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PECTIN HYDROGELS pH STABILITY AS AFFECTED BY METHACRYLIC GRAFTING TO LOW METHOXYL PECTIN STRUCTURE

EFECTO SOBRE LA ESTABILIDAD AL pH DE HIDROGELES DE PECTINA POR LA ADICIÓN DE METACRILATO A LA ESTRUCTURA DE PECTINA DE BAJO METOXILO

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Abstract

Pectin hydrogels have interesting properties for pharmaceutical and food industries; nevertheless, pH sensitivity is one major drawback. The aim of this research was to evaluate the effect of methacrylate grafting on the structure of low methoxy citrus pectin (P), its gelling ability and pH sensitivity. 1% (w/v) P in phosphate buffer (pH 7) solution was mixed with methacrylic anhydride (1:2) (MP). FT-IR spectra (1737, 1617 and 949 cm^{-1}) suggested an efficient grafting. MP gels had larger pores and were 2.46 times stronger than P gels. Gellation of 3% (w/v) pectin dispersions with 10 mM $CaCl_2$ were photocrosslinked with 10% (w/v) Irgacure^{TM} 2959 (265 nm UV). Bovine serum albumin (BSA) was incorporated into gels by diffusion and diffusion coefficient (D_m) was calculated. At pH 1.5, BSA release from P gels was complete after 1 h of exposure, instead, 5% of BSA was released from MP gels after 7 h of exposure and a D_m value of $2.08\times10^{-7} \pm 1.2\times10^{-8}$ $cm^2{\cdot}s^{-1}$ was inferred. At neutral pH, P and MP gels dissolved into medium tested. Grafting pectins with methacrylic groups and combining photocrosslinking and ionic gelation allowed getting MP gels that overcome acidic pH sensitivity and improved the controlled release capacity.

Keywords: Pectin, photo-crosslinking, gelation, diffusion, pH sensitivity.

Resumen

Hidrogeles de pectina son de interés para industrias farmacéuticas y alimentarias, la sensibilidad al pH es un inconveniente. Se evaluó el efecto de adicionar metacrilato en la estructura de pectina cítrica de bajo metoxilo (P) sobre su capacidad gelificante y sensibilidad al pH. Se mezcló P al 1% (p/v) en búfer de fosfato (pH 7) con anhídrido metacrílico (1:2) (MP). El espectro FT-IR (1737, 1617 y 949 cm^{-1}) evidenció la modificación. Geles MP tienen poros más grandes y 2.5 veces más fuertes que geles P. Geles de pectina al 3% (p/v) inducidos con $CaCl_2$ 10 mM se sometieron a foto-entrecruzamiento con Irgacure^{TM} 2959 10% (p/v) (265 nm UV). Se incorporó suero de albumina de bovino (BSA) por difusión y se determinó el coeficiente de difusión (D_m). A pH 1.5, la liberación de BSA de geles de P fue completa después de 1 h, en cambio, 5% fue liberado de MP después de 7 h (D_m $2.08\times10^{-7} \pm 1.2\times10^{-8}$ cm^2s^{-1}). A pH neutro, los geles P y MP se disolvieron. Adicionar metacrilato a la estructura de pectina y combinar gelificación iónica y foto-entrecruzamiento permitió obtener geles MP con resistencia a pH ácidos y con mejor capacidad de liberación controlada.

Palabras clave: Pectina, foto-entrecruzamiento, gelificación, difusión, sensibilidad al pH.

1 Introduction

Pectins are composed mainly of galacturonic acid units (Wang *et al*., 2014) where carboxyl groups may be naturally esterified by methyl groups or can react with ammonia to produce carboxamide with "branched" regions containing neutral sugars (Nollet and Toldrá, 2012). The gelling capacity is one of the basis for applications of pectin in food, beverage (Chasquibol *et al*., 2008), cosmetic (Chen *et al*., 2015) and biomedical industries (Cabrera *et al*., 2011; Sadeghi, 2011; Wong *et al*., 2011; Zhang *et al*., 2015).

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Pectins with the degree of methoxylation lower than 50%, classify as low methoxy (LM) pectin (Ström *et al*., 2007), contain sufficient unesterified groups to gel with calcium ions, which acts as a bridge between pairs of the carboxyl groups of pectin chains (Farris *et al*., 2009).

Nevertheless, the stability of such gels versus low pH values has been reviewed extensively elsewhere. In this regard, pectins have been combined with other natural or synthetic molecules to improve their properties as carriers for drug delivery to the colon, such chitosan (Bigucci *et al*., 2009) and poly (N-vinylpyrrolidone) (Fares *et al*., 2010). Unfortunately, the pectin/chitosan hydrogels with greater amount of pectin displays lower drug availability, because the formation of highly viscous hydrogels (Bigucci *et al*., 2009), and hydrogels from pectin grafted with poly(N-vinylpyrrolidone) show higher release at pH 5.5 due to porosity (Fares *et al*., 2010).

Chemical grafting with methacrylate groups has been previously reported (Khademhosseini *et al*., 2006; Maior *et al*., 2008; Rouillard *et al*., 2010). Maior *et al*. (2008) obtained films of LM pectin grafted with glycidyl methacrylate by photocrosslinking. The films have the potential for a drug oral administration system applied as a pharmaceutical coating, however, the films showed flaws in some regions as a result of the large relaxation of the polymeric chains. Besides, appropriate selection of reagents and reaction conditions may mitigate the undesirable coproducts from initiators containing acid functionality (e.g. methacrylic acid) by atom transfer radical polymerization (Hoare and Kohane, 2008; París *et al*., 2008).

The photocrosslinking seems an interesting alternative to improve the time control and gelling kinetic of hydrogels (Rouillard *et al*., 2010). In this regard, the use of chemical grafting with methacrylic groups could overcome the pH sensitivity of pectin hydrogels and act as a colon-targeted delivery system with its additional prebiotic benefit.

Thus, this work focuses on the combination of two gelling mechanisms, photocrosslinking and ionic interaction with Ca^{2+} in pectin, exploring the effects on textural and diffusional properties derived from methacrylic grafting on the structure of low methoxy citrus pectin.

2 Materials and methods

2.1 Materials

Commercial low methoxy citrus pectin (Grinsted® pectin LC950 Danisco) was used to graft pectin with methacrylic anhydride. All reagents were obtained from Sigma Aldrich.

2.2 Citrus pectin chemical modify using methacrylic anhydride

The commercial low methoxy citrus pectin (P) was chemically modified with methacrylic anhydride (MP) based on the methodology previously reported by Khademhosseini *et al*. (2006) and Rouillard *et al*. (2010). Briefly, LM citrus pectin at 1% (w/v) in phosphate buffer (pH 7) solution was prepared and mixed with methacrylic anhydride at 1:2 ratio. Low methoxy citrus pectin at 1% (w/v) in phosphate buffer (pH 7) solution was used as a control without modification. The precipitates were dissolved in 80% ethanol and placed at 8º C for 24 h. Then the solutions were filtered through 0.45 μm (Whatman) and dried with acetone at 25º C. Samples were powered and stored at 4° C.

2.3 Characterization of pectin grafted with methacrylic anhydride

2.3.1 Fourier transform infrared (FTIR) spectroscopy

Samples were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) using a Nicolet Protége System 400 E.S.P. FTIR Spectrometer, Thermo Scientific, Waltham Mauga. In short, Pectin 3% film with KBr was fabricated for each sample. Films were formed at 6000 psi pressure in a manually operated hydraulic press (International Crystal Laboratories, 12 Ton E-Z Press). FTIR spectra of the samples in the region of 4000 to 400 cm^{-1} were obtained. The data were plotted using Sigma Plot, Systat Software Versión 10.0.

2.3.2 Neutral carbohydrates composition

Neutral sugars were determined following the alditol-acetate methodology reported by Carvajal-Millan *et al*., (2007). The pectin was hydrolyzed with 9'2 N trifluoroacetic acid at 120° C for 2 h. The

reaction was stopped on ice and the extract was evaporated under air at 40° C. Sample was rinsed twice with 200 μL of water and resuspended with 1 mL of acid water. Then, the sample was filtered through 0.45 μm (Whatman) and analyzed by high-performance liquid chromatography (HPLC) using a Waters e2695 Milford, Ma, USA HPLC System and a Supelcogel Pb column (300×7.8 mm; Supelco, Inc., Bellefont, PA), eluted with 5 mM sulfuric acid (filtered with 0.47 mm, Whatman) at 0.6 mL min^{-1} and 50° C. Sorbitol was used as internal standard.

2.3.3 Pectin gelation by photocrosslinking

P and MP were gelled by ionic and photo-crosslinking using 1-[4-(2-Hydroxyethoxy)phenyl]-2-hydroxy-2-methyl-1-propan-1-one (Irgacure, IRG2959®) as an initiator; as previously reported by Rouillard *et al*. (2010) with slight modifications. Briefly, 10 mM $CaCl_2$ solution was prepared in 0.02% (w/v) sodium azide solution to prevent microbial contamination. 10% (w/v) IRG 2959 in 70% ethanol solution (10% IRG 2959-Ethanol) was prepared and used to trigger the crosslinking reaction. On the side, P and MP 3% (w/v) in phosphate buffer (pH 7) solutions were prepared. Briefly, 2.4 mL of each pectin solution of 3% were poured into a 30 mL beaker (30 mm diameter), 0.6 mL of 10 mM $CaCl_2$ was added by the beaker wall, the solutions were kept at 8° C. After 24 h, 900 μL of 10% IRG 2959-ethanol were added. The solutions were exposed to UV radiation (265 nm) for 5 min. The hydrogels were kept at 8° C during 48 h to allow their maturation.

2.4 Characterization of pectin hydrogels

2.4.1 pH sensitivity test

The stability test was carried out at three pH conditions, pH =1.5, 7 and 13 during 48 h. 2 mL of 1 N HCl, 5 M NaOH and 0.15 M sodium phosphate (pH 7) solutions were placed in 5 mL vials respective. In each solution, a 2 cm *times* 1 cm slice of each gel was added. The samples were observed at 2, 24 and 48 h for integrity.

2.4.2 Protein release

Protein release from gels was studied using the protein of bovine serum albumin (BSA) as a model molecule according to Carvajal-Millan *et al*. (2005). Briefly, 3% (w/v) pectin gels were prepared into a 30 mL beaker (30 mm diameter). Protein of BSA (67 kDa) was loaded in the gels and used. The protein solution (500 μL, 10 mg/mL) in 0.05 M citrate-phosphate buffer pH 5 was placed on the surface of the gels. Protein was allowed to diffuse into the gels for 12 h at 25° C and 90 rpm tangential rotation. The un-loaded protein on the surface was recovered by rapidly rinsing twice with 6 mL of 0.02% (w/v) sodium azide solution for further quantification.

The assay was performed at 25° C and 90 rpm tangential rotation for 9 h. Solutions to diffusion were HCl at pH 1.5 during 7 h followed by phosphate buffer solution at pH 7 during 2 h. Solutions were replaced every half hour. At each period, 1 mL was sampled for protein by Bradford's assay (Bradford, 1976). The BSA release was tested to Fick model (Ec. 1):

$$\frac{M_t}{M_0} = kt^n \tag{1}$$

where M_t is the accumulated mass of protein released at time (t) and M_0 is the mass of protein in the gel at time zero, k is the kinetic constant, and n is the dissolution exponent characteristic of the system (Marquez-Escalante *et al*., 2013). There are three recognized mechanism for water transport in polymer compounds, including Fickian diffusion ($n \leq 5$), relaxation-controlled ($n \geq 1$) and anomalous transport ($0.5 < n < 1$) (Adhikary *et al*., 2008; Li *et al*., 2014).

Protein release from gels was characterized by calculating an apparent diffusion coefficient (D_m). This D_m was estimated from the release kinetics curve, fitted by using an analytical solution of the second Fick's law (Ec. 2), which gives the solute concentration variation as a function of time and distance (Crank, 1979) according to Carvajal-Millan *et al*. (2005).

$$\frac{M_t}{M_0} = (4L^{-1})(D_m t\pi^{-1})^{0.5} \tag{2}$$

where M_t is the accumulated mass of protein released at time (t), M_0 is the mass of protein in the gel at time zero, as mentioned before, L is the sample thickness (0.3 cm) and D_m is the apparent diffusion coefficient. The D_m value can be determined by plotting the relative solute mass released ($M_t M_0^{-1}$) at the time (t), versus the square root of time (t) according to Carvajal-Millan *et al*. (2005) if the coefficient is constant, and the sample is a plate with a thickness (L) with unit of measurement ten times smaller than the diameter of the glass o container. Therefore, the D_m was calculated from the linear part of $M_t M_0^{-1}(t)$ curves.

2.4.3 *Texture profile*

The textural test was made in a Texture Analyzer, Model TA XT2, Stable Micro Systems, Godalming, UK. Gels were deformed by compression at a constant speed of 1.0 mm s^{-1} for 5 s to a distance of 4 mm from gel surface using a cylindrical plunger (diameter 25.4 mm). The peak height at 4 mm compression was called gel hardness; as previously reported (Carvajal-Millan *et al*., 2005). The data were analyzed in the Texture Expert software, Stable Micro Systems Ltd. Version 1.05.

2.4.4 *The microstructure of pectin gels*

Scanning Electron Microscopy (SEM) was used to analyze the microstructure of gels. Lyophilized gels were placed on a 13 mm silver tape, metal-shadowed with gold/palladium (60/40) (Sputter coater SPI-Module; West Chester, PA, USA), and mounted on a brass disk. The samples were analyzed using magnifications of 500 X and 1000 X under low vacuum using a JEOL JSM-5400LV scanning electron microscope (Peabody, MA, USA) at an acceleration voltage of 15 kV.

2.5 *Statistical analysis*

Samples were prepared and tested in triplicate. The results were expressed as a mean ± standard deviation. Data were subjected to one-way analysis of variance (ANOVA) following general model procedures. Comparison of sample means was performed by the Tukey's test ($P \leq 0.05$) with the SAS program (2005 version, SAS Institute, Cary, NC, USA).

3 Results and discussion

3.1 *Characterization of Pectin Grafted with Methacrylic Anhydride*

3.1.1 *Fourier transform infrared (FTIR) spectroscopy*

Molecular identity was analyzed by FT-IR spectrum and showed changes in the spectrum profile that suggest the addition of methacrylic to the pectin structure (Fig. 1). Spectra profiles were similar to previously reported grafting on low methoxy pectin (Maior *et al*., 2008; Villanova *et al*., 2015); as well as apple pectins (Vityazev *et al*., 2017).

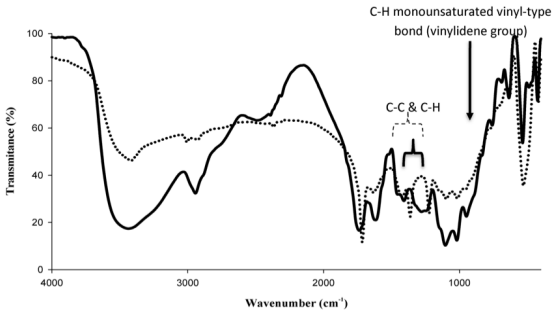

Fig. 1. FTIR spectrum from low methoxy citrus pectin (·····) and methacrylic grafted low methoxy citrus pectin (–).

Both samples showed bands around 1200-1000 cm^{-1} attributed to skeletal C−O and C−C vibrations of glycosidic bonds and a pyranoid ring, considered the "fingerprint" regions of polysaccharides (Vityazev *et al*., 2017).

FTIR spectrum of P displays a band at 3417 cm^{-1} attributed to O-H bond strength vibrations. A prominent band at 1715 cm^{-1} attributed to C=O bond strength vibrations (Ruiz *et al*., 2009). Peaks at 1440-1237 cm^{-1} attributed to asymmetric −C−C− stretching vibrations bonds and −*C*H groups or stretching vibration of methyl ester groups, all typical of pectin molecular identity as reported by Vityazev *et al*. (2017). In addition, changes in FT-IR spectrum profile illustrate the chemical grafted with methacrylic anhydride with a new peak appeared at 949 cm^{-1}, which strongly suggests the presence of the vinylidene group, attributed to C−H monounsaturated vinyl-type link that is characteristic of alkenes and detectable at 990-910 cm^{-1} (Vityazev *et al*., 2017). A structural feature derived possibly from the methacrylate group added to a C-6 position on galacturonic acid. Our results agree with those reported by Reis *et al*. (2006); Maior *et al*. (2008) and Rouillard *et al*. (2010).

In sum, spectrum profile shows the conservation of the main structure backbone of pectin. Additionally, changes in spectra profile and presence of vinylidene group suggest the incorporation of methacrylic moieties onto the backbone of pectin.

3.1.2 *Neutral carbohydrates composition*

The major neutral carbohydrates reported on the side chains structure of pectin are arabinose, galactose, glucose, mannose, and xylose (Nangia-Makker *et al*., 2002; Kaya *et al*., 2014).

Table 1. Neutral carbohydrates composition from low methoxy citrus pectin (P) and methacrylic grafted low methoxy citrus pectin (MP).

Neutral Carbohydrates	**P (%)**	**MP (%)**
Arabinose	21.98 ± 0.02^a	24.33 ± 0.25^a
Galactose	4.07 ± 0.14^a	5.48 ± 0.06^a
Glucose	0.93 ± 0.01^a	0.77 ± 0.01^a
Mannose	ND	ND
Xylose	ND	ND

[1] Values are expressed as mean ± standard deviation. Values followed by different lowercase in the same row are significantly different ($P \leq 0.05$, $n = 2$). ND = No detected.

Furthermore, these neutral sugars are found in branched regions, RGI, and RGII (Maxwell *et al*., 2016). In Table 1 the neutral carbohydrates of interest determined in this study are presented. Though citrus pectins reportedly contain little glucose, xylose, and mannose; in this study xylose and mannose were not detected. The higher content was in arabinose followed by galactose and glucose. Citrus pectins have little-branched regions than pectins from other sources (Carvajal-Millan *et al*., 2007; Urias-Orona *et al*., 2010; Wang *et al*., 2014; Wefers *et al*., 2015; Maxwell *et al*., 2016). In sum, the conditions and extraction method influence the neutral sugar profile (Maxwell *et al*., 2016). Moreover, commercial pectins show variation in neutral sugar content (Georgiev *et al*., 2012). In this research, P and MP displayed similar neutral sugar content between them ($p \leq 0.05$), as occurred in a previous report (Nangia-Makker *et al*., 2002). Our results confirm that grafting with methacrylate does not affect neutral sugar content in the overall.

3.2 Characterization of pectin hydrogels

3.2.1 pH sensitivity test

Gelling capacity of pectins is useful to make hydrogels for drug delivery systems; however, pectin hydrogels have a pH sensitivity feature (Cabrera *et al*., 2011; Sadeghi, 2011); they tend to lose their stability by contact with gastric juices, causing premature release of hauled cells or substances (Cabrera *et al*., 2011). In this regard, pectin was grafted with methacrylic groups to modify its structure and counteract the pH sensitivity of its gels. P gels lost their stability and were de-gelled after 2 h of exposure under neutral and acidic environments. In contrast, P gels subjected to alkaline conditions decreased 25% and 75% of their original size after 24 h and 48 h, respectively, aside of changing its color after 48 h from translucent light beige to transparent white.

Low methoxy pectin gels are the result of ionic linkages via calcium bridges between two carboxyl groups of pectin chains in close contact. The pH must be higher than that of the pKa of carboxylic groups to allow them to take part in ionic interactions. At pH values below the pKa, the carboxylic groups are unionized, which reduces the attraction among pectin with water molecules and calcium ions, and diminishes electrostatic repulsion between pectin molecules, all which triggers hysteresis and pectin aggregation (Sriamornsak, 2003). In contrast, at neutral pH exposure, the pectin chains turn negatively charged, which causes a strong repulsion between the chains of pectin and the stability of the hydrogel structure is lost (Fares *et al*., 2010; Gerola *et al*., 2016).

MP gels lose their stability and were dispersed after 2 h of exposure to neutral pH; conversely, at acidic and alkaline media during 24 h remained firm but changed its color, from translucent light beige to light brown. Finally, after 48 h the size of gels decreased around 15%. This behavior at those pH values is of interest in biomedical applications (Fares *et al*., 2010). Methacrylic anhydride was joined to hydroxyl and carboxyl groups of pectin resulting in MP, forming gels by polymerization through a process of photocrosslinking using IRG-2959, a photo-initiator that triggers a chain reaction of free radicals by UV light (Khademhosseini *et al*., 2006). Due to covalent nature of the methacrylic groups, resulting MP crosslinking was not affected by acidic and basic pH exposure. However, when the gel was exposed to neutral medium the combined effect of methacrylic groups structure and Ca^{2+} might be disrupted by the change in COO^- charge to neutral opening the polysaccharide net-like structure lowering the sensitivity of MP gels. Furthermore, some hydrolysis might have taken place, with the subsequent disaggregation observed in vitro assays. In vivo tests should be assessed to determine if the same

process occurs in living animals. In our study, LM pectin grafted with methacrylic groups reduced the pH sensitivity of control LM pectin gels.

3.2.2 Protein release

Assessment of drug profile release is important to determine the potential as delivery matrix systems. In this regard, protein release profiles from P and MP gels were determined using the protein of BSA (67 kDa) as a model molecule (Table 2 and Fig. 2). Protein loading was highly efficient (2.49 and 2.40 mg BSA mL^{-1} gel) and comparable to previous reports for arabinoxylan gels (Carvajal-Millan *et al*., 2005). Similarly, the transport of protein into the gel is a function of the protein molecular weight and the gel features like polymer concentration and crosslinking degree. P and MP gels were exposed at acidic (pH 1.5) and neutral (pH 7) media. Interestingly, P gels were dispersed after an hour of exposure at pH 1.5, and half an hour of exposure at pH 7. Conversely, MP gels were dispersed afterwards, when exposed to neutral pH medium for 1.5 h. BSA release from P gels was complete at pH 1.5 after 1 h of exposure due instability. Conversely, only, 5.05±0.29% of BSA loaded MP gels was released after 7 h of exposure at acidic medium; the greatest amount (94.41±0.63%) of BSA released was recorded for 1.5 h of exposure to neutral pH medium.

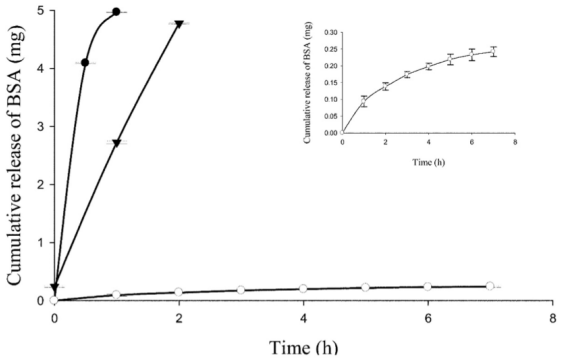

Fig. 2. Cumulative release of BSA from low methoxy citrus pectin (P) and methacrylic grafted low methoxy citrus pectin (MP) gels as a function of time. Closed circle (●) P gels after exposure at pH = 1.5; triangle (▼) and open circle (○) MP gels after exposure at pH = 7 and pH = 1.5, respectively.
Inset: Cumulative release of BSA from MP gels after exposure at pH = 1.5 as a function of time.

These results are comparable to Gerola *et al*. (2016), who developed hydrogels by modified Gum Arabic with glycidyl methacrylate that are more sensitive to neutral pH inducing the maximum amount of the compound released, contrary to the previous reports by Santacruz-Vázquez *et al*. (2013), who found that nano-capsules by Arabic Gum without modification promote a faster and higher compound release at acidic pH than neutral pH, such occurs with P gel without modification.

Table 2. Diffusion kinetics for BSA from low methoxy citrus pectin (P) and methacrylic grafted low methoxy citrus pectin (MP) gels at pH 1.5 and pH 7.0.

	P gels of 5 mg	**MP gels of 5 mg**
Loaded BSA (mg)	4.97 ± 0.008^{a}	4.79 ± 0.005^{b}
Loaded BSA (%)	99.41 ± 0.10^{a}	95.80 ± 0.16^{b}
Loaded BSA (mg BSA /mL gel)	2.49 ± 0.002^{a}	2.40 ± 0.004^{b}
Medium at pH 1.5		
Release BSA (%) after 1h	99.93 ± 0.04^{A}	ND
Release BSA (%) after 7h	ND	5.05 ± 0.29^{C}
Change of medium (pH 7)		
Release BSA (%) after 8h	ND	56.67 ± 0.78^{B}
Release BSA (%) after 8.5h	ND	99.46 ± 0.63^{A}
D_m (cm^2/s) at pH 1.5	NF	$2.08\times10^{-7}\pm1.2\times10^{-8}$
D_m (cm^2/s) at pH 7.0	NF	NF

[1]Values are expressed as mean ± standard deviation. Values followed by different lowercase in the same row are significantly different ($P \leq 0.05$, $n = 3$). Values followed by different capital letter in the same type of determination (release of BSA and D_m, respectively) are significantly different ($P \leq 0.05$, $n = 3$). ND: Non dectected. NF: Non Fickian behavior.

Table 3. Fick model n and k values for methacrylic grafted low methoxy citrus pectin (MP) gels at 3% (w/v) after submission at acidic medium (pH 1.5) for 7 h.

Parameter	Value
k	0.0027±0.0004
n	0.4929±0.024
r^2	0.9960

Protein released decreased over time (Fig. 2), however only the values from MP gels exposed at acidic medium showed a fickian behavior. The n and k values of the Fick model for MP gels at pH 1.5 were calculated (Table 3). The n value was lower to 0.5, indicating that BSA release was due to a Fickian mechanism as the results for diffusion curves of various composites ($n = 0.3 - 0.49$) reported by Adhikary *et al*. (2008) and Li *et al*. (2014) and water extractable arabinoxylan (WEAX) hydrogel ($n = 0.37$), and contrary to no Fickcian mechanism from WEAX aerogel ($n = 0.54$) described by Marquez-Escalante *et al*. (2013). Additionally, lower value of k indicates longer time required to diffusion (Adhikary *et al*., 2008; Li *et al*., 2014) that is in accordance to the results in this research for the BSA release from MP gels exposed to acidic medium. In MP gels lower diffusion is observed, as expected for the concomitant methacrylic-derived crosslinking points, adding to the assembled ionic crosslinking. A linear relationship between cumulative release (M_t/M_0) of BSA and the square root of time were found from MP gels at pH 1.5 (Fig. 3), allowing the inference of apparent diffusion coefficient (D_m). Protein release occurred differently in each medium. Ionic interactions and polymerizations bind for methacrylic groups in the structure, and limits the diffusion of BSA; thus, around 5% of BSA was released from MP gels after 7 h of exposure at pH 1.5 and displayed a $D_m = 2.08\times10^{-7} \pm 1.2\times10^{-8}$ (Table 2) similar to the D_m value of BSA at similar conditions (1.5% w/v in arabinoxylans) calculated by Carvajal-Millán *et al*. (2005).

Additionally, the D_m value is lower than the diffusion coefficient of BSA in water ($D_0 = 6.3\times10^{-7}$ cm^2/s) obtained by Cole *et al*. (1998). When exposed to low pH values, the ionic crosslinking is disassembled and only methacrylic links support the gel structure with larger pores releasing the model BSA protein. Furthermore, based on microbiota degradation reports, we hypothesize that a third release event might be possible if the pH change is in combination with the colon microbiota enzymatic system.

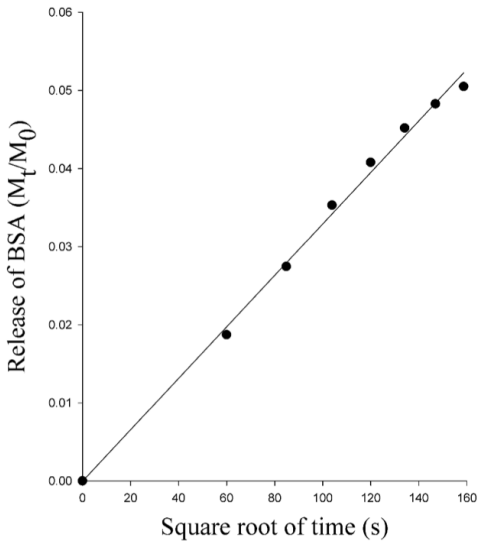

Fig. 3. Cumulative release from BSA from methacrylic grafted low methoxy citrus pectin (MP) gels as a function of root time at pH = 1.5.

Eventually, a three-stage release matrix could be developed for biomedical applications. The methacrylation of polysaccharides is an effective method to make hydrogels with an effective prevention of erosion/degradation (Gerola *et al*., 2016). In addition, the pectin has shown resistance to proteases and amylases from the upper gastrointestinal tract, while selective digestion by the microbiota present in the colon (Wong *et al*., 2011). In theory, the modification of pectin could allow the fabrication of controlled delivery systems to a specific area, providing an advantage over free drugs for prevention of side effects of drugs on healthy patients (Minko, 2004).

3.2.3 Texture profile

In addition, a textural test was carried out to obtain further information on the properties of the hydrogels. The firmness of gels made with pectin depends mainly on the molecular structure and molecular weight, among other features (Fraeye *et al*., 2010; Eshtiaghi and Kuldiloke, 2013). Commercial pectins have structural variations owing to the extraction process and treatments (Beaulieu *et al*., 2001). Additionally, textural properties are related to the extent of the crosslinking reaction (Rodríguez-Huezo *et al*., 2011). Table 4 shows the results of the textural profile test of P and MP gels; interestingly, both samples displayed similar texture values ($p \leq 0.05$).

Table 4. Texture analysis from low methoxy citrus pectin (P) and methacrylic grafted low methoxy citrus pectin (MP) gels at 3% (w/v) without any medium addition and after submission at acidic medium (pH 1.5) for 7 h.

Textural	Without Any Medium Addition		pH 1.5 for 7h	
Characteristics	P gels	MP gels	P gels	MP gels
Hardness (N)	1.23 ± 0.25^{b}	2.63 ± 0.36^{a}	ND	1.74 ± 0.31^{ab}
Cohesiveness	0.41 ± 0.02^{a}	0.31 ± 0.04^{a}	ND	0.31 ± 0.06^{a}
Springiness (mm)	0.93 ± 0.05^{a}	0.82 ± 0.09^{a}	ND	0.88 ± 0.01^{a}
Chewiness (Nmm)	0.73 ± 0.54^{a}	0.57 ± 0.65^{a}	ND	0.71 ± 0.17^{a}
*(N/mm)	1.31 ± 0.65b	3.22 ± 0.08^{a}	ND	1.98 ± 0.34^{b}

[1]Values are expressed as mean ± standard deviation. Values followed by different lowercase in the same row are significantly different ($P \leq 0.05$, $n = 2$). *Force (N) required by the sample for 1 mm depth after compression. ND = No detected.

With the aim of objective comparison of the hardness from gels, the force (N) required by the sample to recover 1 mm of distance after compression was calculated for each sample. It was found that MP gels are 2.46 times stiffer than P gels ($p \leq 0.05$). Eshtiaghi and Kuldiloke (2013) found variations in pectin hydrogels hardness due to environmental conditions, concentration and kind of gelling material. Such occurs in this research, hardness differences between samples are attributed to pectin structures and their gelling mechanisms because of the chemical modification in MP structure. In this regard, chain interactions in MP gels could be closer than that of P gels; while the latter could have some random junction zones, leading to lower hardness (Fraeye *et al*., 2010).

Springiness or elasticity deduces the gel "rubberiness" perception in the mouth, and chewiness deduces the energy needed for masticating (Huang *et al*., 2007). Low methoxy pectin gels break down easily during mastication (Marshall and Vaisey, 1972) which has been used to an improved desirable textural characteristic in products as strawberry jellies with LM pectin (Ciurzyńska *et al*., 2015). This characteristic is in accordance with the results of this research. Both kinds of gels displayed low and similar springiness, cohesiveness and chewiness, indicating that they are easily broken down by an initial compression.

As previously mentioned, gels for colon targeted drug delivery must come into contact with gastric juices with enough firmness to maintain their structure and keep the drug protected until reaching the colon. In this regard, HCl (pH 1.5) was added to a set of P and MP gels, which were stirring (90 rpm) at environmental temperature during 7 h. After the acidic media exposure, the gels were texture analyzed and the results are shown in Table 4. P gels were disintegrated after 1.5 h of contact with the acid medium, thus, the texture profile could not be carried out. MP gels displayed a similar texture profile after and before the HCl addition, although, MP gel hardness decreased after acidic media exposure, consequently, MP gels require significantly lower force to recover.

Rodríguez-Huezo *et al*. (2011) found that calcium alginate capsules with gel core characteristic displayed higher hardness and chewiness, lower cohesiveness, and similar springiness than calcium alginate capsules with liquid core characteristic. Interestingly, P and MP gels in this research showed higher hardness and chewiness, lower cohesiveness, and springiness a behavior comparable to reports on gel and liquid core alginate capsules. Which support the gel characteristics, particularly by the MP gels, due the P gels were disintegrated after their exposure at acidic medium. Pectins can produce diverse types of gels with various maximum firmness, depending on the environmental conditions (Eshtiaghi and Kuldiloke, 2013), in this regard, it is important to mention that P and MP gels showed susceptibility to elevated temperatures (around 50° C). However, this could be not a problem for medical applications, due to corporal human temperature is around 36-37° C and environmental conditions seldom reach such levels.

3.2.4 The microstructure of pectin gels

Fig. 4 shows the micrographs obtained in SEM from pectin gels. The samples displayed differences in their internal structures. MP gels had bigger and less homogenous pores than P gels. Differences in internal structure in both types of gels explain the diffusion kinetics observed at pH changes described above. In theory, compact pH sensitive ionic crosslinking of unmethylated galacturonate residues would unwind, leaving only the pH stable covalent crosslinking of methacrylated bonding.

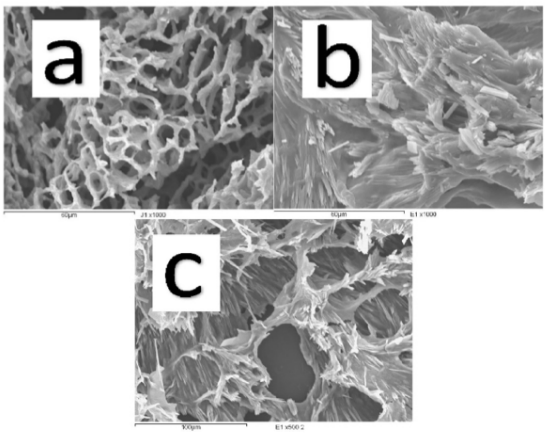

Fig. 4. SEM micrographs from low methoxy citrus pectin (a) and methacrylic grafted low methoxy citrus pectin (b and c) gels.

The latter structural changes would explain the higher release measurements recorded at different pH values. Internal structures of MP gels have large pores to trap and hold drugs; additionally, with an efficient acidic pH resistance, their internal nanostructured network would allow their pass through the gastrointestinal tract until reaching the colon. In fact, in colon environment with pH values around seven, hydrogels would hopefully tend to lose their stability, be biodegraded by microbiota, and release the bioactive compound. Conversely, P gels lost their stability at pH values similar to the stomach, which makes them prone to release the active compound in superior sections of the gastrointestinal tract. Furthermore, microbiota degradation suggests that a third release event may be possible. Eventually, a three-stage release matrix could be developed for biomedical applications.

Conclusions

Grafting pectins with methacrylic groups is an interesting strategy to overcome pH sensitivity of pectin gels. In this regard, the possibility of combining photocrosslinking and ionic gelation is very appealing to design new pectin hydrogels with potential as colon targeted drug delivery systems. The microstructure of low methoxy-methacrylated pectin benefits from the combination of ionic and covalent interactions between polymer chains rising new interesting matrices.

The authors declare no conflict of interest in the present study.

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Abbreviations

LM	Low methoxy
P	Commercial low methoxy citrus pectin
MP	Commercial low methoxy citrus pectin chemically modified with methacrylic anhydride
D_m	Diffusion coefficient
t	Time
M_t	Accumulated mass of protein released at time
M_0	Mass of protein in the gel at time zero
M_t/M_0	Relative solute mass released
L	Sample thickness (0.32 cm)
SEM	Scanning Electron Microscopy
BSA	Bovine serum albumin

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