

Injectable pectin hydrogels produced by internal gelation: pH dependence of gelling and rheological properties

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ABSTRACT

The production of injectable pectin hydrogels by internal gelation with calcium carbonate is proposed. The pH of pectin was increased with NaOH or NaHCO₃ to reach physiological values. The determination of the equivalence point provided evidence that the pH can be more precisely modulated with NaHCO₃ than with NaOH. Degradation and inability to gel was observed for pectin solutions with pH 5.35 or higher. Therefore, pectin solutions with pH values varying from 3.2 (native pH) to 3.8 were chosen to produce the gels. The increase of the pH for the crosslinked hydrogels, as well as the reduction of the gelling time and their thickening, was dependent upon the amount of calcium carbonate, as confirmed by rheology. Hydrogel extracts were not cytotoxic for L-929 fibroblasts.

On the overall, the investigated formulations represent interesting injectable systems providing an adequate microenvironment for cell, drug or bioactive molecules delivery.

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1. Introduction

In the fields of drug delivery and regenerative medicine, injectable biomaterials hold great promise, mostly due to the implantation by minimally invasive surgery and the possibility to avoid systemic administration (Kretlow, Klouda, & Mikos, 2007; Salgado, Sanchez, Zavaglia, Almeida, & Granja, 2012). In drug delivery, large drug or protein doses are usually required for the treatment of a specific site, due to the uptake of the active molecules by the surrounding tissues. The degradation or deactivation of drugs or proteins by enzymatic reactions may further lower their therapeutic efficacy. Injectable systems provide par-parenteral administration or localized injection to the affected site. Injectable materials as cell carriers for regenerative medicine share

the same advantages as those used in drug delivery and, in addition, are able to conform to the shape of a tissue defect, avoiding the need of specific scaffold prefabrication (Bidarra et al., 2011; Kretlow et al., 2007; Salgado et al., 2012).

Natural polymers possess highly organized structures, some of them containing functionalities capable of binding cell receptors, thus inducing cell adhesion (Cheung, Lau, Lu, & Hui, 2007; Swetha et al., 2010). Furthermore, anionic polysaccharides such as alginate, carboxymethyl cellulose, gellan, hyaluronic acid and pectin are good mucoadhesive materials (Lee, Park, & Robinson, 2000; Munarin, Tanzi, & Petrini, 2012), and therefore, as carriers, may prolong the residence and the exposure time of drugs, allowing their improved absorbance (Liu, Jiao, Wang, Zhou, & Zhang, 2008). In addition, the crosslinking of anionic polysaccharides, their pH and time of gelation can be controlled, leading to the fabrication of systems with tunable properties. The control of the pH plays a key role in tissue engineering and drug delivery. For drug delivery applications, the modulation of the pH of polysaccharide solutions may provide the binding of specific drugs, an optimized release profile and the activation/inactivation of the drugs. In the case of tissue engineering, maintaining the pH in the physiological

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range is required for the production of cytocompatible injectable systems.

Anionic polysaccharides carrying carboxylic groups exhibit specific physicochemical properties due to their ability to form ionotropic gels (Bidarra, Barrias, Barbosa, Soares, & Granja, 2010; Evangelista et al., 2007; Munarin, Tanzi, et al., 2012). Due to the inherent biocompatibility and soft tissue-like behavior of anionic polysaccharides, these biomaterials may be exploited to design injectable systems with slow crosslinking kinetics, allowing in situ gel-formation for injectable therapeutic delivery of drugs, proteins or cells (Gutowaska, Jeong, & Jasionowski, 2001). The crosslinking by intermolecular attraction with multivalent cations, such as Ca^{2+} or Al^{3+} , provides means of modulating viscoelastic properties of gels and, therefore, their injectability (Munarin, Petrini, Tanzi, Barbosa, & Granja, 2012; Munarin, Tanzi, et al., 2012).

Among negatively charged natural polymers, pectin is a very promising biomaterial. Pectin is a biocompatible anionic polysaccharide that constitutes 30% of plants cell wall (Harholt, Suttangkakul, & Scheller, 2010), widely used as thickener, gelling agent, stabilizer and emulsifier in food products (Tho, Kjøniksen, Nyström, & Roots, 2003). It is almost entirely composed of three polysaccharidic domains: homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). HGA is the major component of pectic polysaccharides and contains α -(1 \rightarrow 4)-D-linked galacturonic acids (1,4- α -D-GalA) that are partially methyl-esterified and sometimes partially acetyl-esterified. Low methoxyl pectins (degree of methylation, DM < 50%), requires the presence of calcium ions or other divalent metal ions that form intermolecular ionic junction zones responsible for gel formation (Kastner, Einhorn-Stoll, & Senge, 2012; Munarin, Tanzi, et al., 2012; Ravanat & Rinaudo, 1980). The calcium bridges between dissociated carboxyl groups, thus forming well organized structures known as the “egg-box model” (Braccini & Pérez, 2001; Naumenko & Altenbach, 2007; Morris, Nishinari, & Rinaudo, 2012). Besides the electrostatic interactions, van der Waals interactions and hydrogen bonds stabilize the chain when egg-boxes are formed between neighboring chains (Alonso-Mougan, Meijide, Jover, Rodriguez-Nunez, & Vazquez-Tato, 2002; Braccini, Grasso, & Pérez, 1999; Fraeye et al., 2010; Munarin, Tanzi, et al., 2012). The solubility of pectin crosslinked with Ca^{2+} ions can be controlled by other monovalent counterions (such as Na^+) that can replace calcium ions. This attribute makes pectin suitable for externally tunable systems for the release of immobilized cells or therapeutic agents (Munarin et al., 2011).

Two approaches can be followed for the preparation of pectin gels crosslinked with calcium ions: the external or internal gelation. The external gelation is an ionotropic mechanism commonly achieved by extruding droplets of the polyanionic polymers (i.e. alginate, pectin, gellan) (Ching, Liew, Heng, & Chan, 2008; Ferreira Almeida & Almeida, 2004; Miyazaki, Aoyama, Kawasaki, Kubo, & Attwood, 1999; Santucci et al., 1996; Sriamornsak, 1998) in a crosslinking solution containing soluble calcium salts, such as calcium chloride. This mechanism allows the production of particles and beads, due to the extremely fast gelling reaction (Alhaique et al., 1996; Evangelista et al., 2007; Grellier et al., 2009; Munarin, Petrini, et al., 2012; Munarin et al., 2011). The internal gelation is suitable for the preparation of scaffolds for tissue engineering and matrices for drug release, as it produces homogeneous gels (Chan, Lee, & Heng, 2006). In the case of the internal gelation, the polymeric solution is mixed with a poorly soluble calcium salt, such as calcium carbonate (CaCO_3), and the crosslinking starts after acidification, with H^+ ions promoting the progressive pH-controlled dissolution of CaCO_3 . For this reason, when used for the internal gelation of anionic polysaccharides, CaCO_3 is combined with D-glucono- δ -lactone (GDL), a chemical compound able to lower the pH of the polymeric solutions (Baldursdóttir & Kjøniksen, 2005;

Bidarra et al., 2011; Fonseca, Bidarra, Oliveira, Granja, & Barrias, 2011; Fonseca et al., 2013). In this work, we hypothesize the possibility of using CaCO_3 for the preparation of pectin hydrogels by internal gelation, and to do this avoiding the use of GDL. At present, no reports exist so far regarding the preparation of pectin hydrogels crosslinked with CaCO_3 without the addition of GDL. Though GDL is a non-toxic compound already employed to produce alginate and pectin hydrogels for biomedical applications (Baldursdóttir & Kjøniksen, 2005; Fonseca et al., 2011, 2013), avoiding the use of the additive can be more convenient, due to the formation of a self-gelling system and to the reduced time of production.

The gelling kinetic could be easily controlled avoiding the use of GDL. In the presence of GDL, two phenomena are partially correlated: GDL time-dependent hydrolyzation to lower the pH of the polymeric solution, and the solubility of CaCO_3 in the solution, whose pH is changing with time due to the hydrolysis of GDL. Furthermore, in order to obtain fast gelling kinetics, higher amounts of GDL are required, but this leads to a severe acidification of pectin solutions, reaching its maximum values when GDL is completely hydrolyzed, that may cause damages to the cells possibly entrapped or to the host tissues.

The acidic pH of pectin solutions and gels can limit their application as injectable systems for biomedical applications. To increase the pH, different bases can be employed, including NaOH, NaHCO_3 , TEA (tris-ethanol-amine) and others. NaOH is known to precipitate and degrade the poly-GalA chains of pectin, when pectin is at alkaline pH for long time (Eder & Lütz-Meindl, 2008; Hotchkiss et al., 2002; Hunter & Wicker, 2005; Renard & Thibault, 1996). The use of NaHCO_3 , as a pH-modifier, could be an alternative to raise the pH of pectin solutions avoiding the occurrence of polysaccharide depolymerization.

The formation of hydrogels with different gelling kinetics and mechanical properties may be obtained by finely modulating pectin pH, varying it over a specific pH range, prior to the addition of the cross-linking agent.

The gelling kinetic is a fundamental parameter in designing homogeneous gels and composite gels, injectable systems, cell loaded gels, in vivo gelling systems, and for an effective drug loading prior the formation of the gel. To this end our aim was to obtain the internal gelation of pectin by fine-tuning of NaHCO_3 and CaCO_3 content, to keep a tight control over the pH of the hydrogels thus controlling their gelling kinetics.

Pectin-based injectable systems were developed to: (i) have slow gelling kinetics, to avoid premature gelling and allow to obtain homogeneous gels; (ii) possess tunable crosslinking kinetics, to tailor different surgical procedures and anatomical sites; (iii) are shear-thinning, possessing low viscosity at high shear to allow the injection through a needle; (iv) are easily prepared with the possibility to scale-up the preparation process to the industrial setting.

2. Experimental

2.1. Materials

Low methoxyl pectin from citrus fruits (CU701, DE = 38%) was kindly provided by Herbstreith & Fox (Neuemburg, Germany) in a dry powder form. Sodium bicarbonate (NaHCO_3), sodium hydroxide (NaOH) and calcium carbonate (CaCO_3) were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification.

2.2. Characterization of pectin

2.2.1. Molecular weight by gel permeation chromatography (GPC)

Solutions of 2.4% (w/v) pectin with a pH ranging from 3.2 to 11.0 were prepared by dissolving pectin powder in solutions of NaHCO_3

Table 1
Experimental set up of the rheological characterization.

Experiment	Measured parameters	Temperature (°C)	Stress (Pa)	Strain (%)	Frequency (Hz)	Time (min)
Oscillation amplitude	G' , G'' , $\tan \delta$, LVR (linear viscoelastic region)	25	0.1–100	–	0.1	–
Time sweep	G' , G'' , $\tan \delta$, time of gelling (gel point)	25	5	–	0.7	30
Frequency sweep	G' , G'' , $\tan \delta$	25, 37	5	–	0.01–100	–
Temperature sweep	G' , G'' , $\tan \delta$	20–60 (2.5 °/min ramp)	–	0.1	0.1	16
Creep	Strain	25	10	–	–	10

and NaOH with different molarity (0–23 mM) and left overnight under stirring. The molecular weight of pectin solutions was determined using a GPC system consisting of a Waters 1515 isocratic HPLC pump with a differential refractive index detector (Waters 2414). The mobile phase (0.1 M NaNO₃) was eluted through Waters Ultrahydrogel™ 1000, 500 and 250 columns with a flow rate of 0.8 mL/min at 25 °C. Samples were prepared by dissolving 2.4% (w/v) pectin solutions in 0.1 M NaNO₃ for a final pectin concentration of 0.2% (w/v). After filtration through RC syringe filters (0.45 μm porosity), 200 μL of the solutions were injected in the GPC apparatus.

2.2.2. Determination of the equivalence point

The equivalence point of pectin was evaluated by chemical titration, as previously reported (Farris, Mora, Capretti, & Pierviviani, 2012), with slight modifications. Briefly, pectin aqueous dispersion (0.1% w/v) was treated with 7.5 mL of 0.1 N HCl, to completely neutralize the negative charge distributed along the unprotonated carboxylic groups of pectin backbone. Then, 0.1 N NaHCO₃ or NaOH were added under fast stirring. The electrical potential and the pH were evaluated with a Jenway 4510 conductivity meter after sequential injections of NaHCO₃ or NaOH. The titrant was dropped approximately every 60 s to allow solution equilibrium, reducing its volume (from 0.5 to 0.1 mL) while approaching to the equivalence point.

The first derivative of the data on all points of region 2 of titration curves was calculated to access the slope between the two buffering regions of titration curves, for titration curves comparison.

2.3. Preparation of pectin hydrogels

Pectin hydrogels were prepared using calcium carbonate and adjusting the pH of 2.4% (w/v) pectin solutions with 0 mM (native pH), 13 mM and 23 mM NaHCO₃. Calcium–pectin hydrogels were obtained adding 12.5 mM, 25 mM and 50 mM CaCO₃ suspensions (corresponding to the stoichiometric ratio $R = [Ca^{2+}]/2[COO^-]$ of 1, 2 and 4, respectively) to pectin solutions, to reach a 2% (w/v) final pectin concentration. Each mixture was kept under fast stirring (1100 rpm) at room temperature until a gel started to form. Then, solutions were transferred to Petri dishes and left overnight at room temperature to stabilize the gel networks.

2.4. Characterization of pectin hydrogels

2.4.1. Rheological characterization

Rheological characterization of hydrogels was performed with an AR 1500ex rheometer (TA Instruments, Italy), equipped with parallel-plate geometry (diameter = 20 mm, working gap = 1200 μm). Oscillatory experiments (amplitude, time, frequency and temperature sweeps) and creep tests were performed on hydrogels, to evaluate their injectability and stability. The parameters set up for each experiment are indicated in Table 1.

2.4.2. Swelling studies

To determine the swelling capacity of pectin hydrogels they were incubated at room temperature in distilled water and swelling

was investigated at different time points, up to 24 h, by calculating the percent weight variation using the following equation (Eq. (1)):

$$\Delta W = \left[\frac{w_t - w_0}{w_0} \right] \times 100 \quad (1)$$

where w_t is the wet weight of gels at time point t and w_0 is the initial weight of the gels, measured immediately after their preparation. The measurements were made in triplicate, the results are presented as average weight variation and the error was calculated with standard deviation.

2.4.3. pH measurements

The pH of pectin–calcium carbonate hydrogels was determined using a Cyberscan pH 110 pHmeter (Eutech Instruments) with a special penetration electrode (Hamilton double pore slim).

2.5. Cytocompatibility evaluation of pectin hydrogels through indirect cell viability assay

Mouse Fibroblasts L929, from the American Type Culture Collection (Rockville, MO) below passage 15, were cultured in 75 cm² flasks and grown in RPMI-1640 culture medium (pH = 8.2), supplemented with 10% (v/v) fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin and were incubated at 37 °C, in 5% CO₂ atmosphere. The reagents employed for the cell culture were provided by Gibco (Invitrogen). An indirect cytocompatibility test was performed on pectin gels, where distilled water, previously incubated with gels for 24 h or 7 days (gel extract), was diluted (1:10) with fresh medium and then added to the seeded cells. Fibroblasts seeded at density 10⁴ cells/well were then incubated with each diluted culture medium of gel extracts for further 24 h. At the end of incubation, 200 μL 10% (w/v) MTT solution (RPMI serum free) were added for 3 h, and the absorbance was measured at 595 nm in a iMark Microplate Absorbance Reader (Bio-Rad Laboratories) (Munarin, Bozzini, Visai, Tanzi, & Petrini, 2013). All assays were done in triplicate and their significance was calculated by Student's t -test.

2.6. Statistical analysis

All measurements were made in triplicate, and data are presented as mean values ± standard deviation. Student's t -test was performed and differences were considered statistically significant when p values resulted lower than 0.05.

3. Results

3.1. Effect of the pH on pectin molecular weight

Gel permeation chromatography (GPC) was performed to determine the molecular weight (M_w , M_n) of pectin solutions at different pH values, adjusted or not with NaHCO₃ and NaOH. The results of the GPC analyses are shown in Table 2. The values of M_w and M_n of the pectin solution at its native pH increased when increasing the pH of the solution both with NaOH and NaHCO₃, and this effect

Table 2
Molecular weight analysis of pectin solution as determined by GPC.

Solutions	pH	M_w (kDa)	M_n (kDa)	M_w/M_n
Pectin	3.22	317	74	4.3
	3.62	387	164	2.3
	3.88	341	117	2.9
Pectin + NaHCO ₃	4.63	344	107	3.2
	6.25	99	32	3.1
	8.07	93	44	2.1
	3.63	398	151	2.6
	3.86	n.a.	n.a.	n.a.
Pectin + NaOH	4.21	355	68	5.1
	4.72	374	131	2.8
	5.35	328	135	2.4
	11.0	118	28	4.1

was retained up to pH 5.35. This result suggests that the hydrodynamic volume of pectin chains increased due to the salification of the carboxyl groups and the consequent electrostatic repulsion of the carboxylates (Kong, Kaigler, Kim, & Mooney, 2004; Munarin et al., 2013). Above a certain limit (Table 2), such as an increase of pH above 5.35, a reduction of M_w occurs, due to depolymerization by β -elimination of the pectin backbone (Munarin, Tanzi, et al., 2012), leading to chain scission responsible for polysaccharide degradation. A 50% M_w decrease was previously reported in similar conditions (Hunter & Wicker, 2005).

3.2. Determination of the equivalence point

The equivalent point, defined as the equal stoichiometric number of moles of the titrant and the analyte, provides a quantitative measure of the volume of titrant required to deprotonize the carboxyl groups in pectin backbone. Plots of electrical potential and pH versus the volume of titrant for dilute aqueous pectin dispersions are shown in Fig. 1.

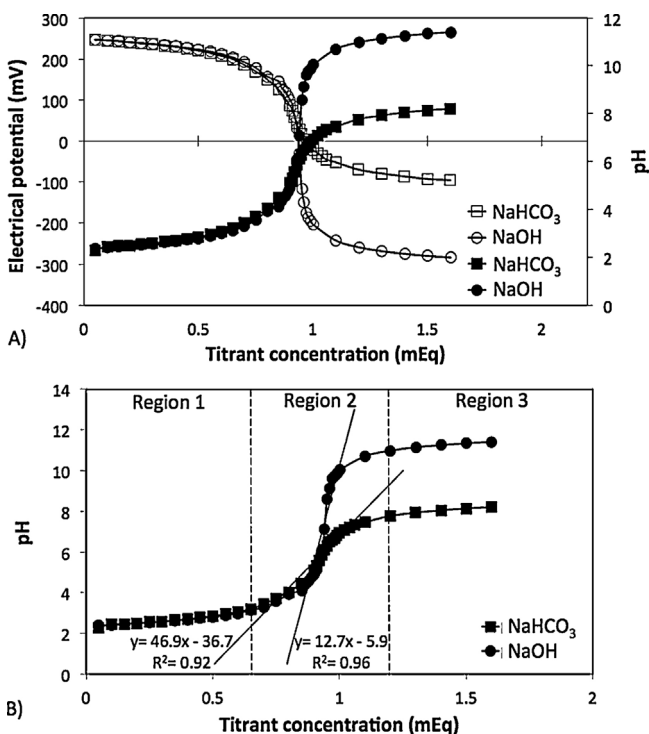


Fig. 1. Titration curves of (A) pectin solutions titrated with NaHCO₃ and NaOH and (B) first derivative of titration curves.

Table 3
Equivalent point and derivative properties.

Titrant	Equivalent point		Derivative line	
	Concentration (mEq)	pH	Slope	Angle (°)
NaOH	0.94	6.59	46.9	88.8
NaHCO ₃	0.98	6.59	12.7	85.6

The curves clearly display three distinct regions (Fig. 1B). As the equivalent point occurs when the electrical potential is zero, the pH of the equivalent point of pectin titrated with NaHCO₃ is the same of that of pectin titrated with NaOH (Table 3). Absolute values of slope and angle of the first derivative of the curves in region 2 are steeper in the case of NaOH than in the case of NaHCO₃ (Fig. 1B and Table 3), meaning that the full neutralization of the acid groups along the pectin backbone is faster when adding NaOH. As a result, modifying the pH of pectin solutions becomes easier with NaHCO₃ due to lower pH buffering of pectin solutions (pH 8 for NaHCO₃ and pH 11 for NaOH) and slower neutralization of carboxyl groups, leading to tighter control of the pH of pectin solutions.

3.3. pH and gel point of pectin solutions as function of CaCO₃ concentration

According to the previous results, NaHCO₃ was added to pectin solutions to partially neutralize the carboxyl groups of pectin backbone and to reach an adequate pH for the physiological environment. The evolution of the pH of the gels with the NaHCO₃ concentration is shown in Table 4.

Increasing the concentration of NaHCO₃, an increase of the pH of pectin solutions can be observed, as well as a decrease of the solid-like morphology of the gels (Fig. 2).

The time-point when materials start to exhibit viscoelastic solid behavior is rheologically defined as “gel point”. The values of G' and G'' at the gel point are the same because of the cross-over of the two components ($\tan \delta \approx 1$) (Munarin, Petrini, et al., 2012; Santucci et al., 1996). Aqueous low methoxyl pectin (LMP) solutions undergo sol-to-gel transformation in the presence of crosslinking cations, in this case Ca²⁺ derived from CaCO₃. An increase in the pH was observed after the addition of CaCO₃, when gel formation occurred. The amount of Ca²⁺ available in solution, derived from CaCO₃ dissolution, is correlated with the final increase of pH, as solubility of CaCO₃ is pH-dependent (Table 4). As seen in the GPC results (Table 2), a pH raise over a certain limit (pH > 5.35) leads to a high reduction of the molecular weight and, therefore, chain scission.

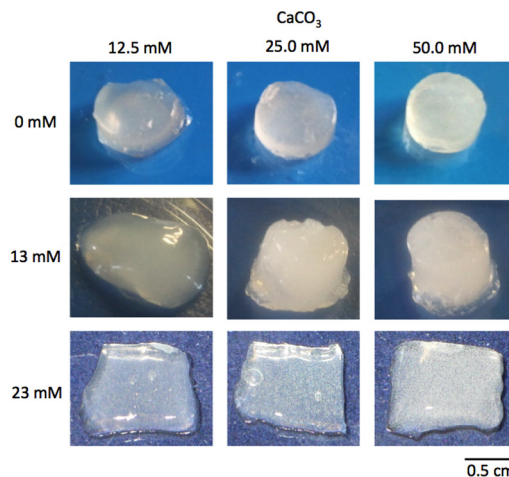


Fig. 2. Pectin–calcium carbonate hydrogel formulations with different concentrations of CaCO₃ and NaHCO₃.

Table 4
Gelling characteristics of pectin formulations.

Name	NaHCO ₃ (mM)	pH of pectin solution	CaCO ₃ (mM)	pH of the hydrogels	Gelling time
PNa0Ca12.5	0	3.23	12.5	3.78 ± 0.10	8' 50" ± 12"
PNa0Ca25			25.0	3.98 ± 0.14	3' 48" ± 6"
PNa0Ca50			50.0	4.41 ± 0.12	<2'
PNa13Ca12.5	13	3.62	12.5	5.48 ± 0.10	6' 35" ± 6"
PNa13Ca25			25.0	5.75 ± 0.12	4' 44" ± 18"
PNa13Ca50			50.0	6.30 ± 0.09	<2'
PNa23Ca12.5	23	3.79	12.5	5.75 ± 0.10	17' 00" ± 18"
PNa23Ca25			25.0	6.02 ± 0.07	7' 44" ± 6"
PNa23Ca50			50.0	6.43 ± 0.46	3' 13" ± 6"

Gel formation is not accomplished for pectin solutions with initial pH higher than 5.35 due the low solubility of CaCO₃ at neutral pH and consequent low availability of Ca²⁺ in solution. Therefore the maximum pH of the initial pectin solution was set to 3.79, corresponding to a 23 mM NaHCO₃ concentration. Different hydrogel formulations were prepared varying the amount of NaHCO₃ (0, 13 and 23 mM) and the concentration of the cross-linker CaCO₃ (12.5, 25 and 50 mM).

After gelling, the pH of the formed hydrogels increases up to physiological values (Table 4), as a consequence of the ionic complexation of the carboxyl groups of pectin.

In a typical curve to determine the gel point (Fig. 3) both G' and G'' moduli increase as a result of the increased crosslinking density, but the elastic component rises more sharply than the viscous component, according to the literature (Chambon, Petrovic, MacKnight, & Winter, 1986).

Gelling time was correlated with CaCO₃ concentrations and pH (Table 4), as the time requested to form the gel decreased when raising CaCO₃ concentration (remaining below its solubility limit) or lowering the pH of the gelling mixture.

3.4. Rheological characterization

3.4.1. Oscillation amplitude test

The linear viscoelastic region (LVR) is defined as the region where the viscoelastic properties of the gels are independent of imposed stress or strain levels, i.e., storage and loss moduli are kept constant while varying oscillation stress or strain. All the rheological experiments should be conducted in the LVR to avoid any disruption of the sample structure. In Fig. 4 the oscillation amplitude test of gels with lower amount of crosslinking ions is represented as an example, to identify the LVR.

Samples kept constant storage and loss moduli for a wide range of oscillation stress (0–80 Pa). Therefore, the subsequent

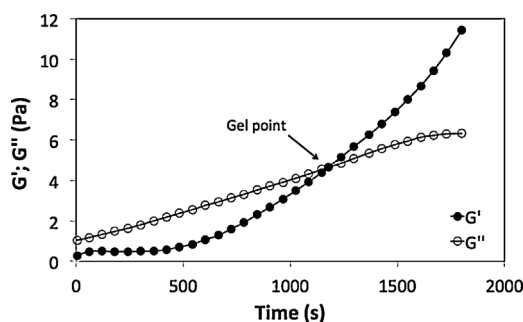


Fig. 3. Evolution of the storage (G') and the loss (G'') moduli of PNa23Ca12.5 (i.e. Pectin and 23 mM NaHCO₃ crosslinked with 12.5M CaCO₃). In this example, the gel point, i.e., the crossover of storage and loss moduli, is reached 17 min after gel preparation.

rheological characterization was performed with oscillation stress below 80 Pa (Table 1) to maintain the samples in the LVR.

The storage modulus was found to decrease when increasing the amount of NaHCO₃ used to adjust the pH of the gels. The same trend was observed for the other stronger gel formulations that were composed of higher amounts of crosslinking ions (data not shown).

3.4.2. Frequency sweep test

The variation of G' and G'' with frequency is known as the “mechanical spectrum” of a material. Typical mechanical spectra for polysaccharide solutions and gels have a solid-like character (G') predominant over liquid-like, viscous response (G'') (Alonso-Mougan et al., 2002; Dobies, Kozak, & Jurga, 2004). The logarithmic representation of the conservative moduli – G' – as a function of frequency (Fig. 5A) shows the characteristic spectrum of pectin gels. It was observed that the absolute values of G' , when comparing different NaHCO₃ concentrations, were higher in the gel with higher calcium content, PNa0Ca50 and PNa13Ca50, following a linear behavior. Given the similarity of viscosity and storage modulus values of PNa23Ca12.5, PNa23Ca25 and PNa23Ca50, the calcium content on gels does not seem to affect their characteristics.

The loss factor $\tan \delta$ (Fig. 5B) reveals the ratio between the viscous and the plastic portion of the viscoelastic deformation behavior. When $\tan \delta < 1$ ($G' > G''$), a confirmation of the solid state of gels is obtained (Fig. 5B). On the contrary, when $\tan \delta > 1$, the liquid state prevails, as observed for PNa0, PNa13 and PNa23 solutions (data not shown). Gels with higher $\tan \delta$ values resulted to be softer. All the gels exhibited a shear-thinning behavior, associated to the decrease of viscosity with the increase of frequency (Fig. 5C). Moreover, when the dynamic measurements are repeated on gels at 37 °C the mechanical spectra showed the same results (data not shown), regarding storage, $\tan \delta$ and viscosity values, being G' frequency independent and always higher than G'' .

3.4.3. Creep test

Creep tests were set up to evaluate the behavior of gels under constant stress. According to previous studies on the viscoelastic behavior of alginate and pectate gels (Mitchell & Blanshard, 1974, Mitchell & Blanshard, 1976a, 1976b; Clark & Rossmurphy,

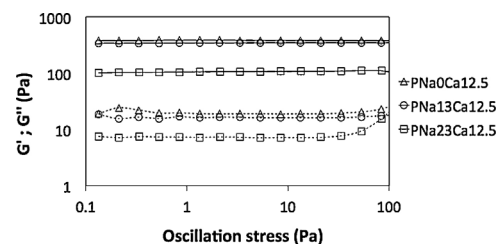


Fig. 4. Oscillation amplitude test results of P0C1, P13C1 and P23C1 hydrogels. Filled symbols represent G' , void symbols represent G'' .

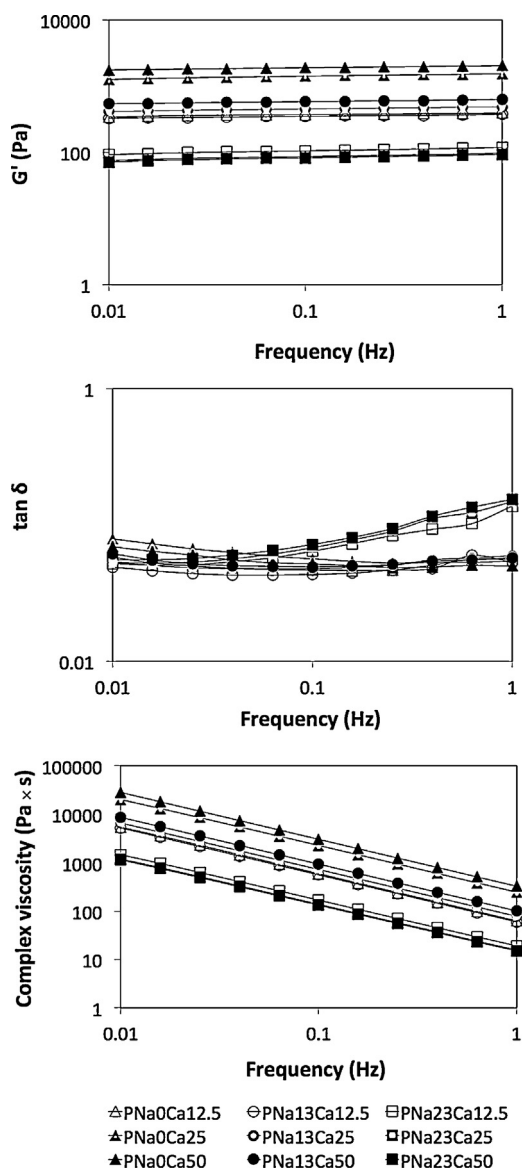


Fig. 5. Average frequency sweep test results of pectin–calcium carbonate hydrogels. (A) Storage modulus, (B) $\tan \delta$ and (C) complex viscosity are represented as function of frequency.

1987), increasing the amount of the cross-linker (Fig. 6), as well as decreasing the pH of the gels, strain and complex compliance decreased, resulting in an improved stiffness of the material. These observations were consistent with the results of the oscillatory

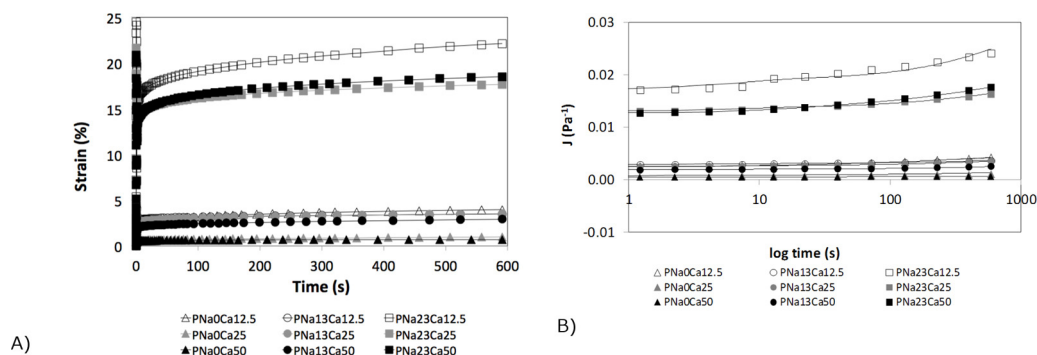


Fig. 6. Creep test results of pectin–calcium carbonate hydrogels. Strain (a) and creep compliance (b) of the produced formulations.

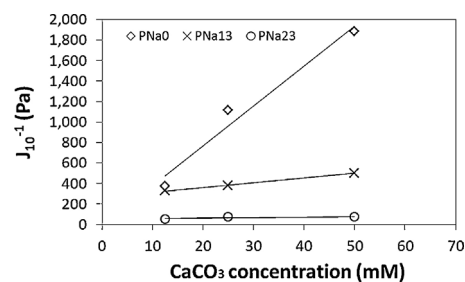


Fig. 7. Linear relationship between the reciprocal of creep compliance measured at 10 s at different NaHCO_3 and CaCO_3 concentrations.

frequency sweep tests (Fig. 5). Fig. 6b gave the evidence of the presence of slow changes in creep compliance at long times, especially for PNa23 hydrogels. This may indicate that the crosslinks are not permanent, but that they can break or reorganize when the gel is deformed. Considering the values of creep compliance obtained in this work, the produced hydrogels appeared stiffer than other pectin gels produced with GDL and CaHPO_4 (Mitchell & Blanshard, 1974, 1976a).

Fig. 7 shows an approximately linear relationship between the reciprocal of 10 s compliance and CaCO_3 concentration. The slope of the linear interpolation was found to decrease increasing the pH of pectin solutions.

The creep curves, obtained as reported in Section 2.4.1, were fitted with a viscoelastic model constituted by Maxwell element in series with three Voigt elements, as described by Eq. (2).

$$J(t) = J_0 + \sum_i J_i (1 - e^{-t/\tau_i}) + \frac{t}{\eta_N} \quad (2)$$

where $J(t)$ is the measured creep compliance, η_N is the Newtonian viscosity and τ_i are the retardation times associated with the Voigt elements. The parameters obtained fitting the curves of the produced hydrogels are reported in Table 5.

The recovery was calculated according to the Boltzman principle, shown in Eq. (3):

$$J_r(t) = J(t) - J(t - t_1) \quad (3)$$

where t_1 is 600 s, the time at which the stress is removed. The recovery was calculated using $J(t)$ as obtained with Eq. (2) and with the parameters of Table 5. A comparison between the experimental results and the calculated model is reported in Fig. 8 as an example.

As shown in previous works (Mitchell & Blanshard, 1976a, 1976b), the first part of the experiment is in good agreement with the model, while in the recovery phase the exponential curve is lower than the calculated results. This may suggest that more Voigt elements are required for an optimal fitting of the curves.

Table 5
Rheological parameters calculated from creep compliance time responses.

Parameters	PNa0Ca12.5	PNa0Ca25	PNa0Ca50	PNa13Ca12.5	PNa13Ca25	PNa13Ca50	PNa23Ca12.5	PNa23Ca25	PNa23Ca50
J_0 (Pa ⁻¹)	2.5×10^{-3}	8.0×10^{-4}	5.0×10^{-4}	2.9×10^{-3}	2.5×10^{-3}	1.9×10^{-3}	1.7×10^{-2}	1.3×10^{-2}	1.1×10^{-4}
J_1 (Pa ⁻¹)	1.6×10^{-4}	8.0×10^{-5}	3.6×10^{-5}	1.9×10^{-4}	2.3×10^{-3}	1.5×10^{-4}	2.0×10^{-3}	7.5×10^{-4}	1.4×10^{-2}
J_2 (Pa ⁻¹)	1.8×10^{-4}	1.3×10^{-4}	5.9×10^{-5}	6.9×10^{-5}	4.7×10^{-4}	1.2×10^{-4}	8.0×10^{-4}	1.5×10^{-3}	2.5×10^{-3}
J_3 (Pa ⁻¹)	8.9×10^{-4}	3.0×10^{-6}	4.7×10^{-5}	4.3×10^{-4}	5.0×10^{-6}	1.4×10^{-4}	4.5×10^{-3}	3.0×10^{-6}	1.0×10^{-5}
T_1 (s)	6	6	8	4	7	6	6	6	6
T_2 (s)	26	43	82	121	233	339	222	205	26
T_3 (s)	151	509	115	344	567	544	501	355	152
η_N (Pa s)	1.05×10^6	1.41×10^6	9.60×10^6	3.02×10^6	1.43×10^6	1.90×10^6	2.95×10^5	4.61×10^5	1.05×10^6

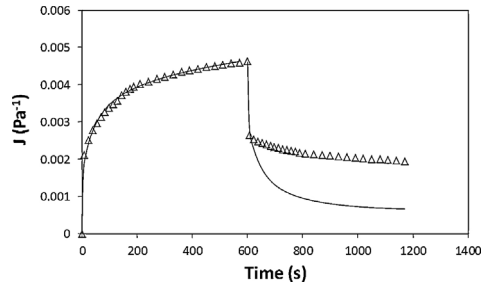


Fig. 8. Measured and calculated creep and recovery curves.

3.4.4. Temperature sweep test

The variation of G' modulus with temperature (Fig. 9) followed the same trend applicable for any polysaccharide gel, characterized with a “plateau” region during an extended temperature range. The decrease of G' and viscosity with temperature, meaning a decrease in the elastic behavior of the samples and an approximation to liquid-like behavior, was visible from a critical temperature, ranging from 55 °C for PNa0 gels, to 36 °C for PNa23 gels (Fig. 9). Some formulations showed an increase of the storage modulus for temperatures higher than 50 °C, which can be due to changes in the macromolecule conformation or different solubilization of CaCO₃ during heating (Gouvêa et al., 2009).

3.5. Swelling studies

All of the hydrogel formulations showed an initial swelling (Fig. 10) due to water absorption. PNa0Ca25, PNa0Ca50, PNa13Ca25 and PNa13Ca50 reached a plateau phase and their weight was retained up to 24 h, whereas PNa0Ca12.5 and PNa13Ca12.5 wet weight slightly decreased with time. The weight of PNa23 formulations instead was rapidly decreasing with time.

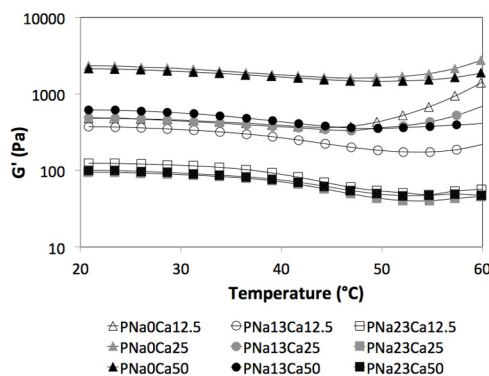


Fig. 9. Evolution of the storage modulus (G') of pectin hydrogel formulations evaluated by temperature ramp test.

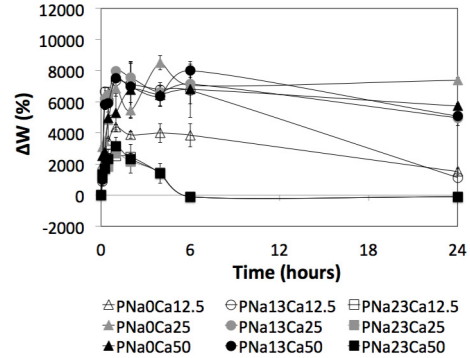


Fig. 10. Swelling behavior of pectin hydrogels incubated in dH₂O at room temperature.

3.6. Cytotoxicity studies

Cytotoxicity tests were performed on pectin hydrogels as preliminary experiments to future trials as injectable systems, where hydrogels with the ability to gel in situ, entrap cells and keep them viable are usually required.

The initial cytotoxicity may be due to the acidic pH of the hydrogels (Table 4), which is probably equilibrated after 7 days of incubation. An increase of the cytocompatibility is observed for PNa23 extracts after 7 days of incubation, with cell viability reaching values comparable with the control (i.e. cells seeded on the tissue culture plate) (Fig. 11).

4. Discussion

The gelation kinetics and the mechanism involved in the ionotropic gel formation are strictly dependent upon the pH of pectin solutions. pH regulates the dissociation – and therefore the availability for ionic complexation – of carboxylic groups, and the molecular weight of the polysaccharides, affecting the final gel properties.

Among the properties of pectin solutions, the equivalence point is important because it constitutes the point at which all the H⁺ of the starting solution are neutralized by the titrant. The curves

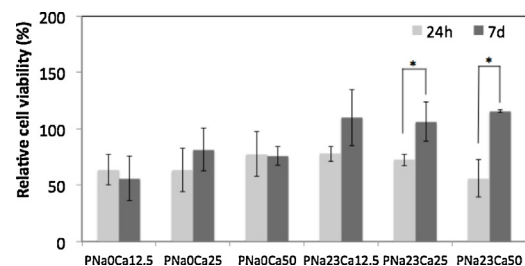
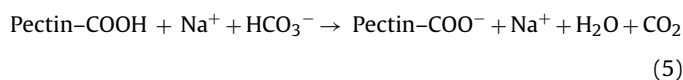
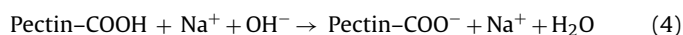


Fig. 11. Indirect cytocompatibility of 24 h and 7 d hydrogels extracts. The metabolic activity read for the control (TCPS) was taken as the reference (100%). Significance values were calculated by t-test (* $p \leq 0.05$).

of electrical potential and pH (Fig. 1) clearly display three distinct zones. First buffering phase (region 1) corresponds to the neutralization of H⁺ ions by HCl. Region 2 is characterized by reaching the equivalence point, where the pH of pectin progressively increases and hence the electrical potential decreases as deprotonation of the carboxylic groups proceeds, due to the increase of titrant ions (OH⁻ and HCO₃⁻ from NaOH and NaHCO₃, respectively) in the dispersion. These ions derive from the neutralization of dissociated hydrogen groups of pectin backbone according to Eqs. (4) and (5).



When the carboxylic groups are completely titrated, pH and electrical potential reach another plateau phase (region 3) (Fig. 1) (Bochek, Zabivalova, & Petropavlovskii, 2001; Farris et al., 2012). The lower pH was reached when titrating pectin with NaHCO₃, possibly due to the release of CO₂ that is known to lower the pH. Also, the lower value of the slope of NaHCO₃, if compared with the slope of NaOH, is typical for a titration of a weak base with a strong acid, and suggests that the conversion of the acidic form of pectin carboxyl groups into the salified form can be more easily controllable with NaHCO₃.

The chosen concentrations of CaCO₃ (12.5, 25 and 50 mM) correspond to the stoichiometric ratios, $R = [\text{Ca}^{2+}]/2[\text{COO}^-]$, desired for the final hydrogels of 1, 2 and 4, respectively. The influence of the concentration of calcium ions on low methoxyl (LM) pectin gelation is related with the amount of non-methoxylated GalA residues. Increasing the concentration of CaCO₃ resulted in a reduction of the gelling time, confirming that the transition rate between the sol and gel states is a function of calcium concentration (Dobies et al., 2004; Fraeye et al., 2010; Gigli, Garnier, & Piazza, 2009).

Frequency sweep results (Fig. 5) showed a linear behavior followed by the hydrogels and. Regarding the calcium content, higher amounts of CaCO₃ available in solution resulted in stronger gels (Iglesias & Lozano, 2004). The stiffness of P₀ hydrogels (Fig. 5A) can be explained by the pectin pK_a of 3.5. LM pectin hydrogels are typically spreadable, with rigidity increasing as pH falls below pectin pK_a. Below the pK_a, less dissociation of H⁺ results in greater hydrogen bonding of the pectin chains, thus originating a stiffer hydrogel. Above the pK_a, acid groups are ionized, the polymeric chains change their conformation and the number of hydrogen bonds diminishes. As a consequence, the polymer acquires the characteristics of a polyelectrolyte, thus allowing for more efficient crosslinking by calcium ions (Aguado, Mulyasmita, Su, Lampe, & Heilshorn, 2012). Above a certain limit, as shown by GPC results (Table 3), such as an increase of pH above 6.25, a reduction of M_w occurs, due to depolymerization by β-elimination of the pectin backbone (Munarin et al., 2012b), leading to chain scission responsible for polysaccharide degradation. A 50% M_w decrease was previously reported, in similar conditions (Hunter & Wicker, 2005).

The formation of a three-dimensional network is shown by the dynamic behavior of pectin hydrogels. The entangled structure of the pectin network is formed by intermolecular electrostatic association of galacturonans and ionic complexation of the carboxyl groups (Sriamornsak, 2003; Ström & Williams, 2003).

The hydrogels showed no frequency-dependence of the storage modulus (Fig. 5A) but decreasing viscosity at higher frequencies (Fig. 5C). According with the literature, (Munarin, Petrini, et al., 2012) values of tan δ lower than 1 indicated the solid-like behavior of each hydrogel formulation (Fig. 5B). When applying physiological temperatures (i.e. 35–42 °C), the rheological properties of the

hydrogels (G' and complex viscosity) remained stable (Fig. 9), thus confirming the suitability of the injectable hydrogels as implantable carriers for regenerative medicine.

Focusing on the injection procedure, a viscoelastic behavior can protect the cells and/or bioactive molecules from being damaged by the mechanical forces experienced during the extrusion through the needle. The flow of stiffer or more compliant gels, such as PNa0 gels through the syringe needle, could damage the cells due to pressure drop, shearing forces (due to linear shear flow) and stretching forces (due to extensional flow) (Aguado et al., 2012). Injectable systems for localized delivery require slow gelling and tunable crosslinking kinetics. Despite homogeneous gels were obtained with PNa23 formulations, the evident softness of these gels caused lower resistance to frequency and temperature (Voragen, Schols, & Visser, 2003). While PNa0 hydrogels appear appropriate injectable formulations for the repair of hard tissues, PNa23 hydrogels seems more indicated for soft tissue regeneration, being softer and easier conforming to the anatomical site.

On the basis of the results obtained with the cytotoxicity assay, it can be concluded that pectin hydrogel extracts were not cytotoxic (Fig. 11) for L929 cells.

These several factors, solubility and concentration of CaCO₃, concentration and pH effect of NaHCO₃, as well as pectin degradation in alkaline pH affect cell viability, as well as all the investigated properties.

5. Conclusions

Pectin-calcium carbonate hydrogels were prepared by inter-nal gelation, avoiding the addition of D-glucono-δ-lactone, which might induce difficulties in the control of the final pH and of the gelling kinetics of the hydrogels. The tight control achieved exclusively over the amounts of NaHCO₃ and CaCO₃, was determinant to promote appropriate gelling kinetics, required to obtain homogeneous hydrogels, as well as to reach pH values compatible with cell viability.

Pectin solutions, modified with NaHCO₃ and NaOH, with a pH of 6 or higher, undergone chain scission, responsible for polysaccharide degradation and consequently they were not adequate to form gels. Also, the control of the pH resulted easier with NaHCO₃ due to its buffer capability.

In view of developing pectin-based injectable systems for cell entrapment in biomedical applications, the results of this work demonstrated that the gelling kinetics and rheological properties of pectin-calcium carbonate hydrogels can be modulated according to the specific agent to be entrapped and to the tissue to be treated. Furthermore, the considered formulations were cytocompatible and could be obtained with inexpensive and easy preparation methods. Injectability was confirmed by rheological analyses, as the gels exhibited a viscoelastic and shear-thinning behavior, thus allowing their injection through a needle.

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References

- Aguado, B. A., Mulyasmita, W., Su, J., Lampe, K. J., & Heilshorn, S. C. (2012). Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue Engineering Part A*, 18(7), 806–815.
- Alhaique, F., Santucci, E., Carafa, M., Coviello, T., Murtas, E., & Riccieri, F. M. (1996). Gellan in sustained release formulations: Preparation of gel capsules and release studies. *Biomaterials*, 17(20), 1981–1986.
- Alonso-Mougan, M., Meijide, F., Jover, A., Rodriguez-Nunez, E., & Vazquez-Tato, J. (2002). Rheological behaviour of an amide pectin. *Journal of Food Engineering*, 55(2), 123–129.
- Baldursdóttir, S. G., & Kjøniksen, A. (2005). Rheological characterization and turbidity of riboflavin-photosensitized changes in alginate/GDL systems. *European Journal of Pharmaceutics and Biopharmaceutics*, 59(3), 501–510.
- Bidarra, S. J., Barrias, C. C., Barbosa, M. A., Soares, R., & Granja, P. L. (2010). Immobilization of human mesenchymal stem cells within RGD-grafted alginate microspheres and assessment of their angiogenic potential. *Biomacromolecules*, 11(8), 1956–1964.
- Bidarra, S. J., Barrias, C. C., Fonseca, K. B., Barbosa, M. A., Soares, R. A., & Granja, P. L. (2011). Injectable in situ crosslinkable RGD-modified alginate matrix for endothelial cells delivery. *Biomaterials*, 32(31), 7897–7904.
- Bochek, A. M., Zabivalova, N. M., & Petropavlouskii, G. A. (2001). Determination of the esterification degree of polygalacturonic acid. *Russian Journal of Applied Chemistry*, 74(5), 796–799.
- Braccini, I., & Pérez, S. (2001). Molecular basis of Ca²⁺-induced gelation in alginates and pectins: The egg-box model revisited. *Biomacromolecules*, 2(4), 1089–1096.
- Braccini, I., Grasso, R. P., & Pérez, S. (1999). Conformational and configurational features of acidic polysaccharides and their interactions with calcium ions: A molecular modeling investigation. *Carbohydrate Research*, 317(1–4), 119–130.
- Chambon, F., Petrovic, Z. S., MacKnight, W. J., & Winter, H. H. (1986). Rheology of model polyurethanes at the gel point. *Macromolecules*, 19(8), 2146–2149.
- Chan, L. W., Lee, H. Y., & Heng, P. W. S. (2006). Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system. *Carbohydrate Polymers*, 63(2), 176–187.
- Cheung, H., Lau, K., Lu, T., & Hui, D. (2007). A critical review on polymer-based bio-engineered materials for scaffold development. *Composites Part B: Engineering*, 38(3), 291–300.
- Ching, A. L., Liew, C. V., Heng, P. W. S., & Chan, L. W. (2008). Impact of cross-linker on alginate matrix integrity and drug release. *International Journal of Pharmaceutics*, 355(1–2), 259–268.
- Clark, A. H., & Rossmurphy, S. B. (1987). Structural and mechanical properties of bio-polymer gels. *Advances in Polymer Science*, 83, 57–192.
- Dobies, M., Kozak, M., & Jurga, S. (2004). Studies of gelation process investigated by fast field cycling relaxometry and dynamical rheology: The case of aqueous low methoxyl pectin solution. *Solid State Nuclear Magnetic Resonance*, 25(1–3), 188–193.
- Eder, M., & Lütz-Meindl, U. (2008). Pectin-like carbohydrates in the green alga *Microcystis* characterized by cytochemical analysis and energy filtering TEM. *Journal of Microscopy*, 231(2), 201–214.
- Evangelista, M. B., Hsiang, S. X., Fernandes, R., Sampaio, P., Kong, H., Barrias, C. C., et al. (2007). Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. *Biomaterials*, 28(25), 3644–3655.
- Farris, S., Mora, L., Capretti, G., & Piervivanni, L. (2012). Charge density quantification of polyelectrolyte polysaccharides by conductometric titration: An analytical chemistry experiment. *Journal of Chemical Education*, 89(1), 121–124.
- Ferreira Almeida, P., & Almeida, A. J. (2004). Cross-linked alginate–gelatine beads: A new matrix for controlled release of pindolol. *Journal of Controlled Release*, 97(3), 431–439.
- Fonseca, K. B., Bidarra, S., Oliveira, M. J., Granja, P. L., & Barrias, C. C. (2011). Molecularly designed alginate hydrogels susceptible to local proteolysis as three-dimensional cellular microenvironments. *Acta Biomaterialia*, 7(4), 1674–1682.
- Fonseca, K. B., Maia, F. R., Cruz, F. A., Andrade, D., Juliano, M. A., Granja, P. L., et al. (2013). Enzymatic, physicochemical and biological properties of MMP-sensitive alginate hydrogels. *Soft Matter*, 9(12), 3283–3292.
- Fraeye, I., Colle, I., Vandevenne, E., Duvetter, T., Van Buggenhout, S., Moldenaers, P., et al. (2010). Influence of pectin structure on texture of pectin–calcium gels. *Innovative Food Science and Emerging Technologies*, 11(2), 401–409.
- Gigli, J., Garnier, C., & Piazza, L. (2009). Rheological behaviour of low-methoxyl pectin gels over an extended frequency window. *Food Hydrocolloids*, 23(5), 1406–1412.
- Gouveia, M. R., Ribeiro, C., de Souza, C. F., Marvila-Oliveira, I., Lucyszyn, N., & Sierakowski, M. (2009). Rheological behavior of borate complex and polysaccharides. *Materials Science and Engineering: C*, 29(2), 607–612.
- Grellier, M., Granja, P. L., Fricain, J., Bidarra, S., Renard, M., Bareille, R., et al. (2009). The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. *Biomaterials*, 30(19), 3271–3278.
- Gutowska, A., Jeong, B., & Jasionowski, M. (2001). Injectable gels for tissue engineering. *The Anatomical Record*, 263(4), 342–349.
- Harholt, J., Suttangkakul, A., & Scheller, H. V. (2010). Biosynthesis of pectin. *Plant Physiology*, 153(2), 384–395.
- Hotchkiss, A. T., Savary, B. J., Cameron, R. G., Chau, H. K., Brouillette, J., Luzio, G. A., et al. (2002). Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *Journal of Agricultural and Food Chemistry*, 50(10), 2931–2937.
- Hunter, J. L., & Wicker, L. (2005). De-esterification of pectin by alkali, plant and fungal pectinmethylesterases and effect on molecular weight. *Journal of the Science of Food and Agriculture*, 85(13), 2243–2248.
- Iglesias, M. T., & Lozano, J. E. (2004). Extraction and characterization of sunflower pectin. *Journal of Food Engineering*, 62(3), 215–223.
- Kastner, H., Einhorn-Stoll, U., & Senge, B. (2012). Structure formation in sugar containing pectin gels: Influence of Ca²⁺ on the gelation of low-methoxylated pectin at acidic pH. *Food Hydrocolloids*, 27(1), 42–49.
- Kong, H. J., Kaigler, D., Kim, K., & Mooney, D. J. (2004). Controlling rigidity and degradation of alginate hydrogels via molecular weight distribution. *Biomacromolecules*, 5(5), 1720–1727.
- Kretlow, J. D., Klouda, L., & Mikos, A. G. (2007). Injectable matrices and scaffolds for drug delivery in tissue engineering. *Advanced Drug Delivery Reviews*, 59(4), 263–273.
- Lee, J. W., Park, J. H., & Robinson, J. R. (2000). Bioadhesive-based dosage forms: The next generation. *Journal of Pharmaceutical Science*, 89(7), 850–866.
- Liu, Z., Jiao, Y., Wang, Y., Zhou, C., & Zhang, Z. (2008). Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*, 60(15), 1650–1662.
- Mitchell, J. R., & Blanshard, J. M. V. (1974). Viscoelastic behaviour of alginate gels. *Rheological Acta*, 13, 180–184.
- Miyazaki, S., Aoyama, H., Kawasaki, N., Kubo, W., & Attwood, D. (1999). In situ-gelling gelatin formulations as vehicles for oral drug delivery. *Journal of Controlled Release*, 60(2), 287–295.
- Morris, E. R., Nishinari, K., & Rinaudo, M. (2012). Gelation of gellan – a review. *Food Hydrocolloids*, 28(2), 373–411.
- Munarin, F., Guerreiro, S. S., Grellier, M. A., Tanzi, M. C., Barbosa, M. A., Petrini, P., et al. (2011). Pectin-based injectable biomaterials for bone tissue engineering. *Biomacromolecules*, 12(3), 568–577.
- Munarin, F., Petrini, P., Tanzi, M. C., Barbosa, M. A., & Granja, P. L. (2012). Biofunctional chemically modified pectin for cell delivery. *Soft Matter*, 8, 4731–4739.
- Munarin, F., Tanzi, M. C., & Petrini, P. (2012). Advances in biomedical applications of pectin gels. *International Journal of Biological Macromolecules*, 51(4), 681–689.
- Munarin, F., Bozzini, S., Visai, L., Tanzi, M. C., & Petrini, P. (2013). Sterilization treatments on polysaccharides: Effects and side effects on pectin. *Food Hydrocolloids*, 31(1), 74–84.
- Naumenko, K., & Altenbach, H. (2007). *Modeling of creep for structural analysis*. Berlin, Heidelberg: Foundation of Engineering Mechanics, Springer.
- Ravanat, G., & Rinaudo, M. (1980). Investigation on oligo- and polygalacturonic acids by potentiometry and circular dichroism. *Biopolymers*, 19(12), 2209–2222.
- Renard, C. M. G. C., & Thibault, J. (1996). Degradation of pectins in alkaline conditions: Kinetics of demethylation. *Carbohydrate Research*, 286, 139–150.
- Salgado, C. L., Sanchez, E. M. S., Zavaglia, C. A. C., Almeida, A. B. A., & Granja, P. L. (2012). Injectable biodegradable polycaprolactone–sebacic acid gels for bone tissue engineering. *Tissue Engineering Part A*, 8(1–2), 137–146.
- Santucci, E., Alhaique, F., Carafa, M., Coviello, T., Murtas, E., & Riccieri, F. M. (1996). Gellan for the formulation of sustained delivery beads. *Journal of Controlled Release*, 42(2), 157–164.
- Sriamornsak, P. (1998). Investigation of pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads. *International Journal of Pharmaceutics*, 169(2), 213–220.
- Sriamornsak, P. (2003). Chemistry of pectin and its pharmaceutical uses: A review. *Silpakorn University International Journal*, 3(1), 206–228.
- Ström, A., & Williams, M. A. K. (2003). Controlled calcium release in the absence and presence of an ion-binding polymer. *The Journal of Physical Chemistry B*, 107(40), 10995–10999.
- Swetha, M., Sahithi, K., Moorthi, A., Srinivasan, N., Ramasamy, K., & Selvamurugan, N. (2010). Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering. *International Journal of Biological Macromolecules*, 47(1), 1–4.
- Tho, I., Kjøniksen, A. L. A., Nyström, B., & Roots, J. (2003). Characterization of association and gelation of pectin in methanol water-mixtures. *Biomacromolecules*, 4(6), 1623–1629.
- Voragen, F., Schols, H., & Visser, S. (2003). *Advances in pectin and pectinase research*. The Netherlands: Kluwer Academic Publishers.
- Mitchell, J. R., & Blanshard, J. M. V. (1976a). Rheological properties of pectate gels. *Journal of Texture Studies*, 7, 341–351.
- Mitchell, J. R., & Blanshard, J. M. V. (1976b). Rheological properties of alginate gels. *Journal of Texture Studies*, 7, 219–234.