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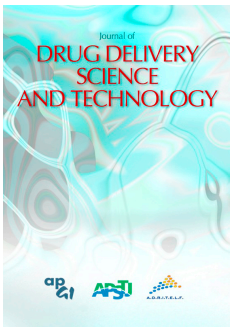

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PII: S1773-2247(18)31178-X

DOI: https://doi.org/10.1016/j.jddst.2019.03.004

Reference: JDDST 967

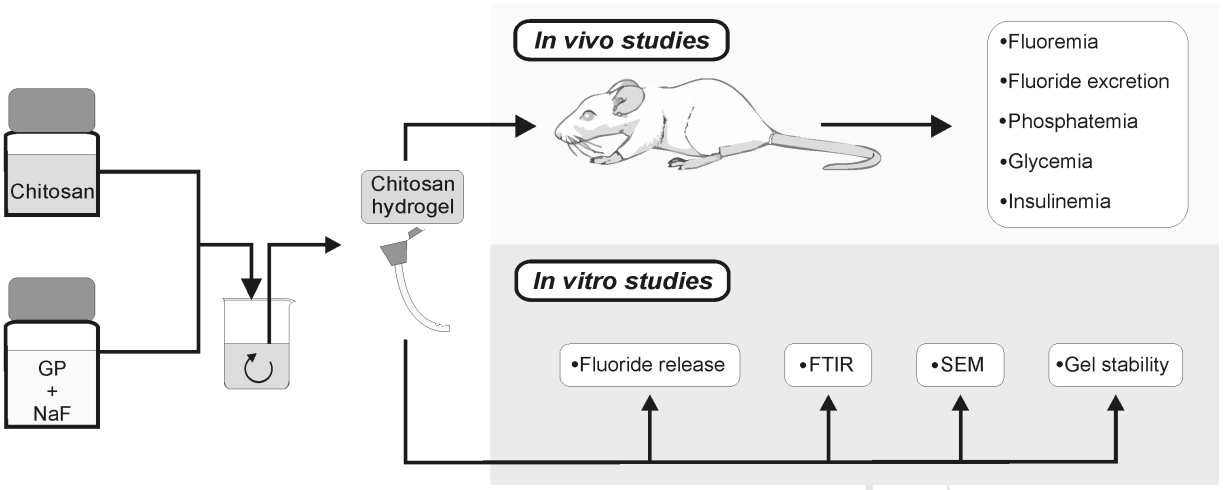
To appear in: *Journal of Drug Delivery Science and Technology*

Received Date: 10 October 2018

Revised Date: 1 February 2019

Accepted Date: 6 March 2019

Please cite this article as: F.A. Fookes, L.N. Mengatto, A. Rigalli, J.A. Luna, Controlled fluoride release for osteoporosis treatment using orally administered chitosan hydrogels, *Journal of Drug Delivery Science and Technology* (2019), doi: https://doi.org/10.1016/j.jddst.2019.03.004.



Controlled fluoride release for osteoporosis treatment using orally administered chitosan hydrogels

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Abstract

Chitosan - β-glycerophosphate hydrogels have been widely studied for biomedical applications in recent years. In the current study a Chitosan - β-glycerophosphate hydrogel was evaluated as a platform for sodium fluoride release in the gastrointestinal tract. For this purpose, microscopy observation, infrared spectroscopy, stability, rheology studies and in vitro release assays were carried out. The hydrogel was stable for at least 48 h when exposed to an aqueous media in a pH range from 4 to 7 and the release of sodium fluoride was controlled for more than 6 h.

In vivo studies were carried out in order to evaluate the oral administration of sodium fluoride using the hydrogel in comparison to a water solution. Fluoride pharmacokinetic was similar when the drug was administered with both formulations. Nevertheless, fluoride absorption was greater when the drug was given with the hydrogel, and further, drug-related side effects were absents. These results suggest that Chitosan - β-glycerophosphate hydrogel could be a good candidate for sustained release of fluoride in oral formulation.

Keywords: Chitosan hydrogel; fluoride; osteoporosis; gastrointestinal delivery.

Abbreviations: API, active pharmaceutical ingredients; CS, Chitosan; GP, β-glycerophosphate; SEM, scanning electron microscopy; IR, infrared spectroscopy; GI, gastrointestinal tract.

Introduction

Hydrogels are physically or chemically crosslinked polymer networks with a high number of hydrophilic groups that are capable of absorbing large amount of water. Due to their structure, composition, porosity and similarities with soft tissues they have been considered as biomimetic systems(Caló and Khutoryanskiy, 2015; Lim et al., 2014). Hydrogels can be formed by natural or synthetic polymers. Natural polymers generally used for hydrogel formulation are proteins (collagen, gelatin and fibrin), polysaccharides (hyaluronic acid, agarose, dextran and chitosan) and hybrid protein/polysaccharide system (collagen-hyaluronic acid, laminin-cellulose, gelatin-chitosan, fibrin-alginate)(Z. Modrzejewska et al., 2014; Yan et al., 2008).

When administered systemically some active pharmaceutical ingredients (API) present poor activity, toxicity or low bioavailability and blood concentration can quickly drops below the minimum effective value. This can lead to requiring several administrations, decreasing compliance with the treatment by patient and an increasing in the risk of suffering an overdose (Bhattarai et al., 2010). Controlled delivery systems can modulate the bioavailability to maintain adequate API blood concentration over time. Depending on the drug delivery formulation, release time may be from few hours to years(Caló and Khutoryanskiy, 2015; Lim et al., 2014). Due to their properties,

hydrogels have attracted noticeable interest in the field of controlled released systems.

Chitosan (CS) is a natural random copolymer formed by D-glucosamine and N-acetyl glucosamine obtained from the partial deacetylation of chitin. CS is biocompatible and has a good mucoadhesion. This polymer forms hydrogels that has been proposed to different biomedical application like scaffolds in tissue engineering, wound dressing and drug release systems(Jayakumar et al., 2010). CS hydrogels have been prepared with a variety formulations that can be divided into two classes: those formed by irreversible covalent links (chemical hydrogels) and those formed by various reversible links (physical hydrogels). Covalent crosslinking forms gels showing enhanced mechanical properties. However, cross-linking agents, such as glutaraldehyde; are often associated with significant toxicity(Muxika et al., 2017). For these reasons, physically crosslinked hydrogels have gained increasing attention.

CS and β-glycerophosphate disodium salt (GP) solutions present low viscosity at room temperature but at physiological conditions (37 °C) suffer a phase transition (sol-gel) without any external stimulation(Y. Peng et al., 2013). CS/GP hydrogels has been studied as a drug delivery system for a wide range of API such as antineoplastic (paclitaxel), antidepressant (venlafaxine), antiepilepsy (ethosuximide), hormones (insulin) and are mainly used in parenteral applications (Hsiao et al., 2012; Q. Peng et al., 2013; Zhou et al., 2015). In view of CS properties, CS/GP hydrogel is a good candidate for drug release systems for enteral application.

Biodegradability of chitosan hydrogels by human colonic bacteria has been deeply studied by McConnell et al (Mcconnell et al., 2008). These authors concluded that chitosan hydrogels similar to the CS/GP hydrogels studied in the current work can be digested by human colonic bacteria.

CS/GP hydrogels act as an API reservoir with different release profiles. The most influent parameters in API diffusion through hydrogel are gel formulation, drug properties (such as molecular weight and hydrophilicity) and the release environment. While drugs with low hydrodynamic radii are expected to be released in less than 24 hours, drugs with greater radii could reach a sustained release effect for months or even years (Zhou et al., 2015). Modelistic studies predict that the release as a function of time of an API from hydrogel have a rate-limiting step for controlled release(Bhattarai et al., 2010). Diffusion-controlled release through the hydrogel is the primary mechanism of many drugs from hydrogels. In swollen state, typical mesh sizes reported for biomedical hydrogels range from 5 to 100 nm. Macromolecules, because of their hydrodynamic radii, will have sustained release for long period of time, whereas diffusion of small molecules is moderately retarded in this kind of matrix. In any case the desirable period of time for controlled release depend on the kind of pharmaceutical application.

Fluoride is the ionic form of fluorine, the most electronegative element, is recognized for stimulate osteoblast differentiation and for its anticariogenic properties (National Research Council, 2006; Ullah and Zafar, 2015). When administered in low dose, bone mass is increased reducing the risk of vertebral fractures in patients with osteoporosis

(Rubin et al., 2001). For systemic therapy, oral administration of fluoride, is the only possible way (Rigali et al., 2003). However, there are several works that discuss the potentially negative effect of fluoride when applied in high doses or chronically (as the case of fluoridated drinking water). Manifestations of fluoride toxicity may include hormonal disorder, dental and skeletal fluorosis(Buzalaf et al., 2008; Menoyo et al., 2005; Sharma et al., 2017; Ullah and Zafar, 2015).

Several works has proposed fluoride delivery system based on CS. Nguyen et al(Nguyen et al., 2017) develop CS based nanoparticle sized between 100 and 400 nm prepared by ionic gelation. This nanoparticles showed a slightly controlled release effect in in vitro assays at pH 5 and 7 with cumulative percentual released between 60 and 90 % of API in the first 2 h (if compared with the total API released in 24 h). Keegan et al(Keegan et al., 2012) has manufactured spray dried CS-fluoride microparticles with or without the inclusion of glutaraldehyde as a crosslinker. In these works, fluoride release is studied at three different values of pH (4, 5.5 and 7) and the percentage released was always greater than 50 % at 2 h.

The aim of the present study was to investigate CS/GP hydrogels as a system for controlled release of fluoride orally administered. Stability and drug release were studied at different pH. In vivo studies were carried out to compare pharmacokinetic parameters and insulin levels when the API was applied in a gel or in an aqueous solution.

Materials and methods

Materials

CS was obtained from Easter Group (China), β-glycerophosphate disodium salt (GP) was provided by Surfactan (Argentina). Sodium Fluoride (NaF) and Acetic Acid were purchased from Cicarelli (Argentina). Milli-Q quality water was used in every assay. Buffer solutions compositions are listed in Table 1. All other reagents were of analytical grade.

Preparation of CS/GP and CS/GP/NaF gel

Gels were prepared according to the work of Mengatto et al(Mengatto et al., 2016). In brief, 2 g of CS were dissolved in 98 ml of an acetic acid solution (0.14M), then was mixed with a solution containing GP (35 % w/w) and NaF at two different concentrations in order to get hydrogels with API concentration of 0.625 or 2.5 % w/w. The resulting solution was heated at 37 °C for 10 min in order to allow solution to gel transition.

Gel stability at different pH

In order to study the stability of the gel at different pH, 500 mg of preformed gel with or without NaF were placed in a flask containing 50 mL of buffer solution at different pH (37 °C). Macroscopic integrity was observed over time until fully gel disaggregation and photographs of the gels were taken at different periods of time to visualize their evolution.

Infrared spectroscopy studies

Structural properties of gels with or without NaF were analyzed before and after conditioning in distilled water for 48 h. Afterwards, gels were frozen at -80 °C and lyophilized (TELSTAR CRYODO –80) for 24 h to obtain a solid residue. Subsequently, samples (2 mg) were mixed with 100 mg of dry KBr (potassium bromide) and the mixture were then ground into a fine powder before compressing into a disc. The spectra were obtained using a SHIMADZU FTIR-8201PC

apparatus in the frequency range of 400-4000 cm^{-1} (spectral resolution: 4 cm^{-1}, number of scans: 40).

Release experiments

Dissolution studies were performed in 4 different buffers with pH from 4 to 7 using an orbital shaker at 100 rpm incubated at 37 °C. Before the release experiments, gels were prepared into cylindrical shape using orogastric tube (inner diameter of 2.3 mm). Approximately 400 mg of preformed gel were immersed into a flask containing 50 mL of dissolution media. Aliquots of 1 mL were withdrawn at time intervals and were replenished with an equal volume of fresh medium. All experiments were performed in triplicate and results are expressed as the mean ± S.E. of cumulative fluoride (%) at given sampling time.

Scanning electron microscopy

The structure of gels was studied by scanning electron microscopy (SEM). Gels were freeze-fractured in liquid nitrogen, put over an aluminum stub and subsequently lyophilized. All the samples were examined using an acceleration voltage of 15 kV, in a Phenom ProX microscopy.

Rheological measurements

Rheological measurements were carried out using a Haake RheoStress RS80 rheometer (Haake Instruments Inc., Paramus, NJ, USA). Strain and frequency sweeps were carried out to compare rheological behavior of CS/GP-NaF hydrogels exposed to different conditions using parallel plate geometry cell (20 mm) with a gap between plates of 1 mm (Soares et al., 2014). The temperature (37 °C) of the lower plate was maintained by circulating water from a water bath. Measurements were carried out in triplicate.

In vivo studies

Female Sprague–Dawley rats (110 – 150 g) were employed. Animals were isolated in individual metabolic cages with water and food *ad libitum*. Feces and urine were collected for 24 h. Fluoride dose was orally administered with an orogastric tube either in an aqueous solution or in a gel. Fluoride effects on plasma glucose, phosphorus and insulin levels were also studied. Blood samples (100 µL) were collected from the tail vein at designated time intervals. Fluoride, insulin, phosphorous and glucose levels were measured in plasma. In addition, fluoride content was determined in feces and urine as described below.

Determination of fluoride on in vitro assays

Fluoride was measured by direct potentiometry using an ion selective electrode ORION 94-09 and a reference electrode of Ag/AgCl connected to a digital-analogical converter. A five-point calibration curve was made between 1 and 100 ppm. Samples and standards were treated with a 10 % of an acetic acid-sodium acetate (2 M) buffer to adjust pH to 5.5 and the ionic strength.

Fluorine determination in biological samples

Urine concentration of fluoride was measured by direct potentiometry using an ion selective electrode ORION 94-09 and a reference electrode of Ag/AgCl. Plasma and ashes of feces were treated previous to the measurement. Acid labile fluorine was isolated from 50 µg of the sample by isothermal distillation and the sample treated with phosphoric acid 98 % w/w at 60 °C for 1 day. During this time, the hydrofluoric acid released from the sample is recovered by sodium hydroxide placed in the cup of the distillation chamber. Subsequently, the sodium hydroxide trap is adjusted to pH 5.5 with acetic acid 17.5 M. Standards in the range of 10^{-3} - 10^{-6} M were simultaneously processed. Results are expressed as ppm.

Insulinemia

Measurement of plasma insulin levels were carried out by radioimmunoassay using a commercial kit (RIA kit Rat insulin, Millipore Corporation, Billerica, MA, USA). The handling of radioactive material was carried out according to the standard regulations set by the Nuclear Regulatory Authority Argentina (RNA standard radiation safety 10.1.1).

Glycemia and phosphatemia

Glucose concentration and inorganic phosphorus were spectrophotometrically measured with a commercial kit (Wiener Laboratorios, Rosario, Argentina) in a Perkin Elmer lambda 11 spectrophotometer.

Results and discussion

Gel stability at different pH

Cylinder-shaped gels with or without NaF were formed in a 2.3 mm inner diameter tube. Subsequently, they were subjected to a stability study at 5 different pH (37 °C) until fully disaggregation. Samples at the lower pH (2) were entirely disaggregated after 30 min (fig.1). Gels kept at pH 3 started to dissolve after 10 min of the beginning of the assay and after 40 min were completely disaggregated. On the other hand at pH 4, 5, 6 and 7 gels were stable for more than 6 days. Fig. 1 shows the images that represent time evolution of the gels exposed to buffers of pH 2, 4 or 6. The pH of every media was measured before and after the assay and the difference was never greater than 0.1.

Infrared spectroscopy studies

Infrared spectra of CS, GP and gels without and with NaF (CS/GP and CS/CS/NaF respectively) were obtained before and after conditioning in distilled water for 24 h (CS/GP-24) (Fig.2a).

198 The strong and broad band in the range of 3600 – 3000 cm^{-1} centered at about 3400 cm^{-1} is
199 observed in CS, CS/GP, CS/GP/NaF and CS/GP-24h spectra is a result of the overlap of the –OH
200 and –NH stretching vibrations. GP spectrum also presents a broad band in the same range
201 which corresponds to –OH oscillations. This groups are involved in the formation of inter-
202 and/or intramolecular hydrogen bonds, which play an important role in the solution-to-gel
203 transition. Two bands at 1643 and 1591 cm^{-1} related to C=O stretching vibration in amide I and
204 NH_2 bending are present in CS spectrum. In CS/GP, CS/GP/NaF and CS/GP-24h the peak at
205 1591 cm^{-1} displays a slightly shift to 1550 cm^{-1}. This effect could be due the protonation of the
206 amino groups of CS or to electrostatics attraction between these protonated amino groups and
207 phosphate groups of GP (Deng et al., 2017; Zofia Modrzejewska et al., 2014). GP, CS/GP/NaF
208 and CS/GP spectra show a band at 980 cm^{-1} characteristic of $–PO_4^-$ group and a band at 780 cm^{-1}
209 that could be attributed to aliphatic stretching P-O-C. Is noticeable that the FTIR spectra
210 obtained for gels after been conditioned in water (CS/GP-24h) do not shows the peaks related
211 to phosphate which could indicate that GP was released to the medium, furthermore, in
212 CS/GP-24h only characteristics band of CS are present.
213 This assay was carried out to identify the presence of different functional groups and to
214 determine possible interactions between the components of the gel, mainly electrostatic
215 interactions between amine group of CS and fluoride. This interaction was reported as a
216 decrease in the intensity of the peak related to NH_2 at 1591 or 1550 cm^{-1} (Huang et al., 2012).
217 In this work, this effect was absent in samples with fluoride, then it could be suggested that
218 interactions between CS and fluoride were not present or could not be detected.

219 ***Scanning electron microscopy***

220 SEM micrographs of CS/GP/NaF and CS/GP-24h are showed in Fig.2b and Fig.2c respectively.
221 Before being exposed to water, hydrogels presented a closed structure with the presence of

small crystalline structures (Fig2.b). These crystals could be related to GP and NaF which were released to the media during the 24 h conditioning, for this reason could not be observed in Fig.2c. In addition, a more open structure was recognized after the immersion in water during one day.

Rheological measurements

The rheological behavior of CS/GP/NaF without treatment and exposed for 24 h to a buffer of pH 6 (CS/GP/NaF-pH6) and for 5 min to a buffer of pH 2 (CS/GP/NaF-pH2) was studied. Storage modulus (G´) is a measure of the energy stored and recovered per cycle of deformation (elastic component) and loss modulus (G'') is a measure of the energy dissipated or lost as a heat per cycle of deformation (viscous component) (Olivares et al., 2012). Complex viscosity (η*), G' and G'' were measured.

In the range of deformation studied (0.1 to 10 % at 1 Hz) all the samples showed to be in the linear viscoelastic regime. Frequency sweep tests were performed at a deformation of 2 % (within the linear viscoelastic region) in a range from 1 to 100 Hz. Results displayed in Fig.3 show that at low frequencies all the hydrogels have a gel-like behavior (G' greater than G´´) (Supper et al., 2014). According to Schorsch et al. (1997) CS/GP/NaF and CS/GP/NaF-pH6 can be considered as "true gel" due to its G'/G'' ratio is greater than 10. Despite CS/GP/NaF-pH2 showed a predominant elastic behavior, G'/G'' ratio is lower than 10 (Schorsch, C., Garnier, C. and Doublier, 1997). Complex viscosity in CS/GP/NaF and CS/GP/NaF-pH2 was at least one order of magnitude lower than CS/GP/NaF-pH6. This could be a consequence of a lower amount of GP in CS/GP/NaF-pH6 in comparison with the other two hydrogels. This component could reduce CS/CS interactions or acts as a plasticizer (Chenite, 2001; Supper et al., 2014).

At higher frequencies G´ and η* decreased, showing an inflection point. Then G´, G´´ and η* increased with the frequency (Baxter et al., 2008). This could be due to a breakage of the

hydrogel and a subsequent rearrangement of polymeric chains building a new microstructure. It is worth mention that this breakage point occurs at 10 Hz in CS/GP/NaF and CS/GP/NaF-pH6 while in CS/GP/NaF-pH2 this point take place at approximately 3 Hz. These results show that the structure of CS/GP/NaF hydrogel is weaker when is exposed to a high acidic medium.

Release experiments

In the gastrointestinal tract (GI), the pH of the environment changes from acidic in the stomach to around neutral in the lower tract. In order to evaluate CS/GP gels as extended release matrix, gels with two different concentration of NaF were immersed in 50 mL of medium at pH 4, 5, 6 or 7 and fluoride release over the time was recorded. The results were expressed as percentage of cumulative drug released over the time. Fig. 4 shows the profiles obtained at every condition assayed. Gels with 0.625 % w/w of NaF showed a sustained release for at least 6 h where between 55 and 70 % of the initial charge was released (Fig.4a). The exception was the gel at pH 5, which released about 90 % of the load (Fig. 4a). On the other hand, gels with a higher NaF concentration (2.5 % w/w) also showed a sustained release, but after 6 h only between 40 and 55 % of the API was released (Fig.4b).

Several works have developed fluoride release matrices; nevertheless, due the small size of the ion and its water solubility, long period of controlled release could not be reached (Keegan et al., 2012; Nguyen et al., 2017).

In vivo studies

Pharmacokinetic of fluoride

Effects of oral administration of fluoride have been widely reported (National Research Council, 2006). In the present work, in vivo pharmacokinetic studies were performed to contrast the effect of fluoride administration by a gel (CS/GP/NaF, API concentration equal to

0.625 % w/w) in comparison to a water solution (NaF solution). The given dose was 0.84 mg API / 100 g of body weight). Fasted female Sprague–Dawley rats were used for this study. Enzymatic content and pH change over the intestinal tract, in such nutritional condition, average rat stomach pH was reported to be between 3 and 4. Meanwhile, intestinal pH is approximately 6.6(McConnell et al., 2008). Gels showed to be stables and fluoride release rate is slow in the range of pH between 4 and 7; however, some variables that were not studied *in vitro*, as enzymatic CS degradation, could affect the behavior in vivo. A visual summary of in vivo assays are display in Fig.5.

Fluoride blood concentration was followed for 24 hours after treatments administration. In every rat studied, basal fluoride content was greater than expected, but at the end of the assay (24 h after of fluoride administration) this value was close to 0 for all the animals in both treatments.

In the first 2 h fluoremia showed to be similar between treatments, nevertheless, after 3 h considerable differences appeared between them (Fig.6). Animals treated with a NaF solution presented greater fluoride blood content than those which received CS/GP/NaF, 3.62 ± 1.49 ppm and 0.68 ± 0.19 ppm respectively. In both cases, fluoremia levels started to decay until reach a value close to 0 which remain constant for at least 24 h.

Fecal and urinary excretion of fluoride

Fluoride metabolism has been studied in many works(National Research Council, 2006). After an oral dosage, this ion is absorbed in the gastrointestinal tract or excreted in feces. Once absorbed, fluoride is cleared from blood through two mechanisms, uptake by bone or excretion in urine. In order to compare treatments, feces and urinary excretion were collected for 24 h and total fluoride content was determined. Results were expressed as the ratio between fluoride content and the amount of fluoride given to each animal.

Fig.7 shows urinary fluoride excretion ratio in animals treated with CS/GP/NaF and a NaF solution. Excretion was significantly lower in rats that received the drug in the gel in comparison to the administration in the solution (unpaired Student´s t- test $p<0.05$). It should be noted that gel treated animals showed a lower dispersion on excretion/dose in comparison to those treated with NaF solution (0.13 vs 0.49).

Fecal fluoride excretion was also studied and there was no difference between both treatments (unpaired Student´s t- test $p> 0.05$). An explanation could be that the fluoride absorption at the GI tract when the API is given in the gel is similar to the absorption when provided in the solution; in consequence non absorbed and excreted fluoride is similar between treatments.

Insulinemia and glycemia

It has been reported that fluoride has effects on serum glucose regulation by inhibition on insulin secretion. These effects were studied by comparing plasma insulin and glucose levels before and after an hour of oral administration of fluoride, where insulin levels decrease significantly and consequently an increase of glycemia were observed(Menoyo et al., 2005). In the present work this effects was studied by measuring insulin levels before and after 60 min of API dosage (2.5 mg NaF / 100 g of body weight). A comparison of the values of insulinemia and glycemia after and before API administration was done (Table 2). As expected, in those animals where fluoride was provided in an aqueous solution, insulin level was significantly diminished ($p<0.05$). On the contrary, this effect was not observed in rats treated with the gel. In order to evaluate if the difference in fluoride administration also affects glycemia, glucose blood levels were also determined. Although animals treated with a fluoride solution showed an increase in glycemia ($p<0.05$), glucose blood levels in gel treated animals showed no difference with basal values ($p>0.05$). This effect is concordant with those descript by Menoyo

et al(Menoyo et al., 2005). Probably, fluoride slow release from CS/GP/NaF collaborates to keep fluoremia levels under a threshold that helps to avoid insulinemia decrease and the resulting increase in glucose blood concentrations.

Phosphatemia

Di Loreto et al (Di Loreto et al., 2006) observed that animals treated with a sodium fluoride dose between 1.26 and 3.40 mg/ 100 g of body weight showed an increase in plasma phosphate levels after an hour of the administration. Chronically fluoride treated animals showed a decrease in bone phosphorus which is related to the increase in phosphatemia. In the current work a comparison of the values of phosphatemia after and before API administration (2.5 mg NaF / 100 g of body weight) was done. When fluoride aqueous solution was administered, variation in phosphatemia was greater than 0($p<0.05$); similar results were obtained for Di Loreto et al. When fluoride was provided in a gel, no significant differences were observed in plasma phosphate levels after and before the treatment (Table 2).

Fluoride is a small highly water-soluble ion which is used in the treatment of osteoporosis but presents some side effects when is given in high doses. In addition, due to its small size and hydrofilicity reach a controlled release in a water media is a challenge. CS/GP hydrogel has been proposed in the current work as a matrix to reach a sustained released of this API in order to be orally administrated with the consequent reduction on dose dependent undesirable effects.

In the present paper is showed that, even though relatively high fluoride charge was reach (0.625% - 2.5%), CS/GP/NaF hydrogels showed a sustained release in a wide range of pH for at least 6 h without a burst effect. In contrast, CS containing fluoride particles developed in other works showed a marked rapid release was reached in the first 120 minutes. Keegan et al, observed a rapid release of fluoride from spray dried CS-microparticles. These authors proposed that dried particles quickly swelled and fluoride situated near to the surface was fast

342 released to the medium. The remaining fluoride was slowly released due to the formation of
343 an outer gel phase that reduced the hydration rate in the center of the particles and increased
344 the diffusion path length(Keegan et al., 2012). Nanoparticles manufactured by Nguyen at al.
345 did also show a rapid release which can be partially explained by the rapid diffusion of the API
346 through the matrix and a high surface/volume ratio(Nguyen et al., 2017). Sustained release
347 observed in CS/GP/NaF hydrogels could not be associated to the previously proposed
348 electrostatic binding between fluoride and CS because of the absence of evidence in the
349 infrared spectroscopy studies (IR) carried out in this work. CS/GP/NaF hydrogels were not
350 dried before the in vitro release studies and gels did not swell significantly, so a hydration
351 process cannot explain the release rate. In addition to a low surface/volume ratio, the
352 diffusion rate through the hydrogel matrix is probably decreased due to the presence of
353 channels formed by the gel mesh that increase the matrix tortuosity.

354 In this work API effects on animals were also studied, after fluoride administration in a water
355 solution or in a CS/GP hydrogel. Fluoride fecal excretions results showed that values obtained
356 in animals treated with a hydrogel were not higher than those obtained in animals treated
357 with a NaF solution. This result indicates that GI absorption of fluoride when was given in a
358 CS/GP hydrogel presented the same magnitude than when was given in a water solution. In
359 addition, lower urinary fluoride excretion and fluoremia level observed in CS/GP/NaF
360 hydrogels treated rats suggest a higher fluoride accumulation in bone tissue.

361 Insulinemia, glycemia and phosphatemia results suggest that previously reported fluoride
362 negative effects are diminished when is given in a hydrogel. Since fluoride is trapped in a slow-
363 release matrix, the absorption rate is lower leading to lower values of fluoremia which are the
364 responsible for the disturbance of glycemia, insulinemia and phosphatemia. A remarkable

aspect observed in this work is that no changes in health and behavior were observed in treated animals after the application of the API in a solution or a hydrogel.

Further studies should be carried out to confirm the effects of the API applied in a hydrogel, for example the administration of the API in a CS/GP hydrogel and in a water solution for long periods of time analyzing different bone characteristics such as the fluoride content and mechanical properties.

The CS/GP hydrogel showed to be effective at controlling fluoride release. Hence its use as a platform to control the delivery of small and highly water soluble API for gastrointestinal tract application could be proposed. Future studies to evaluate different dosage forms, for example the use of the gel as a filler of soft capsules could also be carried out.

Conclusions

In this paper the feasibility of using CS/GP hydrogel as a matrix to gastrointestinal controlled release of fluoride was studied. This matrix showed to be stable over a long period of time (more than 48 h) in a wide range of pH (from 4 to 7). IR and SEM results indicated that remaining three-dimensional networks of the hydrogel after 48 h of being exposed to water is mainly composed of CS. Fluoride release from this matrix was sustained for at least 6 hours and without the presence of the typical burst expected for small highly hydrophilic molecules such as fluoride.

In vivo studies were conducted to compare the performance of CS/GP/NaF hydrogel with NaF solutions. Fluoremia after a fluoride dosage were similar in both treatments with the difference that when the API was given in a solution a peak appears after 3 h of received the treatment. Taking into account that gastrointestinal API absorption was similar in both treatment and fluoride urinary excretion was lower in CS/GP/NaF animals could be suggested

388 that API dosage in a hydrogel increase fluoride bone absorption. In addition CS/GP hydrogel
389 help to avoid some undesirable side effect related to high fluoride doses.

390 Finally, it is worth mention that CS/GP hydrogel can be an interesting biocompatible
391 formulation intended to control the release of small hydrophilic drugs in the gastrointestinal
392 tract.

393 ## Acknowledgements

394 The authors thank CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas,
395 Argentina) and UNL (Universidad Nacional del Litoral, Argentina) for the financial support.

396 **Declarations of interest:** none

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Figure captions

Fig. 1. Images of time evolution of CS/GP (top) and CS/CS/NaF (bottom) hydrogels exposed at different pH.

Fig.2. Hydrogel characterization: a) IR spectra. SEM images of CS/GP/NaF (b) and CS/GP-24h (c).

Fig.3. Mechanical spectra of CS/GP/NaF (a), CS/GP/NaF-pH6 (b) and CS/GP/NaF-pH2 (c).

Fig.4. Fluoride release profiles from CS/GP/NaF hydrogels with 0.625 % (a) and 2.5 % w/w of NaF (mean ± E.D., n=3).

Fig.5. Schematic representation of in vivo assays.

Fig.6. Fluoremia-time profiles obtained after a fluoride treatment with a NaF solution or a CS/GP/NaF hydrogel (n = 8).

Fig.7. Urinary fluoride excretion ratio in animals treated with CS/GP/NaF and a NaF solution (n = 8).

Tables

Table 1. Composition for 1000 ml buffer solution.

Table 2. Variation in insulinemia, glycemia and phosphatemia in animals treated with a NaF solution or CS/GP/NaF.

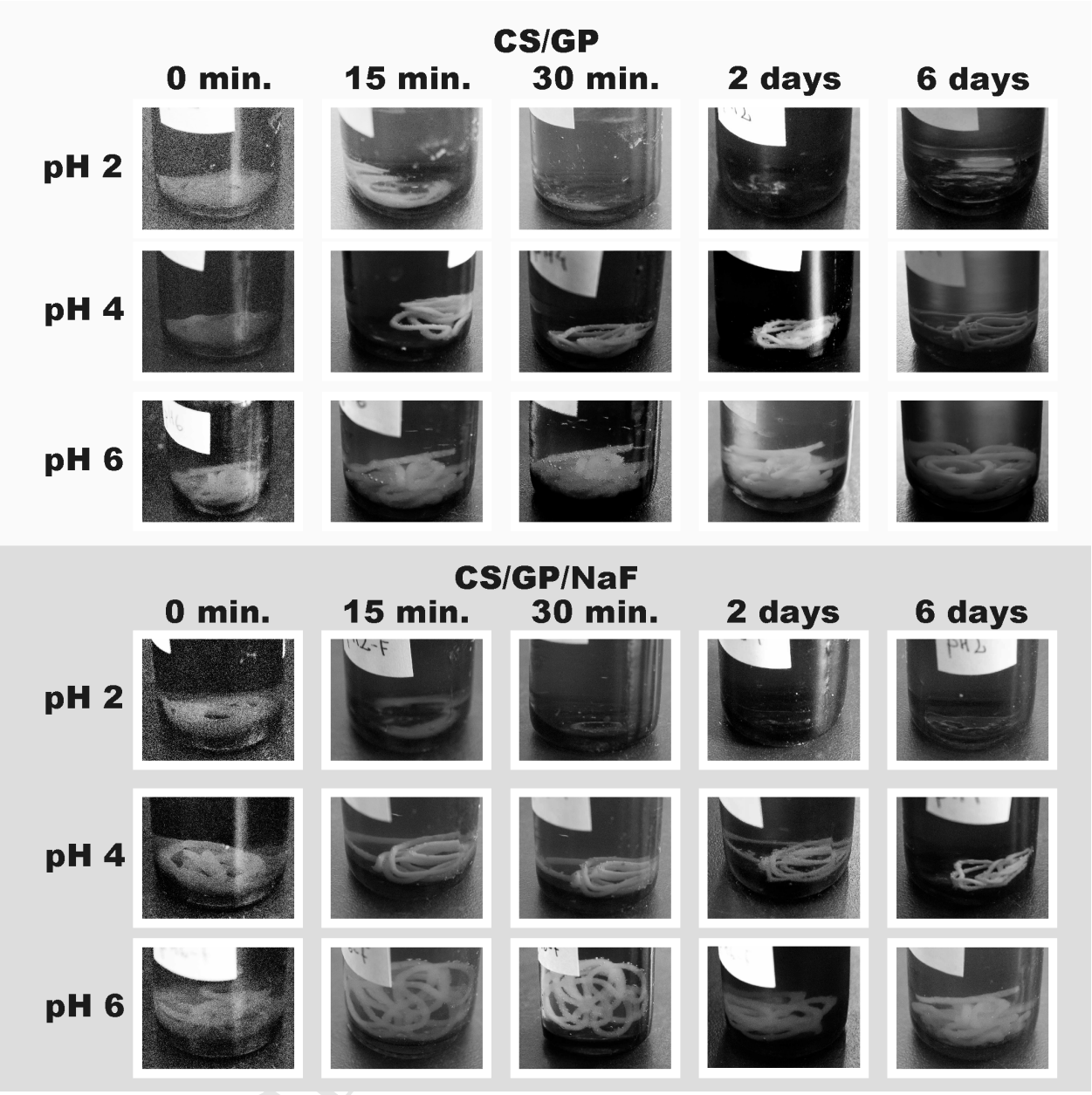
Table 1. Composition for 1000 ml buffer solution

pH	KCl (g)	$C_8H_5KO_4$ (g)	HCl 0.1 M (ml)	KH_2PO_4 (g)	NaOH 0.1 M (ml)	water
2	3.725	-	130	-	-	q.s.
3	-	10.21	223	-	-	q.s.
4	-	10.21	1	-	-	q.s.
5	-	10.21	-	-	226	q.s.
6	-	-	-	6.81	56	q.s.
7	-	-	-	6.81	291	q.s.

Table 2. Variation in insulinemia, glycemia and phosphatemia in animals treated with a NaF solution or CS/GP/NaF.

	treatment	*mean*	*s.d.*	*p.value*
insulinemia	NaF solution	-33.31	28.62	0.03
(ng/l)	CS/GP/NaF	53.83	39.88	0.02
glycemia	NaF solution	0.11	0.08	0.02
(g/l)	CS/GP/NaF	0.01	0.13	0.46
phosphatemia	NaF solution	1.97	1.78	0.035
(mg/dl)	CS/GP/NaF	-0.88	2.07	0.19

Results were considered statistically significant if p<0.05.


CS/GP
0 min.
15 min.
30 min.
2 days
6 days
pH 2
pH 4
pH 6
CS/GP/NaF
0 min.
15 min.
30 min.
2 days
6 days
pH 2
pH 4
pH 6