids, mimicking pharmacokinetic properties of these compounds in humans, for potential applications in (1) investigative delivery and in (2) therapeutics, especially for improving anticancer efficacy in skin.

The proposed system consists of dry alginate films, where flavonoids are encapsulated prior to film application. Several studies were made to develop and characterize this system, from production of different films to investigation of epicatechin release in varied conditions.

The pharmacokinetics of EGCG in humans was characterized with compartmental models, exhibiting an absorption rate of  $1.10 \pm 0.70 \text{ h}^{-1}$  and an elimination rate of  $0.28 \pm 0.12 \text{ h}^{-1}$ . Films were prepared by solvent evaporation, where additives such as glycerol and chitosan increased their flexibility and hydrophobicity, respectively. Copper ions interact with EGCG, but not calcium, barium or zinc that were used to gel alginate for flavonoid encapsulation. Epicatechin could be encapsulated in alginate films, but not EGCG, and glycerol-containing films may increase the encapsulation efficiency. The release of epicatechin from alginate films depends on the crosslinking ion and film thickness, with diffusion being the predominant release mechanism as suggested by Weibull equation modelling. Release studies on Franz cells showed kinetics of release of epicatechin by alginate films similar to the absorption of EGCG in humans, suggesting a potential utility for investigative delivery studies of flavonoids. Calcium-alginate films were able to deliver epicatechin to skin *ex vivo*, achieving therapeutically relevant concentrations of antioxidant functional epicatechin.

**Keywords:** Alginate, Flavonoids, Controlled release, Investigative delivery, Skin

## 1. Introduction

Polyphenols in general are recognized antioxidant and anti-inflammatory agents, showing in varying degree anticancer, neuroprotective and other health promoting effects [1; 2; 3]. There is a great diversity of polyphenols, from phenolic acids such as gallic acid, to curcuminoids and stilbenes (resveratrol). Flavonoids are a major group of polyphenols, including leading compounds such as quercetin and the tea catechins (epi)catechin and epigallocatechin-3-gallate (EGCG). A recent cohort study has shown that daily intake of flavonoids is inversely associated with all-cause mortality, cardiovascular-related mortality and, in particular, cancer-related mortality, reaching a plateau at a daily intake of 500 mg [4]. Furthermore, human trials involving premalignant injury or chemoprevention show that favorable results are achieved with daily oral intake of at least 500 mg of EGCG or green tea extracts [5; 6]. In dermal applications, *in vitro* and animal studies, namely for cytoprotection of fibroblasts against UVA damage and for anti-proliferation of melanoma, show that low concentrations of catechins ( $<50 \mu\text{M}$ ) are effective [7; 8].

Alginate is a polyanionic polymer obtained from algae that can be cross-linked by divalent ions forming hydrogels [9]. Alginate biomaterials are well accepted due to its good biocompatibility in most applications, relative low cost and the amenable gelation of the polysaccharide with divalent cations [10]. Therapeutically, alginate is mostly used in wound healing [11], due its versatility to be formulated in various soft and non-brittle forms, namely in foams by freeze-drying or films by the casting/solvent evaporation method and the flexibility of alginate films can be improved by the addition of glycerol or other plasticizers [12].

Various types of delivery system formulations aimed at improving polyphenol pharmacokinetic (PK) properties, as these compounds generally have low bioavailabilities. Such delivery systems have successfully increased the bioavailability of the administered drug both in animal models [13; 14] and in human trials [15; 16]. Some formulations have been trialed in cancer patients and showed good tolerability and beneficial outcomes (i.e. partial tumor marker responses and decreased lesion incidence), but still not a clinically accepted efficacy [1]. A hy-

pothesis is that despite the improved bioavailability of the drug, the delivery system may have a PK profile significantly different from the drug in the diet, which chemoprevention capacities have been shown in epidemiological studies. In the evaluation of delivery systems often PK parameters, such the kinetics of absorption is not carefully analysed. It is also noticeable that *in vitro* investigation of pharmacological activities of polyphenols is typically carried by exposing cells to a constant concentration of the compound, whereas the physiological absorption of polyphenols from the diet leads to a gradual increase in blood concentration.

A more careful consideration of the PK of polyphenols in the diet and the development of delivery systems that can mimic such kinetics could have important applications for investigative purposes and, perhaps, improved therapeutic efficiencies. As such, the main objective of this work is to develop alginate-based films for the controlled release of flavonoids, mimicking their pharmacokinetic properties in humans, for potential applications in (1) investigative delivery and in (2) therapeutic applications, especially chemoprevention in skin. The proposed system consists of dry alginate films, where flavonoids are encapsulated before release through the gelation of alginate with divalent ions. The gelation prior to use is important in the case of flavonoids since these compounds are easily degraded with light or at higher temperatures.

## 2. Materials and Methods

### 2.1. Pharmacokinetic Data Analysis

Empirical PK data from human trials was searched in the Dimensions.ai database using the terms, ("*green tea*" OR "*polyphenon E*" OR "*EGCG*") AND *pharmacokinetics* AND (*human* OR (*clinical trial*)), yielding 64 initial results of which 17 results contained unique PK data of EGCG in humans. The results were further filtered to 6 studies, following the criteria of exclusion of having less than 7 sampling time points, not capturing the absorption phase adequately, or using doses smaller than 400 mg/day. The data was extracted from plots using WebPlotDigitizer [17] when not available and both 1-compartment and 2-compartment models were fitted to the selected data.

In a 1-compartment model the time evolution concentration of drug in a central compartment (blood) is defined as:

$$C_c(t) = \frac{F \cdot Dose \cdot k_a}{V_c \cdot (k_a - k_e)} \cdot (e^{-k_a \cdot t} - e^{-k_e \cdot t}) \quad (1)$$

, where  $F \cdot Dose$  is the bioavailable dose of drug administered,  $V_c$  is the volume of distribution of the central compartment and  $k_a$  and  $k_e$  are the drug absorption and rate constants respectively.

While in a 2-compartment model it is defined as:

$$C_c(t) = \alpha_1 e^{-\beta_1 t} + \alpha_2 e^{-\beta_2 t} - (\alpha_1 + \alpha_2) e^{-k_a t} \quad (2)$$

, with the following auxiliary constants:

$$\begin{cases} \alpha_1 = \frac{Dose \cdot F}{V_c} \cdot \frac{k_a \cdot (k_r - \beta_1)}{(k_a - \beta_1)(\beta_1 - \beta_2)} \\ \alpha_2 = \frac{Dose \cdot F}{V_c} \cdot \frac{k_a \cdot (k_r - \beta_2)}{(k_a - \beta_2)(\beta_1 - \beta_2)} \\ \beta_1 = \frac{k_e + k_d + k_r + \sqrt{(k_e + k_d + k_r)^2 - 4k_r k_e}}{2} \\ \beta_2 = \frac{k_e + k_d + k_r - \sqrt{(k_e + k_d + k_r)^2 - 4k_r k_e}}{2} \end{cases} \quad (3)$$

, where  $k_d$  is the distribution rate constant, that is related with the movement of drug from the central compartment to a peripheral compartment, while  $k_r$  is the redistribution constant, that is related to the opposite movement of drug.

### 2.2. Materials and Reagents

Sodium alginate used was from Sigma (catalog number A2033, medium viscosity, from *Macrocystis pyrifera*) and chitosan was from Roig Farma. Glycerol (GL0026005P, >99.5%) was from SCHARLAU. Metal chlorides were obtained from Panreac, Acros or Carlo Erba. Polyphenols gallic acid (G7384, >97%), epicatechin (E1753, >90%) and EGCG (93894, >98%), and cytochrome c (C7752) were from Sigma-Aldrich. Caffeine (27600, >99%) was from FLUKA. The silicone membrane was kindly offered by Lintec Co., Ltd.

### 2.3. Fabrication of Dry Alginate Films

Dry alginate films of three different thicknesses were prepared by the casting/solvent evaporation. Solutions of sodium alginate (2% w/v) were prepared by powder dissolution in distilled water, under magnetic agitation for at least 12 h ensuring complete dissolution. Afterwards, the solution was distributed on a mold in three volume/area ratios (0.79, 1.19 and 1.59 mL cm<sup>-2</sup> of base). After drying for 6 days at 40°C, alginate films with 85, 139 and 202 μm thicknesses and, respectively, with 1.5, 1.5 and 1.2 mg mm<sup>-3</sup> apparent density were obtained and identified as thin, intermediate and thick films.

Glycerol has been used before as a plasticizer of dry alginate films [18]. Alginate films with glycerol were produced by adding to the previously prepared alginate solution 15 and 30% (w/w) of glycerol, based on the weight of alginate.

Films of chitosan and alginate have also been explored for drug delivery purposes [19]. The direct mixing of alginate and chitosan, a polycationic polymer, originates polyanion-polycation (polyelectrolyte) complexes creating a hydrogel [19], which is incompatible with the proposed rationale of encapsulating the drug before usage. From preliminary tests, it was found that stock solutions of chitosan with concentrations lower than 0.03% (w/v)

did not create visible alginate-chitosan complexes when added to the alginate solution (2% w/v) in equal volume ratios, regardless of the concentration of acetic acid or the ionic strength (100 mM of NaCl). From the resulting solutions (with and without NaCl) thin films were prepared by the aforementioned method.

#### 2.4. Measurement of Contact Angles

The contact angles of dry alginate films with and without additives were studied using the sessile drop technique using water as a probe liquid. A camera look down angle used of  $5^\circ$  was employed to ease measurements by bringing the image to a sharp focus. Immediately after dropping a 5  $\mu$ L water drop an image was captured. The contact angle was determined using the LB-ADSA plugin [20] for ImageJ [21].

#### 2.5. Quantification and stability of polyphenols in presence of alginate-crosslinking metals

Stock solutions of the polyphenols were routinely prepared in dimethyl sulfoxide (DMSO) and diluted to the target concentrations just before use. Different methods were used to analyze the polyphenols: UV-Vis spectrophotometry, polyphenol assay and HPLC. Epicatechin was measured by molecular absorption at 278 nm using a Varian Cary 50 spectrophotometer. The total polyphenol assay was adopted from Ganesan et al. [22], briefly, 100  $\mu$ L of sample was added to 2 mL of Na<sub>2</sub>CO<sub>3</sub> (2% m/v), homogenized and, after resting for 2 min, 100  $\mu$ L of Folin-Ciocalteu reagent (50% v/v) was added, homogenized and the absorbance at 720 nm measured once incubated for 30 min at room temperature in the dark [46]. EGCG and epicatechin were also quantified by phase reverse HPLC in a Agilent 1100 system equipped with a C18 column, and using a mobile phase consisting of triethylammonium phosphate:water:acetonitrile 1:83:16 (pH 4.3), flux 0.6 mL/min, and UV detection at 278 nm.

The stability of EGCG in presence of metal ions (Ca<sup>2+</sup>, Ba<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup>) was studied with 100  $\mu$ M solutions in saline medium (NaCl 150 mM). Assays were carried with 100  $\mu$ M metal ions (1 min reaction time) and 500  $\mu$ M metal ions (5 min reaction time) to examine possible changes in the absorption spectra or chromatograms of EGCG. Difference spectra of EGCG in presence of metals were obtained by subtracting the absorption spectra of the metal solutions (at the corresponding concentration) from the spectra of the mixture (EGCG + metal ion).

In other set of stability tests, EGCG at a concentration of 10 mM was mixed with metal ions (200 mM) and incubated for 20 hours, at 4 or 20°C. Initial and final mixtures were analyzed by HPLC. Chloride salts of the metal cations were used in all

the stability studies as in the gelation of alginates.

#### 2.6. Polyphenol Encapsulation

Epicatechin, EGCG or gallic acid were encapsulated by addition to the crosslinking metal solutions used to gel the alginate films. For gelation, the dried alginate films were immersed in a small volume of calcium, zinc or barium chloride 164 mmol L<sup>-1</sup>, for 18 h at 4 °C. Blank alginate matrices were gelled in the absence of epicatechin, while epicatechin-loaded films were gelled with crosslinking metal solutions containing 3.8 nmol of the polyphenol per mm<sup>2</sup> of the film's total surface area. The encapsulated efficiency was determined by quantifying the amount of polyphenol in the residual encapsulation solution using HPLC.

#### 2.7. Distribution of the Encapsulated Epicatechin

The evaluation of the distribution of the epicatechin encapsulated in the films was accomplished using an adapted version of the total polyphenol assay. Small disks ( $\phi = 5.5$  mm) were punched from alginate films and 0.18  $\mu$ mol of epicatechin was encapsulated in each disk. After the 18 h the disks were removed from any residual liquid from encapsulation, 20  $\mu$ L of Na<sub>2</sub>CO<sub>3</sub> (2% w/v) was deposited on the discs for 2 min. Afterwards, the remaining solution of Na<sub>2</sub>CO<sub>3</sub> was removed and 20  $\mu$ L of Folin-Ciocalteu reagent (50% v/v) was deposited in the disc for 10 min in the dark at room temperature. Finally, the remaining Folin-Ciocalteu was dried and the disks were photographed.

#### 2.8. Epicatechin Release Studies

The kinetics of release of epicatechin by alginate was studied with alginate films having a total surface area of 143 mm<sup>2</sup> in total, gelled using different crosslinking gelation ions (Ca<sup>2+</sup>, Ba<sup>2+</sup> and Zn<sup>2+</sup>) as previously described.

After gelation and encapsulation, the films were washed (thrice for 1 s) in 1 mL of saline, and placed in 5 mL of saline solution (NaCl 150 mmol L<sup>-1</sup>) for monitoring epicatechin release under gentle agitation. Samples of the release medium were withdrawn various times and epicatechin quantified by UV/Vis spectroscopy at 278 nm. Blank alginate matrices gelled with each ion were also assayed in parallel and the absorbance values of the blank films were subtracted to the polyphenol-loaded films. Release assays for each ion were done in triplicate both for blank and epicatechin-loaded films.

The kinetics of epicatechin release was studied by fitting the experimental data to the first order, Korsmeyer-Peppas and Weibull models [23]. The mathematical models were fitted and the adjusted coefficient of determination ( $R_{adj}^2$ ) was calculated with MATLAB<sup>®</sup>.

## 2.9. Permeation Studies with Franz Cells

The permeation of different compounds was studied using a silicone membrane from LINTEC Co., Ltd. that has been previously showed by Uchida *et al.* [24] to have some validity as model of human skin permeation. The permeation studies were made with gallic acid, as well as with caffeine replicating studies by Uchida *et al.* [24]. Saturated solutions of the aforementioned compounds were pipetted into the donor chamber at volumes of 600  $\mu\text{L}$ , as to simulate an infinite donor, that is, an amount that does not deplete in the duration of the assay. Before each assay the membrane was equilibrated in saline for 30 min and then rinsed in 1 mL of fresh saline before being placed in the Franz cell. The permeation kinetics were followed for 6 hours with UV/Vis spectroscopy at 254 nm for caffeine and 260 nm for gallic acid.

The permeability coefficient ( $P$ ), the diffusion parameter ( $DL^{-2}$ ), and partition parameter ( $KL$ ) were calculated from the permeation kinetic profiles using Equation 4 [24]:

$$\begin{cases} Flux = P \cdot C_v \\ DL^{-2} = \frac{1}{6 \cdot T_{lag}} \\ KL = 6 \cdot T_{lag} \cdot P \end{cases} \quad (4)$$

, where the permeability coefficient was calculated from the  $Flux$ , which is the slope of the linear phase of the permeation profile divided by the effective permeation area, and from the concentration of the donor solution ( $C_v$ ). The  $T_{lag}$  was obtained from the intersection of the linear trendline of the linear phase with the x-axis, and was used to calculate the partition and diffusion parameters.

## 2.10. Catechin Release in Franz Cells

The Franz cells were also used as a support for drug release studies, enabling the study of drug release in conditions similar to that of a skin patch. An alginate film with total surface area of 800  $\text{mm}^2$  was loaded with epicatechin as described in Chapter 2.6 and placed between the donor and receptor chambers. The receptor chamber was filled with 5 mL of saline ensuring contact with loaded film and through the sampling port 30  $\mu\text{L}$  of the receptor liquid was sampled and replenished with saline at various time intervals. The concentration of the samples was then determined through HPLC with detection at 278 nm.

## 2.11. Epicatechin Delivery on Skin

Skin delivery was studied with *ex vivo* pig skin collected in a local abattoir and used less than 6 h after animal sacrifice. Calcium alginate films with a total surface area of 800  $\text{mm}^2$  were prepared as described in Chapter 2.6. The skin was placed in a horizontal surface, a small superficial incision was

made, 100  $\mu\text{L}$  of saline were pipetted on the incision and, then, a catechin-loaded film was applied to completely cover the incision surface. Control assays with a blank (no-catechin) film were run in parallel. After 1 h, the films were gently removed, the incision covered by the film was sampled with 100  $\mu\text{L}$  of saline rapidly collected to a microtube and frozen or maintained refrigerated for less than 3 h until analysis. The samples were centrifuged at 10 000 g for 5 min, and the supernatant analyzed for the catechin concentration by HPLC and antioxidant bioactivity. Antioxidant bioactivity was measured by the cytochrome c (cyt c) reduction assay [25], briefly, the samples were added to potassium phosphate 10 mM pH 8.0 buffer supplemented with cytochrome c (20  $\mu\text{M}$  final concentration), and the kinetics of reduction of the protein was followed at 550 nm.

## 3. Results

### 3.1. Pharmacokinetics of Green Tea catechins in humans

For all the PK studies analysed, the 1-compartment model describes well the observed data (Figure 1-A). On the other hand, the 2-compartment model, seems to overfit the PK data, since the goodness-of-fit ( $R^2_{adj}$ ) never increased when the additional explanatory variables, the distribution ( $k_d$ ) and redistribution ( $k_r$ ) rate constants, were added to the model (Figure 1-A). Furthermore, the values of  $k_r$  estimated by the model were unrealistically large, and  $k_d$  values often tended towards zero. The low number of time points of the pharmacokinetic curves for the high number of variables increases the uncertainty of the fitting and may be the cause of such unrealistic values. However, the overall explanation may be that the distribution of epicatechin to peripheral tissues is not relevant. The biodistribution of EGCG has been previously studied with Wistar rats by positron emission tomography, the labeled EGCG administered orally accumulated in the digestive tract and a portion was absorbed to the blood and accumulated in the liver, with negligible amounts accumulating in the peripheral tissues (brain and heart) [26].

The rate constants obtained from the 1-compartment model were used to characterize the PK of EGCG in humans and are summarized in Figure 1-B, where  $k_a$  averages  $1.1 \pm 0.7 \text{ h}^{-1}$  and  $k_e$  averages  $0.28 \pm 0.12 \text{ h}^{-1}$ . There is a high variability in the absorption rate, which could be caused by inaccuracies in measuring the start of the experiment, especially in the cases of teas and solutions, where drinking the tea may take some minutes. Nevertheless, it is curious that the elimination rate has a lower variability. Importantly for the present work, the analysis of the PK after administration of doses similar to those found therapeutically effective in

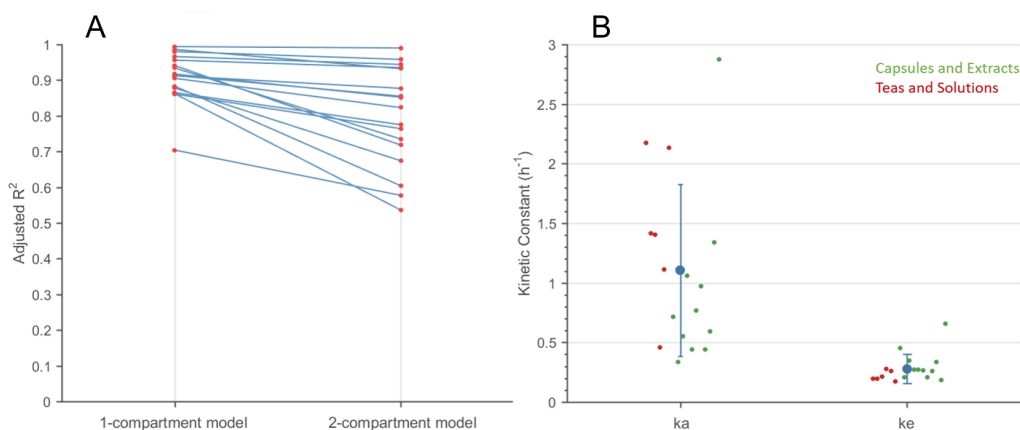


Figure 1: (A) Adjusted  $R^2$  of the fitted curves using 1-compartment and 2-compartment models. (B) Average and SD of absorption ( $k_a$ ) and elimination ( $k_e$ ) rate constants from the 1-compartment model.

trials points to  $k_a$  values of  $1.1 \text{ h}^{-1}$ . With future flavonoid therapies in mind, delivery systems with release kinetics that approach the PK of such drugs may be useful for investigative delivery and perhaps therapeutic purposes.

### 3.2. Production and Characterization of Alginate Films

As potential matrices to support catechin delivery dry alginate films were prepared by casting/solvent evaporation method with and without additives, namely with added glycerol and chitosan. The alginate films with additives were compared with normal alginate films regarding their transparency and flexibility and water contact angles were measured using the sessile drop technique (Table 1).

The addition of glycerol to the alginate increased the flexibility of the film. Furthermore, the film with higher content in glycerol 30% (w/w of alginate) was more flexible than the film with lower glycerol content (15% w/w of alginate). The transparency of the glycerol-containing films remained similar and the measured contact angles did not change significantly.

Despite the low concentration of chitosan (0.03% w/v), the chitosan supplemented films had their hydrophobicity increased, as shown by the increase of contact angle by  $\approx 7^\circ$ , regardless of presence of NaCl. However, the presence of NaCl did decrease the transparency and flexibility of the alginate films, while the chitosan alone did not show any clear difference in these properties.

### 3.3. Catechin Stability in Presence of Alginate-Gelling Cations

The construction of polyphenol-loaded alginate films requires the selection of gelation ions compatible with polyphenols. Some ions and polyphenols are far from chemical inert species, so this point can be critical for the design of delivery systems.

Having in mind the redox behavior of polyphe-

nols, it is plausible they reduce certain oxidizing cations. In addition, formation of flavonoid-ion complexes has been described [27], so the interaction between polyphenols and these cations was studied by HPLC and UV/Vis spectroscopy.

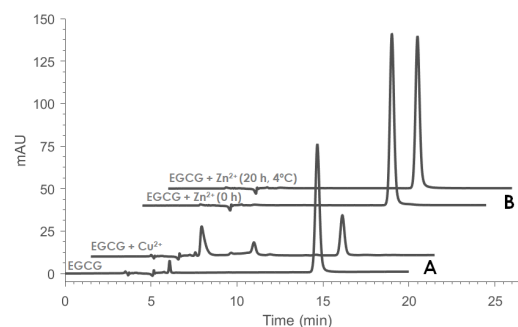





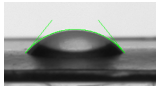
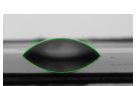
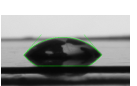
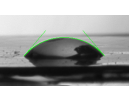
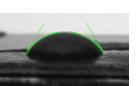


Figure 2: (A) Chromatograms of EGCG (100  $\mu\text{M}$ ) in saline medium (NaCl 150  $\mu\text{M}$ ) with and without the addition of  $\text{Cu}^{2+}$  (100  $\mu\text{M}$ ) of the initial screening tests. (B) chromatograms of EGCG (10 mM) with a  $\text{Zn}^{2+}$  (200  $\mu\text{M}$ ) at 0 h and after 20 h at 4  $^\circ\text{C}$ . The latter chromatograms were diluted before HPLC measurements.

HPLC analysis revealed no changes in the chromatograms for the addition of  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  or  $\text{Zn}^{2+}$ , while the addition of  $\text{Cu}^{2+}$  decreased the peak of EGCG at 15 min retention time alongside the appearance of two additional peaks at earlier retention times corresponding to unidentified degradation products (Figure 2-A). EGCG difference spectra by UV/Vis spectroscopy also remained unchanged in the presence of  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  or  $\text{Zn}^{2+}$ , but not with  $\text{Cu}^{2+}$  (not shown).

After this screening  $\text{Cu}^{2+}$  was excluded from being a potential gelation ion for drug delivery of catechins, where high concentrations of polyphenol and cation are needed. Further stability tests with  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  over a longer time and at varied temperatures were carried to confirm the viability of these ions. After 20 h, HPLC analysis reveals no anomalies in the chromatograms when  $\text{Ca}^{2+}$  or  $\text{Zn}^{2+}$  are

Table 1: Physio-mechanical properties of alginate dry films with additives, where Alg G15 and Alg G30 is alginate with glycerol 15% (w/w of alginate) and 30% (w/w of alginate), respectively; and Alg Chi is alginate with added chitosan 0.03% (w/v). The transparency and flexibility of alginate films were qualitatively compared with the alginate film with no additives. The contact angles show average and standard error of different films (n=4), as well as a representative picture of the contact angle measurement.

Dry films	Alginate	Alg G15	Alg G30	Alg Chi	Alg Chi (w/NaCl)
<b>Transparency</b>	—	Similar	Similar	Similar	Lower
<b>Flexibility</b>	—	Higher	Higher	Similar	Lower
<b>Photograph</b>					
<b>Contact Angle (°)</b>	47.4 ± 1.0	47.2 ± 1.2	49.0 ± 1.0	54.7 ± 0.9	55.0 ± 1.7
					

present (Figure 2-B). However, some degradation of EGCG appears to be promoted by the ions, albeit the change expressed in relative area of the peak is generally small (less than 8% with  $\text{Ca}^{2+}$  and 13% with  $\text{Zn}^{2+}$ ). Although untested,  $\text{Ba}^{2+}$  should not behave very differently from  $\text{Ca}^{2+}$  ions as they are chemically similar, with barium having lower reduction potential than calcium.

On these grounds, the hypothesis that calcium, barium or zinc ions react substantially with EGCG was excluded and they were selected as good gelation candidates for alginate systems carrying catechin flavonoids.

### 3.4. Polyphenol Encapsulation in Alginate Films

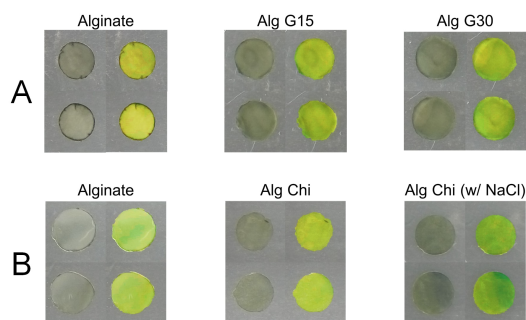


Figure 3: Distribution of epicatechin loaded into alginate-based films. (A) Intermediate thickness films with and without added glycerol 15% (w/w) - Alg G15; and 30% (w/w) - Alg G30; (B) Thin alginate films with and without added chitosan or chitosan and NaCl. On the left is the original picture, while the right pictures had their color saturation enhanced by 400% for better visualisation. Green/blueish regions correspond to the regions with encapsulated epicatechin, while yellow regions have very little or no epicatechin.

Polyphenol encapsulation studies were done by gelling alginate films with  $\text{Ca}^{2+}$  solution containing gallic acid, epicatechin or EGCG. Overall the encapsulation efficiency of polyphenols into the alginate films is low, only around 30% for epicatechin and gallic acid. Surprisingly, the encapsulation

of EGCG was very low or nonexistent given that epicatechin and EGCG are very similar molecules. Alginate hydrogels are typically nanoporous with around 5 nm pore size [10] and the dry alginate films should have even smaller pore sizes. Possibly, the size difference between the two molecules, with EGCG being an abnormally large flavonoid (with an additional aromatic ring), hinders the entry of EGCG through the nanosized pores of the alginate film, preventing its encapsulation.

To evaluate the homogeneity of epicatechin encapsulation, intermediate thickness films with and without glycerol, as well as, thin alginate films with and without chitosan were encapsulated with  $0.18 \mu\text{molL}^{-1}$  of epicatechin. Afterwards the regions with epicatechin were made visible with a procedure based on the total polyphenol assay (Chapter 2.7).

Figure 3 shows that the encapsulation of epicatechin is not homogeneous, as some regions of the alginate film display a green/blueish color, whereas others appear to contain very little or no epicatechin, indicated by the yellow color. One explanation is that some regions of the alginate film have tighter pores than others, disabling the epicatechin from getting encapsulated. Furthermore, both the films supplemented with glycerol and the films supplemented with chitosan seem to allow a wider distribution of epicatechin, possibly pointing toward a larger porosity or a more uniform distribution of pores in the film.

### 3.5. Epicatechin Release Studies

The release of epicatechin from alginate films gelled with calcium, barium and zinc ions was studied by immersing the films in saline under gentle agitation and following the release by UV/Vis spectroscopy.

Independently of the gelation metal ion used, the thin films released epicatechin faster than the thick films (Figure 4). Noticeably, the calcium-gelled matrices exhibited a higher cumulative release than zinc and barium alginates. In gen-

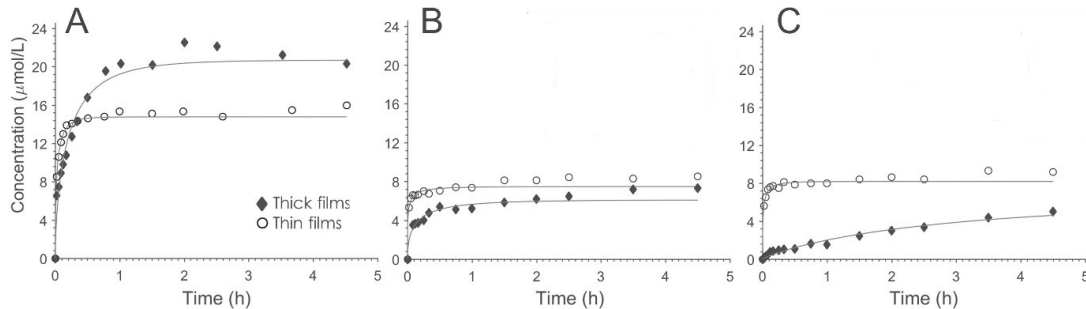


Figure 4: Profiles of epicatechin release by calcium (A), zinc (B) and barium (C) alginate films in saline medium. The kinetic profiles shown are representative of triplicate encapsulation and release assays. Lines in the main plots are the best nonlinear regression fits of the experimental data to the Weibull kinetic model.

eral, most of the flavonoid was released in the first hour, although the thick barium films prolonged significantly more the kinetics of release (Figure 4). It should be noted that the release assays of epicatechin-loaded films were always run along with a blank film prepared in the same way, but without the flavonoid, to control eventual interferences with epicatechin analysis. Overall, the results demonstrated that the delivery systems here conceived are able to afford micromolar concentrations of flavonoids, as required for accepted pharmacological activity for dermal applications.

First order, Korsmeyer-Peppas and Weibull models were fitted to all the triplicate release assays for the combinations of each ion with each film thickness used. The average values of the parameters and the lowest  $R_{adj}^2$  values are reported in Table 2. According to the correlation coefficients, the Weibull model is the best fitting the experimental data, with the lowest  $R_{adj}^2$  observed 0.877. The values of the Weibull parameter  $b$  inferior to 0.75 obtained for most films (Table 2) indicate the release mechanism is Fickian diffusion [23]. The thick barium films are a clear exception, with  $0.75 < b < 1$  pointing to a combined mechanism of Fickian diffusion and case II transport. Korsmeyer-Peppas parameter  $n > 0.5$  also suggests a combined mechanism occurring with thick barium films, but this result is limited as the model could not be fitted only to the first 60% of release [28].

Remarkably, the films exhibited release properties dependent on the crosslinking metal, opening a possibility to tune alginate delivery systems for specific applications. Apart from the clear slow release from the barium thick films, the kinetic rate constants,  $k$  of the first order model and, to some extent, the  $k$  of Korsmeyer-Peppas model and the parameter  $a$  of the Weibull model, indicate a slower release from zinc films when compared with the calcium films. A previous study with alginate microparticles also indicated that crosslinking with  $Zn^{2+}$  delayed diffusional drug release compared to calcium alginate matrices [29].

Since the absorption in the compartmental models follows first order kinetics, the values obtained can be compared with the first order model of epicatechin release from films. The films that better approximate the  $1.1 \text{ h}^{-1}$  absorption rate constant are the thick films with release rates ranging 0.33 to  $6.80 \text{ h}^{-1}$ .

### 3.6. Drug Permeation in Franz Cells

Permeation studies were also carried using Franz cells and a silicone membrane previously reported to model skin permeability [24]. Caffeine could permeate the silicone membrane and the acidification of the saturated caffeine solution did not alter the permeation kinetics (Figure 5), suggesting pH does not influence the permeability of the membrane. From the caffeine permeation profile, the permeability coefficient ( $P$ ), the diffusion parame-

Table 2: Kinetic model parameters for epicatechin release by different alginate films. First order, Korsmeyer-Peppas and Weibull models were tested by nonlinear regression and  $R_{adj}^2$  is the lowest (adjusted) coefficient of determination obtained from the curves for each film type.

Condition	First order		Korsmeyer-Peppas			Weibull			
	$k \text{ (h}^{-1}\text{)}$	$R_{adj}^2$	$k \text{ (h}^{-n}\text{)}$	$n$	$R_{adj}^2$	$a \text{ (h}^{-b}\text{)}$	$b$	$R_{adj}^2$	
Ca	Thick	6.80	0.826	0.89	0.21	0.898	2.87	0.79	0.934
	Thin	25.60	0.963	0.99	0.11	0.764	10.21	0.68	0.988
Zn	Thick	3.83	0.819	0.85	0.21	0.869	2.66	0.69	0.877
	Thin	22.52	0.879	0.95	0.13	0.871	6.10	0.57	0.908
Ba	Thick	0.33	0.949	0.28	0.69	0.947	0.34	0.94	0.946
	Thin	40.76	0.882	1.01	0.07	0.918	6.76	0.46	0.886

ter ( $DL^{-2}$ ) and the partition parameter ( $KL$ ) were obtained ( $P=0.18\times 10^{-6}$   $\text{cm s}^{-1}$ ;  $DL^{-2}=0.13\times 10^{-2}$   $\text{s}^{-1}$ ;  $KL=1.59\times 10^{-4}$   $\text{cm}$ ). The obtained parameters are similar with the results from Uchida *et al.* [24].

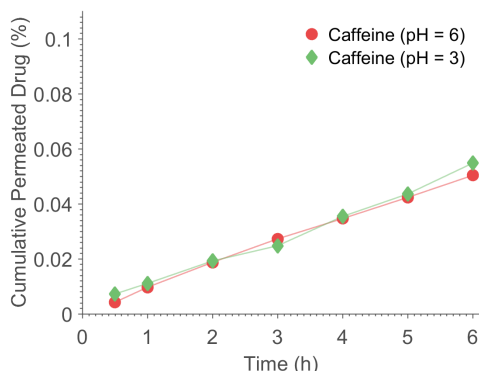


Figure 5: Permeation of caffeine through a silicone membrane, with an initial donor of 600  $\mu\text{L}$  of saturated solutions of the compound in saline.

Permeation assays were carried with epicatechin and gallic acid solutions. Epicatechin was quickly discarded as it reacted with the silicone membrane. In gallic acid assays in concentrations ranging from 12  $\text{mmol L}^{-1}$  to saturation, permeation to the receptor chamber could not be detected in assays up to 24 h. The permeation of a gel formulation of gallic acid through the skin of Sprague-Dawley rats has been studied before in Franz cells, where it was able to permeate in small amounts ( $<1\%$  after 12 h), with most of the gallic acid not permeating or accumulating in the stratum corneum [30]. As such, the silicone membrane here studied might not be adequate to study the permeation of gallic acid in skin.

### 3.7. Drug Release in Franz Cells

Drug release was also studied with Franz cells since this would better approximate the release conditions from skin patches.

The results in Figure 6-A with intermediate thickness calcium films afforded similar maximum concentrations to the previous studies with immersed films. However, considering the release rate ( $k$ ) of first-order model, the release is slower than the previous assays, likely due to the release in Franz cells happening only from one side of the film.

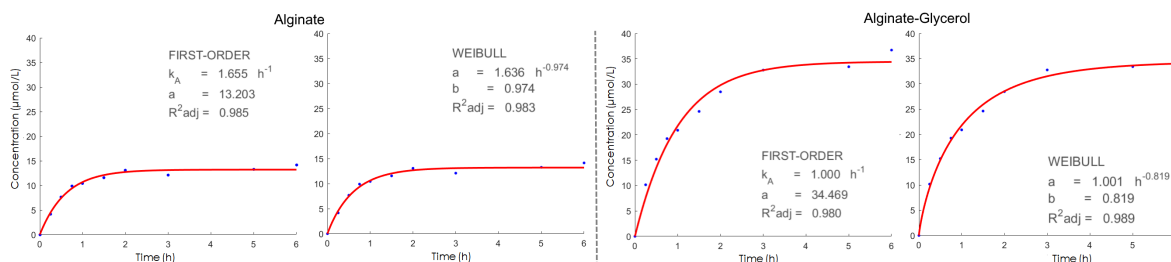


Figure 6: Franz cell release profiles of epicatechin release by a calcium alginate film and film with added glycerol 30% (w/w). Lines are the nonlinear regression fits of the experimental data to first order and Weibull models.

The Weibull models seem suitable to describe the release kinetics in these conditions (Figure 6), and parameter  $b$  suggests a complex release mechanism, but further studies are necessary.

Remarkably, the release rate of glycerol-supplemented films (Figure 6) is slower than the unsupplemented films and is very close to the average absorption rate constant of EGCG. Furthermore, the glycerol-alginate films afforded higher concentrations than the simple alginate films, as well as, higher encapsulation efficiency (47% for the glycerol-containing film *vs* 26% for the alginate film).

### 3.8. Epicatechin Delivery on Skin

A proof-of-concept of the system devised in previous section was planned on skin *ex vivo*. Since calcium alginate films afforded the higher concentrations than films gelled with other metal cations (Chapter 3.5), calcium was chosen to crosslink the alginate films tested in skin. Alginate films were loaded with epicatechin and applied on porcine skin (Figure 7-A). A small incision was made in the skin at the site for patch application so that released epicatechin could contact with injured dermis. Dermal fibroblasts are known to metabolize epicatechin and its metabolites retain capacity to protect against oxidant skin-damaging conditions [7], so the presence of unmodified and antioxidant functional epicatechin was evaluated 1 h after topical application by HPLC and Cyt *c* assay (Figure 7-B).

Panel C summarizes the quantification of the flavonoid ( $50.0\pm 17.2$   $\mu\text{mol L}^{-1}$ ) and antioxidant bioactivity measured in the samples from skin treated with epicatechin-loaded films. The antioxidant activity of these samples seems tightly related to their epicatechin concentration, because blank samples spiked with the flavonoid prior to Cyt *c* assay, in concentrations similar to those measured in skin of epicatechin films, yielded reduction rates almost identical (Figure 7-B). It should be noted that reduction of Cyt *c* is a potential mechanism of action of flavonoids in cells [25], so the present results demonstrate that the alginate patch is able to deliver significant amounts of functionally active epicatechin on skin.

Due to the similarity of epicatechin with other

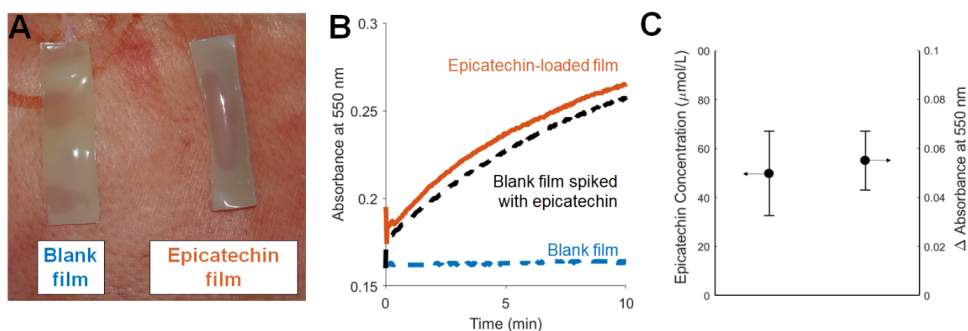


Figure 7: Topical delivery of antioxidant epicatechin on porcine skin *ex vivo*. Epicatechin-loaded calcium alginate film applied on skin (A), and cyt c reducing activity of skin surface sampled 1 hour after film application in comparison to skin treated with unloaded (blank) film (B). A kinetic curve of cyt c reduction by a blank sample spiked with 60  $\mu\text{M}$  epicatechin was also registered. Panel C presents the quantification of epicatechin levels measured by HPLC and the cyt c reductive activity (absorbance increase after 10 min assay) of the samples from skin surface after 1-hour application of epicatechin-loaded films. The results shown are representative experimental traces or the mean  $\pm$  SE from delivery assays using skin from three different animals ( $n=3$ ).

flavonoids, the delivery system here proposed is probably useful for the topical application of flavonoids in general. The epicatechin levels reached on skin surface suggest the future testing of the system in anticancer (melanoma) therapies for which simulation models predict skin surface concentrations below  $100 \mu\text{mol L}^{-1}$  can be effective [1; 31].

#### 4. Conclusions

Based on the previous results, it can be concluded that:

- The PK of EGCG in humans (therapeutic relevant doses) can be described by a 1-compartment model with an absorption rate constant ( $k_a$ ) of  $1.10 \pm 0.70 \text{ h}^{-1}$  and an elimination rate constant ( $k_e$ ) of  $0.28 \pm 0.12 \text{ h}^{-1}$ .
- The addition of glycerol improved the flexibility of alginate films prepared by the casting/solvent evaporation method and the addition of a low concentration of chitosan (0.03% w/v) increases the hydrophobicity of the films without gelling alginate.
- Copper ions should be avoided in the production of EGCG-loaded materials, but calcium, barium and zinc can be used in alginate encapsulating EGCG.
- The encapsulation degree of epicatechin and gallic acid into alginate films is low ( $\approx 30\%$ ) and EGCG could not be encapsulated, possibly due to being a larger molecule. Furthermore, the distribution of encapsulated epicatechin is not homogeneous, but improves in glycerol-containing films.
- Epicatechin loaded into alginate films crosslinked with  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Zn}^{2+}$  is released by a kinetics dependent on the crosslinking ion and the film thicknesses. Diffusional release is the predominant mechanism and kinetics can be modeled using the Weibull equation.

- Using Franz cells, the permeation of caffeine through a skin-mimicking silicone membrane could replicate data published in the literature [24], but the membrane reacts with epicatechin and gallic acid could not permeate through it.
- Release studies on Franz cells showed a kinetics of release of epicatechin by alginate films similar to the absorption of EGCG in humans, suggesting a potential utility for investigative delivery studies of flavonoids.
- The calcium alginate films were able to deliver epicatechin on skin, achieving therapeutically relevant concentrations of antioxidant functional epicatechin.

In future work, increasing the encapsulation degree should be a priority to avoid wastage of polyphenols, this could be achieved by using glycerol-alginate films or by using alternative production methods, such as freeze-drying, which should result in films of higher porosity. Further studies of skin therapeutics should be preceded by alternative permeation assays, as flavonoids need to permeate the skin to reach the target cells (i.e. fibroblasts and melanocytes).

**Acknowledgement:** This document was written and made publically available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biomedical Engineering of the author at Instituto Superior Técnico. The work described herein was performed at the School of Technology and Management of the Polytechnic Institute of Leiria (Leiria, Portugal), during the period February-November 2019, under the supervision of Prof. Ricardo Lagoa, and within the frame of the research project "MBSTox - Multifunctional biomolecular systems for new methods of decontamination, protection and toxicological assessment", PTDC/BIA-MIB/31864/2017, funded by "Fundação para a Ciência e Tecnologia" (FCT - Portugal). The thesis was co-supervised at Instituto Superior Técnico by Prof. Frederico Ferreira.

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