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Influence of Homogenization Technique and Blend Ratio on Chitosan/Alginate Polyelectrolyte Complex Properties

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Abstract Polyelectrolyte complex (PEC) films were prepared from chitosan (CHI) and alginate (ALG) which are polymers of opposite charge. Two homogenization techniques and two ratios of ALG CHI blends were compared: mechanical agitation under vacuum (ALG CHI ST) or agitation by high turbulence (ALG CHI UT) and 50/50 or 63/37 ratios. Surface and structure of PEC films are affected by the homogenization technique while the swelling percentage is only affected by polymer ratio. The homogenization ratio does not seem to influence in vitro cell proliferation. Results show that the UT homogenization technique with a 63/37 ratio, which gives films with a smooth, homogeneous surface and a higher rate of enzymatic resistance, is more efficient for cell proliferation and viability. These first results confirm the potential use of ALG CHI films for surgery application.

Keywords Chitosan · Alginate · Polyelectrolyte complex · Film

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1 Introduction

Polysaccharides, nucleic acids and proteins constitute three major classes of biopolymers. Polysaccharides represent the most commonly used biopolymer in the family. They are components of most cells (plant, animal, and microbial). They present suitable characteristics for all biomedical applications. These are based on their properties including non-toxicity, biocompatibility and biodegradability. Several polysaccharides such as pectin, cellulose, chitosan and alginate have been reported to have potential uses in the pharmaceutical and biomedical fields such as drug delivery systems and cell encapsulation [1].

Alginate is a linear polysaccharide obtained from brown algae. It is composed of alternating blocks of α-L-guluronic and β-D-mannuronic acid residues [2]. Alginate was proved to display biocompatibility, non-toxicity, biodegradability and antimicrobial activity [3]. It is known to be simply gelled with divalent cations as calcium ions [4]. It was shown that 90% of sodium ions contained in a sodium alginate solution can be easily moved by calcium ions. Indeed, the calcium ions are able to crosslink the alginate polymers because they can form two bonds, as opposed to monovalent ions such as sodium, which can only form one bond [5].

Chitosan, obtained by the partial deacetylation of chitin, is a polysaccharide consisting of glucosamine (GA) and *N*-acetyl-glucosamine (NAc-GA) linked by β-1 → 4 glucosidic bonds [6]. It has found numerous applications in various fields such as waste-water treatment, agriculture, cosmetics, food processing, packaging industries, textile, electronics and pharmaceuticals [7]. It possesses numerous physicochemical and biological properties: due to its biocompatibility, biodegradability and bioactivity, it is more and more considered as a very interesting substance for

diverse applications such as biomaterial [8]. In normal conditions, clinical tests carried out to promote chitosan-based biomaterials do not report any inflammatory or allergic reaction following implantation, injection, topical application or ingestion in, to or by the human body [9]. Chitosan degradation is influenced by molecular weight (Mw) and the acetylation degree [10]. It is easily hydrolyzed by lysozyme depending on the acetylation degree [11]. In addition, chitosan, as a bioactive polymer, has a part in functions of the human body. It presents antithrombogenic properties [12] and it also stimulates the immune system against viral and bacterial infections [13, 14]. Finally, it possesses wound-healing properties and favors both soft and hard tissue regeneration [15 17].

Chitosan films are moisture-sensitive and alginate films have a brittle mechanical behavior and poor thermal stability. In addition, the degradation of alginate occurs easily in physiological conditions, while chitosan showed poor degradation for up to 28 days when used as implants in rats' abdominal cavities [18]. Since the polymer blend strategy allows for the development of new materials with improved properties, chitosan/alginate films could present synergistic properties. Due to the anionic and cationic nature of alginate and chitosan respectively, the complexation of these two polyelectrolytes is possible [19]. The carboxylate moieties on alginate can ionically interact with the protonated amines on chitosan, forming physical cross-linked hydro-gels. This shaping is known as PolyElectrolyte complex (PEC). The PEC reduces the tendency for swelling and the degradation of alginate and improves structural strength [20].

The mechanical stability of PECs is greater than that of alginate films due to the chitosan contribution [21]. Also, the ionic nature of PECs involves high charge densities and allows for good water affinity and bioadhesivity [22, 23]. Both the biodegradability and biocompatibility properties of chitosan and alginate are maintained after PEC formation [23]. Studies have shown that chitosan hydrogels formed by PEC are well-tolerated systems [24] and can be used in various applications such as drug-delivery systems, cell culture, enzyme immobilization for tissue reconstruction and wound-healing management.

Today chitosan alginate PEC systems are used in biomedical application in the form of membranes, as cell encapsulation [25]. Using PEC in gastrointestinal surgery is innovative and has never been studied. More specifically, alginate/chitosan based biomaterial can be envisaged for the prevention of gastrointestinal fistula (GIF). A GIF is an abnormal opening in the stomach or intestines that allows the contents to leak. Gastrointestinal fistula is a common post-operative complication in abdominal surgery. The leaks can go through intestines creating diseases called entero-enteral fistulas. The most serious complication of GIF is sepsis, an illness in which the body has a severe response to bacteria. This condition may lead to dangerous low blood pressure, organ damage, and even death. Thus, a solution would be to use a wound dressing using biopolymers which would be designed with suitable properties. In particular, the use of this type of material as intra-abdominal surgery implies the development of device with sufficient mechanical strength and good absorption ability. Also, film with a biodegradation time of a few weeks is crucial, as it would leave time for organs to heal without requiring a second operation to remove the film. Biocompatibility is also a key point.

In this work, we aimed at developing optimal chitosan-alginate PEC wound dressings for GIF prevention. If the literature emphasizes the influence of polymers ratios on PEC properties, to the best of our knowledge no studies have yet reported the influence of the homogenization technique on the physico-chemical properties of films. Indeed, the homogenization step is a key parameter to provide films with properties for the focused application. The blending step should be optimal to obtain interpenetrating polymer chains networks without affecting the opposite-charged polymer interactions. Different alginate/chitosan blend ratio films were prepared using a casting evaporation method and two different techniques of homogenization were investigated, namely a high shearing mix, using an Ultra turrax (UT), and a soft rotating homogenization under vacuum. The resulting mechanical behavior, swelling properties, degradation rate, surface characteristics and porosity of the films were then studied and their interactions with cells analyzed.

2 Experimental

2.1 Materials

Low viscosity alginic acid sodium salt (CAS: 9005-38-3; Batch: 090M0092 V) and medium molecular weight (MW) chitosan (CAS: 9012-76-4; Batch: MKBH1108 V) were purchased from Sigma Chemical Company (St. Louis, Missouri, United States) and used as received. The main characteristics of the employed materials, designated as ALG and CHI were studied. The chitosan deacetylation degree (DA) was determined by FTIR (following the procedure described by Shigemasa et al. [26], given by Eq:

$$\frac{A_{1595}}{A_{2875}} = 0.0125 \times DA + 0.2 \ (R^2 = 0.99)$$

where A1595 and A2875 = chitosan absorption area bands at 1595 and 2875 cm^{-1}, respectively specific deformation of the NH band at 1595 cm^{-1} and non-specific elongation of 2875 cm^{-1} CH functions.

The viscosimetric average molecular weights (Mw) of the polymers were estimated at 25 °C in 0.1 M NaCl for ALG and at 25 °C; in 0.1 M CH_3COOH for chitosan, using known α and κ values from the literature for chitosan [27] and alginate [28] by the Eq:

$$V = \kappa(M_V)^{\alpha}$$

where V is the intrinsic viscosity of the polymer α and κ are constants which are dependent on the interactions between the polymer and a specific solvent for a given solution.

The fraction of guluronic acid (G) and mannuronic acid (M) in the alginates was determined by NMR (Nuclear Magnetic Resonance) spectroscopy [29].

2.2 Conductimetric Study

The reaction of electrolytic interactions between alginate and chitosan was investigated by conductivity. This technique makes it possible to study the kinetics of the complexation of carboxyl and amine functions. The conductivity measurements were performed at 25 °C in a glass cell, controlled with mechanical stirring at 500 rpm. The conductimeter CDM210 with an error of 0.1 $\mu S.cm^{-1}$ was calibrated before each measurement. The pH was also recorded using a pH meter Calimatic 766 calibrated before each measurement.

Chitosan was dissolved in an excess of 0.1 M hydrochloric acid [30] and filtered through Millipore membranes 0.45 μm cellulose. The alginate was dissolved in distilled water. The chitosan solution (1.5% w/v, pH 1.59) was added to the alginate solution (1.5% w/v, pH 7.48) by 0.5 mL intervals. Conductivity and pH are statements of the stabilization of the measured values [31].

2.3 Biopolymers Films

Chitosan, 1.5% (w/v), was dissolved in water containing 1% (w/v) of acetic acid solution; a sodium alginate 1.5% (w/v) solution was prepared by dissolution in deionized water. Both solutions were stirred separately using a mechanical stirrer overnight.

Each solution was poured into 11 cm diameter petri dishes, dried in an oven at 50 °C for 48 h. Secondary treatment, intended to neutralize free groups was then applied to the dry films. The CHI films were treated with NaOH (0.1 M) solution and ALG films were treated with $CaCl_2$ (1%) solution for 15 min. Then these films were washed with distilled water until neutral pH was obtained. The films were then dried again between two glass plates at 50 °C for 48 h in order to obtain xerogels.

The solvent evaporation process was stopped upon stabilization of the mass weight of the measured films.

2.4 Preparation of Polyelectrolyte Complexe Films

Three films were prepared by casting and evaporation. The corresponding solution of each polymer was prepared as follows: chitosan, 1.5% (w/v), was dissolved in water containing 1% (w/v) of acetic acid solution; sodium alginate 1.5% (w/v) solution was prepared by dissolution in deionized water. Both solutions were stirred separately using a mechanical stirrer overnight.

Chitosan alginate blends were prepared by adding an alginate solution into a chitosan solution at 50/50 or 63/37 massic percentage ratio respectively.

Then these blends were stirred with one of the following homogenization techniques:

i. With an Ultra-turrax homogenizer (IKA T25, Staufen, Germany) at 1000 rpm, for 11 min. The Ultra-Turrax is an instrument for dispersion and homogenization, based on a rotor/stator system.
ii. Or with a Stephan homogenizer (Stephan UMC 5 electronic, Schwarzenbek, Germany), under vacuum at 100 mbars, 500 rpm, for 45 min. Stephan mixes the solution with blades attached to a rotating support under controlled vacuum.

The mixture was poured into an 11 cm Petri dish, and dried in oven at 50 °C for 48 h.

A secondary treatment intended to neutralize free groups was then applied to the dry films.

The films were immerged in a bath of NaOH (0.1 M) and $CaCl_2$ (1%) for 15 min then washed with distilled water until a neutral pH was obtained. The films were then dried again between two glass plates at 50 °C for 48 h in order to obtain xerogels.

The solvent evaporation process was completed for the mass transfer measurements.

2.5 Degree of Swelling of Chi–Alg Membranes

CHI-ALG membranes were weighed individually and immersed in 0.9% NaCl and 37 °C for a period of 24 h. Their weights were measured at different intervals. The degree of swelling was measured using the Equation:

$$\text{Weight change } (\%) = (Ww - Wi)/Wi \times 100\%$$

where Ww and Wi refer to the final weight and initial weight of the membranes respectively.Each sample was analyzed in triplicate.

2.6 Biodegradation of the Complexes

The degradation test was conducted by incubating the samples in 0.9% NaCl containing 3 g/L of porcine pancreatine at 37 °C. Every two days, the pancreatine

solutions were replaced. The experiments were conducted over a period of 14 days. The complexes were washed with distilled water to remove any buffer and lysozyme remaining on the surface and dried to constant weight for weight measurement.

The remaining weight of the complex after the enzymatic degradation was calculated using the following equation:

$$\text{Weight remaining } (\%) = (\text{Wf} - \text{Wi})/\text{Wi} \times 100\%$$

where Ww and Wi refer to the final weight and initial weight of the membranes, respectively.

Five specimens were analyzed for this experiment.

2.7 Mechanical Properties

The mechanical characterization of membranes was performed on H3-type dumbbell specimens with a HSKT Universal Testing Machine (Tinius olsen, Horshaw, USA). The specimen geometry is 70 × 6 × 0.06 mm (length × width × thickness). The samples were conditioned to relative humidity and temperature controlled (60%, 25 °C) before 7 days of testing. They were tested using a tensile testing machine equipped with a 100 N force sensor. The tensile speed is 10 mm/minute, and the measurements were carried out up to the fracture of the sample. The mechanical properties were determined from the stress strain curve: elastic modulus (Em).

Seven dumbbell specimens were analyzed for every film.

2.8 Porosity

The estimation of porosity was performed by measuring the displacement of a liquid through the sample [32]. Dried films were weighed to obtain the initial mass of the sample (m_0). After immersion of the films into ethanol, the liquid started to invade the porous spaces originally occupied by air [33, 34].

Ethanol was chosen as it can diffuse across membranes without the shrinking or swelling phenomenon. The procedure was as follows. Films were immersed in anhydrous EtOH and maintained under vacuum for 20 min. The final mass of the sample (m_f) was measured after the excess of alcohol on the surface was absorbed by a filtration paper. The porosity was then calculated with the equation:

$$\text{Porosity} = \frac{m_0 - m_f}{\rho \times V} \times 100$$

with m_o the initial mass of the sample (g), m_f its final mass (g), ρ its density (g/m^3) and V its volume (m^3).

Each sample was analyzed in triplicate.

2.9 Contact Angle Measurement

Contact angle measurements were performed in ambient conditions with a commercial system (MCAT Digidrop, GBX, France). The sessile drop method was used to determine the contact angle between a water droplet and the substrate. The volume of the water droplet was 1 µl. The contact angles were measured for both the left and right sides of the drop. The average contact angle of a reported substrate was the average of five different drop images on a substrate.

Each sample was analyzed in triplicate.

2.10 Culture of Miapaca2 and Panc1 Cells

The cell lines were chosen because they are derived from human digestive cells.

The MiaPACA-2 and PANC-1 cells were cultured in DMEM supplemented with 10% FCS (Fetal Calf Serum) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere of 5% CO2/95% air at 37 °C. We used 3 mL of medium according to the lineage in each well. The incubation was carried out at 37° C and the culture medium was changed every 72 h.

A sterile plate 6 well was used. A well has a volume of 3 mL and a surface of 9.6 cm^2.

The films were cut to form 2-cm squares.

Before the inoculation, the films were washed for 15 min with the culture medium. They were also treated with a UV sterilization lamp for 10 min to reduce pathogens and resistant organism.

The pH was checked before inoculating the cells into the wells. In all the cases where the pH was not neutral, the film was not used. Indeed, it was necessary to avoid toxic compounds like NaOH inhibiting the growth.

Cells were seeded at 2.5 × 10^5 per film.

The control group was cells cultured on culture plates without film.

To evaluate cell proliferation, the cells were detached from membranes by trypsinization. Automated cell counting was used. Cells were also observed using an optical microscope.

Each sample was analyzed in triplicate.

3 Results and Discussion

Alginate/chitosan films were prepared by casting/evaporation method using two different techniques of homogenization: the Ultra-turrax homogenizer (UT) and the Stephan® homogenizer (ST). While the ST technique is a simple mechanical stirring under vacuum, the agitation generated by the UT is caused by rapidly-rotating sets of metal blades within a cylindrical set of outer blades. This

system is employed when high shear forces are required for homogenization purposes [35]. This method was studied to optimize the dispersion of one polymer in order to obtain composites and it promises to be more efficient, when compared to the ultrasound bath and sonicator [36]. If chitosan is commonly described as a "single" polysaccharide, it is in fact a wide class of different polymers with various degrees of deacetylation and molecular weights. Since these parameters influence chitosan properties, it is important to characterize the polymer before its use. In this purpose, chitosan viscosity-average Mw was evaluated to be 1,200,000 ± 2800 g/mol and the degree of deacetylation (DD) 80.9% as determined by FTIR. Indeed, chitosan DD is an important parameter since the formation of PECs takes place upon the ionic interactions between protonated amines chitosan and carboxylate groups of alginate. Lee et al. [37] examined the influence of chitosan DD on the stoichiometry of the PEC. They showed that at a given pH the composition of the PEC shifted to lower alginate content as the DD of chitosan decreased. Alginate viscosity-average Mw was Mw = 95 500 ± 1768 g/mol as estimed at 25 °C in 0.1 M NaCl. The molar fraction of mannuronate ($F_M = 0.82$) and guluronate ($F_G = 0.28$) were determined by 1H NMR spectroscopy. However, the formation of alginate/chitosan PEC is not affected by the type (F_M/F_G ratio) of alginate used [38], in contrast to the formation of calcium cross-linked alginate hydrogels.

3.1 Characterization of PEC Complexation

The interpolyelectrolyte reaction between chitosan and alginate was followed by conductimetry to determine the ratio for which the complexation between carboxylic groups of alginate and ammonium groups of chitosan is maximal and the formation of the complex is completed.

The complexation between a solution of chitosan chlorohydrate (pH 3.91) and a solution of sodium alginate (pH 6.24) can be described as follows:

$$\wedge\wedge COO^- Na^+ + \wedge\wedge Cl^- NH_3^+ \quad \rightarrow \wedge\wedge COO^- NH_3^+ \wedge\wedge + Na^+ + Cl^-$$

Hydrochloric acid was chosen to obtain the highest ionic concentration and improve the conductivity measurement [39].

This equation shows the production of light ions, i.e., Na^+ and Cl^-, as the complex is formed. Hence an increase of the conductivity occurs in the solution while the chitosan is added. Before the equivalence point, the excess of alginate gives the following equilibrium:

$$\wedge\wedge COO \;\; Na^+ + H_2O \rightarrow \wedge\wedge COOH + OH \;\; + Na^+$$

Figure 1 shows titration curves of ALG-CHI system. Conductivity and pH vary depending on the mixture composition. They are given as a function of the ratio between the total massic concentration of chitosan chlorohydrate and the massic concentration of sodium alginate, as chitosan is added.

The initial value of conductivity corresponds to the sodium alginate solution. For $0 < V_{CHI} < 31$ mL, a linear behavior is observed in relation to the liberation of Na^+ and Cl^+. The low slope is explained by the polyanion consumption.

A change in the slope is observed at $V_{CHI} = 31$ mL, indicating a complete complex formation. After this equivalence point, a higher increase in the specific conductivity is observed. Indeed, the chitosan chlorohydrate excess dissociates and highly mobile protons H^+ are released in the solution:

$$\wedge\wedge NH_3^+ Cl \;\; + H_2O \rightarrow \wedge\wedge NH_2 + H_3O^+ + Cl$$

These results led us to determine the equivalence point, which was found for a massic percentage ratio of ALG CHI: 63/37.

It is known that alginate has low mechanical properties [40] and poor enzymatic resistances. Indeed ionically cross-linked alginate gels can be dissolved by the release of the divalent ions cross-linking the gel into the surrounding media due to exchange reactions with monovalent cations such as sodium ions, or the enzyme as hydrolase and amylase which can cleave the polymer chains with sugar units [41].

In order to improve the above properties PEC films containing a higher amount of CHI were also prepared and compared to the equivalence point blends (ALG CHI 63/37).

Several films were prepared from different polymers blend ratios mixed with two different techniques of homogenization: ALG CHI 50/50 ST, ALG CHI 50/50 UT, ALG CHI 63/37 ST and ALG CHI 63/37 UT.

50/50 and 63/37 represents ALG CHI massic percentage ratio; ST is used for blends mixed with ST and UT with UT.

In aqueous media (pH 5), the polyelectrolyte complex formation is due to the ionic interactions between carboxylate anions of alginate [pKa 3. 38 (G) et 3.65 (M)] and ammonium cations of chitosan (pKa 6.5). The following steps of film elaboration process (casting/evaporation) could dissociate these interactions and polymer/phase separation could occur (addition of calcium chloride salt or solvent evaporation step). In order to study the influence of polymers ratios and homogenization techniques on the interpenetrating polymer network structure, mechanical properties were correlated to theoretical laws (Voigt and Reuss Models) to determine the optimal experimental conditions in Table 1. The different blends present intermediate moduli between the values of both pure polymer ALG and CHI. To estimate the Young's modulus of the

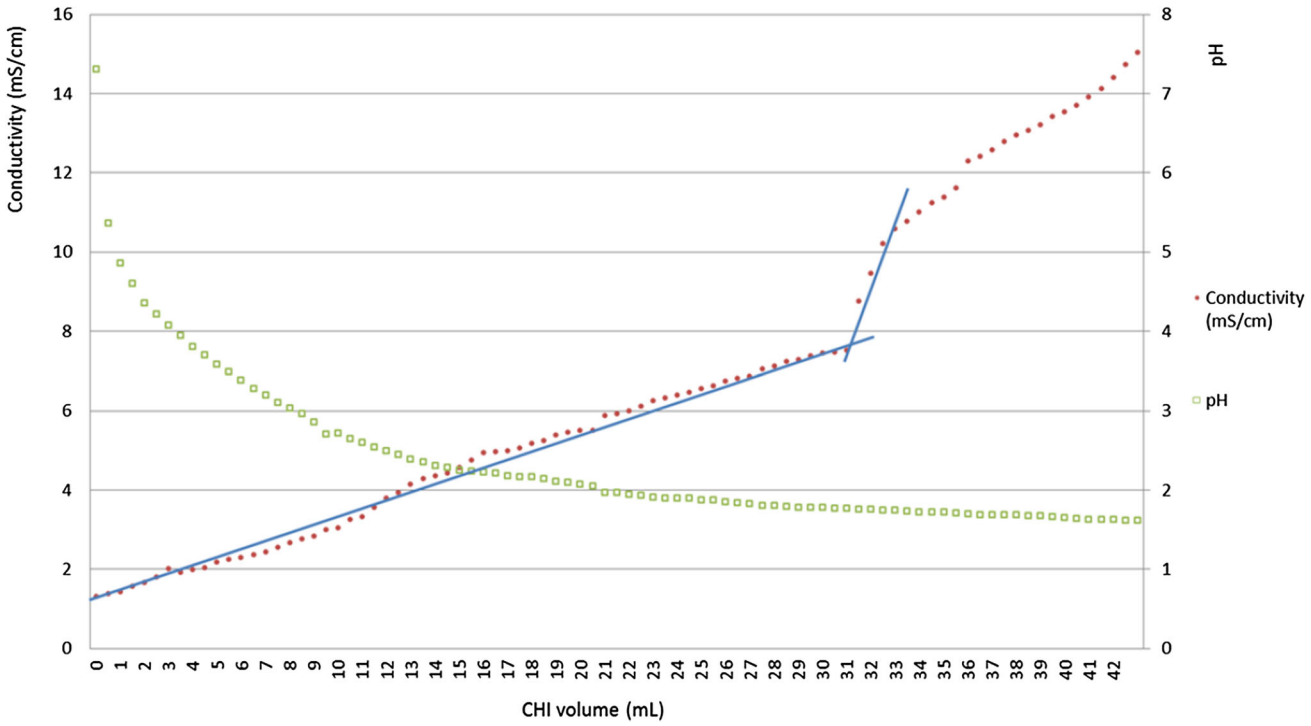

Fig. 1 Conductimetric titration of ALG CHI system

Table 1 Theoretical composition and experimental and theoretical Young's moduli of ALG CHI films

Sample	Theoretical composition (volumic %)		Experimental results	Theoretical results
	Φ_{ALG}	Φ_{CHI}	Young's modulus E (MPa)	Voigt model
ALG	100	0	654.8 ± 46.3	
CHI	0	100	2389.5 ± 82.5	
ALG CHI 50/50 ST	10.8	89.2	744.4 ± 109.2	2202.6
ALG CHI 50/50 UT	10.8	89.2	1409.3 ± 165.0	2202.6
ALG CHI 63/37 ST	40.7	59.3	789.2 ± 68.0	1682.9
ALG CHI 63/37 UT	40.7	59.3	1694.9 ± 256.4	1682.9

Φ $\frac{wi}{wi+\left(\frac{\rho_1}{\rho_2}\right)x(1-wi)}$ (Eq. 3) with w_I the mass fraction of ALG and CHI in the blend and ρ_I the measured density of ALG CHI

PEC, Voigt and Reuss models were applied. The two simple models are the so-called parallel and series models, which should represent the upper and lower bounds of the tensile strength predictions. Equations 1 and 2 give these two bounds in which E and Φ are the modulus and the volume ratio of each polymeric powder respectively. Volume fractions (Φ) are determined according to Eq. (3), using the density (ρ) of ALG = 0.786 and CHI = 0.258. Voight's model is applicable to materials in which the components are connected parallel to one another (Eq. 1) given by the rule of mixtures, so that the applied stress lengthens each component to the same extent. In the lowest lower bound series model, the blend components are arranged in series (Reuss prediction) perpendicular to the direction of the applied force. The Reuss modulus prediction is given by the inverse rule of mixtures (Eq. 2).

Based on Hill's work [42], the bounds for Young's modulus of the composite $E_{composite}$ therefore can be given as:

$$E_{Reuss} \leq E_{composite} \leq E_{Voigt}$$

where

$$1/E_{Reuss} = \Phi_1/E_1 + \Phi_2/E_2 \quad (1)$$

$$E_{Voigt} = \Phi_1 E_1 + \Phi_2 E_2 \quad (2)$$

Φ_i = volumic percent of ALG and CHI in the PEC [43].

The density of films was measured to determine the volumic fractions of polymer powders of each polymer

ALG and CHI. The mechanical properties of ALG-CHI films were studied using tensile strength experiments.

Whatever the composition of the blend, the theoretical volumic fraction of ALG powder is in minority in the film (10.8 40.7% of the global volume).

As already described [44] the chitosan film presents higher Young's modulus than the alginate film, which is mechanically weaker.

PEC films have intermediate mechanical properties. However, the ratio does not significantly influence the Young's modulus values for the same homogenization technique.

On the contrary, the homogenization technique shows an important impact on mechanical properties. For an identical polymers ratio, films homogenized with the UT technique present Young's modulus values twice as high as those homogenized with ST.

Theoretical results for Voigt and Reuss models and experimental data are shown in Fig. 2. Experimental values, as described in Hill's law, are between Voigt and Reuss models.

PEC films prepared from blends homogenized with the UT technique present Young's modulus values closer to Voigt's Model. This result indicates that the homogenization step is the key parameter in the films' process elaboration. Indeed if this step is optimized, the mechanical properties of obtained films display optimal interactions between both polymers. The UT homogenization technique creates an axial movement which draws the mixture of liquid or suspension into the reduced space between the rotor and the stator. In addition, with the slots on the stator, the mixture is subjected in a radial motion to very high shear forces and thrust that provide dispersion and homogenization of the solution.

On the contrary, the ST homogenization technique mixes the solution with blades attached to a rotating support under controlled vacuum.

The Young's modulus of ALG CHI 63/37 UT fits perfectly with the theoretical Voigt model. In conclusion film prepared from ALG CHI ratio of 63/37 and homogenized by the UT technique is the optimal PEC in terms of polymer complexation as expected from conductimetry study.

3.2 Structure and Surface Characterizations

In order to characterize more deeply the ALG/CHI structure and surfaces of films, porosity and contact angle measurements were assessed (Table 2). Whatever the polymers ratio, PEC films present a low porosity since they are xerogel [44]. Indeed, the polymer concentration (or ratio) and viscosity of the mixture have no effect on the porosity of xerogels unlike aerogels. In evaporative drying processes, the tension of the meniscus at the solvent vapour interface draws together the colloidal units of the gel and brings to the formation of xerogels with minimal

Table 2 Porosity and contact angle values of the different films

Sample	Porosity (%)	Contact angle (°)
CHI	1.9 ± 0.3	75 ± 2
ALG CHI 50/50 ST	3.7 ± 0.2	45 ± 2
ALG CHI 63/37 ST	4.1 ± 0.5	45 ± 5
ALG CHI 50/50 UT	2.1 ± 0.4	63 ± 5
ALG CHI 63/37 UT	2.5 ± 0.5	61 ± 5
ALG	ND	32 ± 2

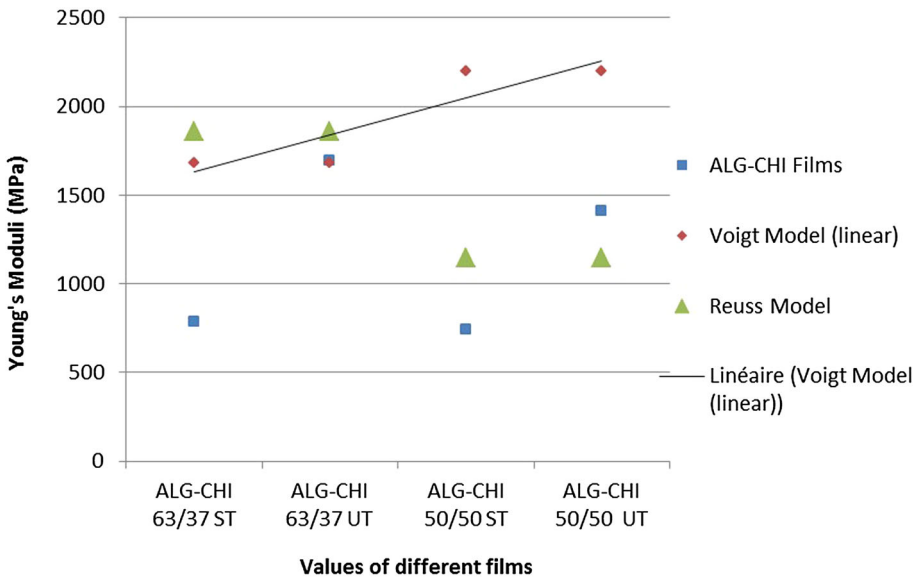

Fig. 2 Experimental values of Young's moduli for the ALG CHI films compared to Voigt and Reuss models

surface area [45]. However, we noticed that values are slightly influenced by the homogenization technique. Indeed, films homogenized with ST are more porous than those homogenized with UT (3.7 ± 0,2% and 4.1 ± 0,5% for 50/50 ST and 63/37 ST; 2,1 ± 0,4% and 2.5 ± 0,5% for 50/50 UT and 63/37 UT, respectively).

Contact angle values can reflect the hydrophilic character of a surface. The contact angles were analyzed using the surface of the films obtained in contact with the air and not the cast. Chitosan film surface presents the most hydrophobic surface while other samples present hydrophilic surfaces as 75 ± 2°. The alginate film surface is the most hydrophilic 32 ± 2°. This result was expected since the pK of guluronic is 3.38 and that of mannuronic 3.65. The pKa of chitosan is 6.5. At a neutral pH, each alginate unit was deprotonated (anionic carboxylate) and desacetylated chitosan units were deprotonated too (neutral amine function). The charge parameter of ALG films explains the hydrophilic character, while uncharged CHI unit are less hydrophilic than ALG.

PECs display intermediate contact angle values. The homogenization technique influences film surface characteristics since ALG/CHI 50/50 UT and ALG CHI 63/37 UT films have higher contact angle values (63° and 61°) than ALG/CHI 50/50 ST and ALG CHI 63/37 ST (approximately 45°). ST films are more hydrophilic than UT films. In addition, the polymers ratio does not influence the hydrophilicity.

In conclusion, structure and surface characteristics are only affected by the homogenization technique.

Since the ratio does not influence the surface characteristics, only ALG CHI 63/37 ST and ALG CHI 63/37 UT were observed by SEM (Fig. 3) to obtain the contact angle value difference. Parts A, B, C and D are upper surface.

Chitosan film presents a smooth surface while Alginate film surface is rough with the presence of polymer agglomerates. ALG CHI films homogenized with ST show significant roughness. They resemble the ALG film surface while the ALG CHI films homogenized with UT have a smooth surface similar to CHI films. As for the ALG CHI film homogenized with ST, the fibrils may be associated with alginate aggregates and correspond to a surface network of alginate formed when the film is dried [46, 47]. This finding corroborates the contact angle value of ALG-CHI films homogenized with ST, which is closer to ALG film value, showing more hydrophilic surfaces.

SEM micrographs of PEC films cross section (Fig. 3) show a compact structure constituted by parallel layers. Similar descriptions of the microstructure of ALG-CHI based films examined with scanning electron microscopy have been reported by other authors [46, 48]. No significant difference was observed on the cross section of ALG CHI-UT and ALG-CHI ST films SEM images.

The degradation test was conducted by incubating films with pancreatic enzyme solutions stirred at 120 rpm and at 37 °C. Figure 4 presents the percentage of degradation as a function of time. As expected chitosan film is much more resistant to enzymes since only 16.6% is degraded in 14 days while alginate film is completely degraded after 6 days. It can be noted that the homogenization technique influences the enzymatic resistance property of PEC films. Films homogenized with UT show a lower degradation percentage (26 and 33% for ALG CHI 50/50 UT and ALG CHI 63/37 UT respectively) than those homogenized with ST (42 and 59% for ALG CHI 50/50 ST and ALG CHI 63/37 ST respectively). It is also obvious than the degradation rate shifted to higher percentage as the content of alginate increased (ALG CHI 50/50 vs. ALG CHI 63/37) for the same homogenization procedure.

The ALG CHI 50/50 UT film presents the best resistance to enzymes degradation. Indeed, a higher chitosan content in films allows for a better enzymatic resistance and the UT technique has already been determined as the optimal homogenization technique for the complexation of polymers. In the literature, authors showed that steric hindrances and electrostatic interactions tend to reduce the degradation of PEC [49].

The absorption capacity of the different films was determined using the immersion method. Matrixes were immersed in 0.9% NaCl and weighed every 10 min having been drained on a sintered glass filter connected to a vacuum pump. The measured weight is based on the initial dry mass by the following formula: $T_t = (m_t - m_0)/m_0$ (with T_t: swelling rate at time t, m_t: mass at time t, m_0: the initial mass). It can be observed on Fig. 5 that the chitosan film presents the lowest swelling capacity (220%) while the alginate film swells ten times as much (2200%). This difference is due to the higher hydrophilic character of alginate. The pH of the NaCl solution is 5.5. Regarding the pKa of ALG and CHI, carboxylic functions are deprotonated and the amine groups are protonated. Swelling properties are optimal in this condition.

PEC films present intermediate swelling properties. The higher the alginate content, the higher the swelling percentage, this, independently from the homogenization technique used. Films with 63% of alginate (ALG CHI 63/37 ST and ALG-CHI 63/37 UT) reached almost 1600% while those containing only 50% of alginate (ALG-CHI 50/50 ST and ALG CHI 50/50 UT) swell less than 1000%. The maximum swelling percentage is reached after 20 min for all samples.

Only the ratio of PEC shows an influence on the percentage of the swelling of the films. Although the homogenization technique impacts the structure and hydrophilicity of surfaces, it is not related to swelling properties.

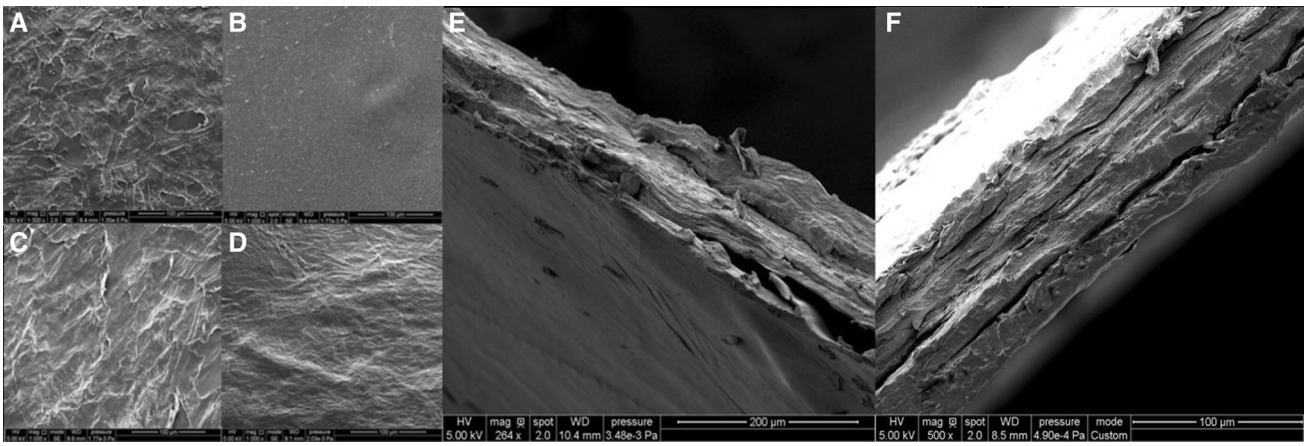

Fig. 3 SEM micrographs *left* ×1000: **a** ALG film, **b** CHI film, **c** ALG CHI ST film, **d** ALG CHI UT film *right* ×264: **e** ALG CHI 63/37 ST xerogel's cross section *right* ×500: **f** ALG CHI 63/37 UT xerogel's cross section

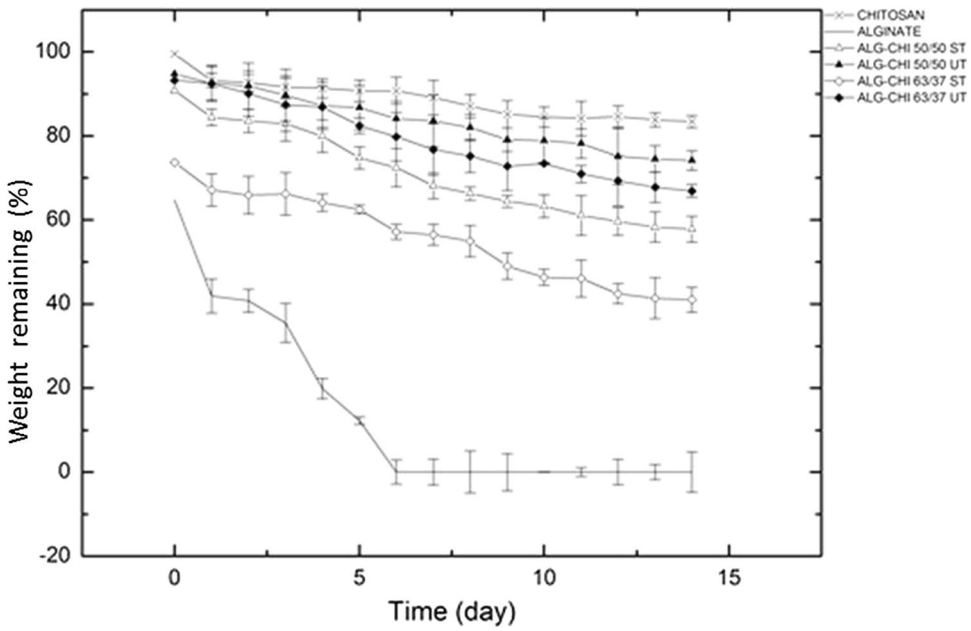

Fig. 4 Degradation kinetic of ALG films, CHI films and ALG CHI films

3.3 Cell Proliferation Study

In order to evaluate the cytotoxicity of our films and to study the influence of their physico-chemical properties on cell response, MiaPACA-2 and PANC-1 cell proliferation was studied. Figure 6 presents the relative growth proliferation of MiaPACA-2 and PANC-1 cells on the different samples in comparison with the control. The control was cells cultured on culture plates without film, and represents 100% proliferation.

The alginate film shows the lowest amount of MiaPACA-2 and PANC-1 cell proliferation (23.2 and 12.4%, respectively). The chitosan film does not present a high percentage of proliferation. It is clear that PEC films allow us to obtain better cell viability than pure alginate or chitosan films. Culture cell assays were done at pH 7.4, at which most amine groups of chitosan are deprotonated and alginate present anionic carboxylic functions. However, cell adhesion needs to be considered in order to allow cell life and proliferation. Thus the ionic parameter can impact cell proliferation.

Direct interactions between cells and the substrate can be affected by the charge of the substrate: it is accepted that cellular membranes are anionic, so a negatively charged surface can lead to the repulsion of cells and then limit their adhesion onto the substrate [50]. It can also be the case for ALG films. On the contrary, cationic substrates can strongly interact with cell membranes and sometimes damage them, as can be the case for chitosan substrates, which bears a part of protonated amines [51]. In this context, PEC films can promote a better cell/substrate interaction due the lower availability of their charges when

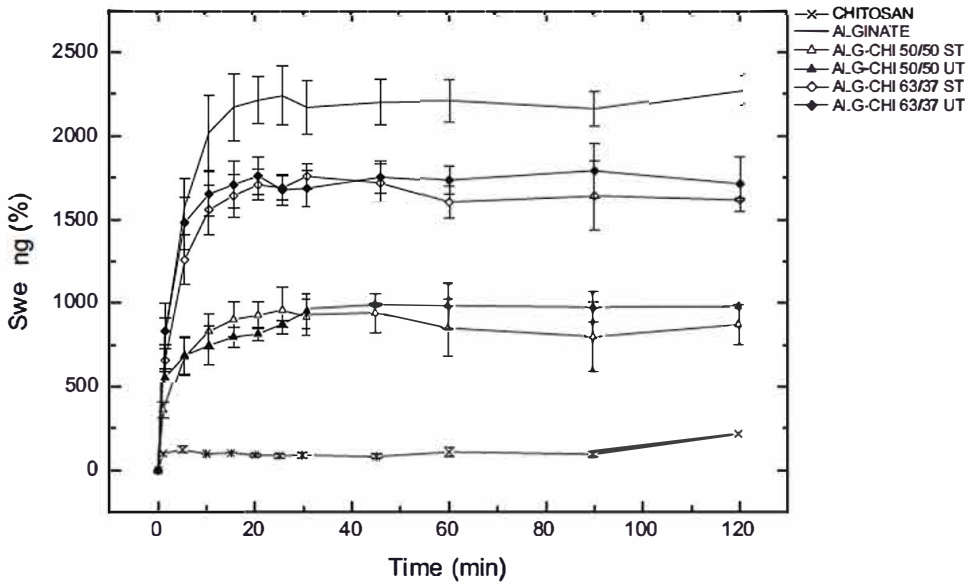

Fig. 5 Swelling degree of ALG films, CHI films and ALG CHI films

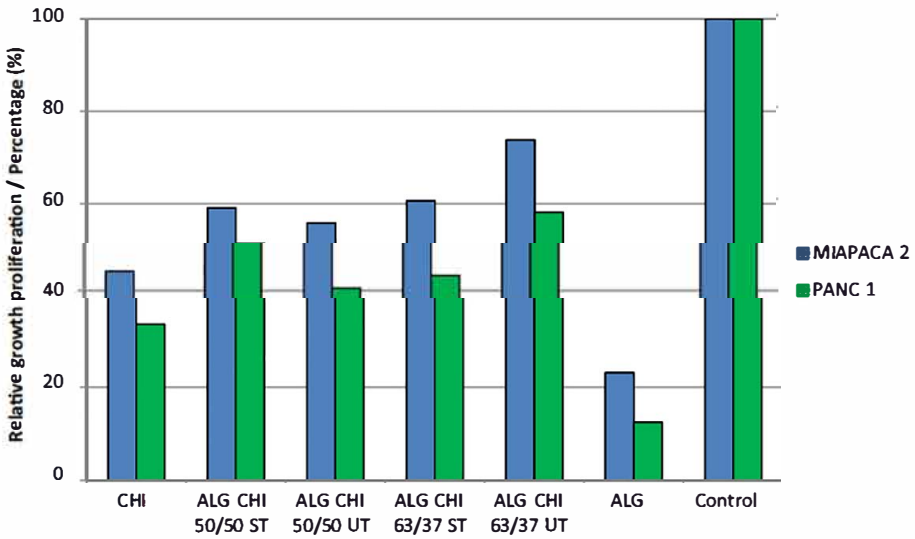

Fig. 6 Relative growth proliferation (%) of MIAPACA 2 and PANC 1 cells with CHI films, ALG films, and ALG CHI films at day 7

wrapped with the other polymer. Additionally, the overall charge of the substrate will be more neutral than that of pure polymers.

Once again, the higher the amount of alginate, the higher cell proliferation percentage (ALG CHI 63/37 vs ALG CHI 50/50). It was shown that the technique of homogenization influences the surface characteristics of PEC films: the ST technique leads to roughness and a more hydrophobic surface than the UT one. These results can explain the more suitable affinity of PANC-1 cells for ALG CHI 50/50 ST than for ALG CHI 50/50 UT and for ALG CHI 63/37 ST than for ALG CHI 63/37 UT. However, the cell growth profile is not exactly the same for MiaPACA-2 cells. We can therefore deduce that cell behavior depends on the type and lineage. The ratio does not have any influence on cell proliferation.

The best cell viability is obtained for samples containing 63% of alginate and homogenized with UT (ALG CHI 63/37 UT) with 70.8% of MiaPACA-2 cell proliferation. In the literature, the rate of cell attachment to polymers with a wet contact angle of 60° to 70° has been reported to be high which is confirmed by the contact angle value of 61 ± 5° for ALG CHI 63/37 UT films [52].

Cell bioadhesion is also driven by an intermediary protein layer. This protein layer is absorbed on the surface. The efficiency of this effect is dependent on the hydrophilicity/hydrophobicity balance of the substrate [53, 54]. Highly hydrophilic surfaces will weakly interact with proteins whereas highly hydrophobic surfaces can denature proteins and then limit their good interaction with cells. If a generality is difficult to address, moderately hydrophilic surfaces are generally considered as suitable. Knowing that, and

considering that hydrophilicity follows this order (by increasing order, Table 2) CHI < ALG CHI 50/50 UT = ALG CHI 63/37 UT < ALG CHI 50/50 ST = ALG CHI 63/37 ST < ALG, we can presume that the medium hydrophilicity of PEC films compared to CHI films and especially ALG films (highly hydrophilic) can favor the substrate/protein/cell interactions and then their proliferation.

Owing to these reconsiderations, we believe that the higher proliferation on PEC films is related to their ability to support cell adhesion due to their "moderate" physico-chemical properties.

Owing to the ISO 10993-5, a reduction by more than 30% is considered a cytotoxin. All films (excepted ALG CHI 63/37 UT for MiaPaca-2 cell line) are potentially moderately cytotoxic.

Presumably the UT films surfaces allow for better cell proliferation compared to ST films. It is also possible that the cell proliferation is limited by the release of ionic charges in the culture medium or that the toxic compounds from the film preparation (as $CaCl_2$) limit the growth even if it did not alter the pH.

To conclude, only ALG CHI 63/37 UT films do not show any cytotoxicity against MIAPACA-2 cells which is an inherent characteristic for all kinds of surgical applications.

4 Conclusion

In the present work two parameters were studied on PEC films properties: polymer (alginate/chitosan) ratio and homogenization technique were implemented. Overall, biodegradation, swelling and cytotoxicity are mostly influenced by the polymer ratio. On the other hand, the structure and the mechanical properties of PECs were mostly influenced by the homogenization technique.

The increase of alginate content allows us to obtain PEC films with higher swelling but less resistance to enzymes and cell affinity. The technique used for the homogenization step is also decisive. High shearing conditions (UT), which permit us to mix more intimately both polymers, lead to PEC films with better enzyme resistance, less porous and less permeable, but with smoother and more hydrophilic surface and cell affinity than the mechanical homogenization under vacuum conditions (ST), all in keeping the same swelling property.

These properties must be confirmed by in vivo studies to validate the potential of these films for surgical application.

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References

1. Alves, N. M., & Mano, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environ mental applications. *International Journal of Biological Macro molecules, 43*(5), 401 414.
2. Augst, A. D., Kong, H. J., & Mooney, D. J. (2006). Alginate hydrogels as biomaterials. *Macromolecular Bioscience, 6*(8), 623 633.
3. Mohandas, A., et al. (2015). Exploration of alginate hydro gel/nano zinc oxide composite bandages for infected wounds. *Int J Nanomedicine, 10*(Suppl 1), 53 66.
4. Eiselt, P., et al. (2000). Porous carriers for biomedical applica tions based on alginate hydrogels. *Biomaterials, 21*(19), 1921 1927.
5. Seely, G. R., & Hart, R. L. (1974). The binding of alkaline earth metal ions to alginate. *Macromolecules, 7*(5), 706 710.
6. Madihally, S. V., & Matthew, H. W. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials, 20*(12), 1133 1142.
7. Chatelet, C., Damour, O., & Domard, A. (2001). Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials, 22*(3), 261 268.
8. Choi, C., Nam, J. P., & Nah, J. W. (2015). Application of chi tosan and chitosan derivatives as biomaterials. *Journal of Industrial and Engineering Chemistry, 33,* 1 10.
9. Muzzarelli, R. A., Nicoletti, C., & Rocchetti, R. (1974). Inter actions of metal ions with alginic acid, polygalacturonic acid, carboxymethylcellulose and cutin. *Ion Exchange Membranes, 2*(1), 67 69.
10. Kean, T., & Thanou, M. (2010). Biodegradation, biodistribution and toxicity of chitosan. *Advanced Drug Delivery Reviews, 62*(1), 3 11.
11. Pangburn, S. H., Trescony, P. V., & Heller, J. (1982). Lysozyme degradation of partially deacetylated chitin, its films and hydro gels. *Biomaterials, 3*(2), 105 108.
12. Graisuwan, W., et al. (2012). Multilayer film assembled from charged derivatives of chitosan: Physical characteristics and biological responses. *Journal of Colloid and Interface Science, 376*(1), 177 188.
13. Wu, Y., et al. (2015). Serum sodium: A reliable and validated predictor for mortality in enteric fistula patients complicated with sepsis. *Journal of Investigative Surgery, 28*(3), 131 139.
14. Wu, C. S., et al. (2015). Antibacterial activity and in vitro eval uation of the biocompatibility of chitosan based polysaccharide/ polyester membranes. *Carbohydrate Polymers, 134,* 438 444.
15. Muzzarelli, R. A., et al. (1999). Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. *EXS, 87,* 251 264.
16. Muzzarelli, R. A., et al. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regenera tion: A review. *Carbohydrate Polymers, 89*(3), 723 739.
17. Busilacchi, A., et al. (2013). Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydrate Polymers, 98*(1), 665 676.
18. Zhao, F., et al. (2002). Preparation and histological evaluation of biomimetic three dimensional hydroxyapatite/chitosan gelatin network composite scaffolds. *Biomaterials, 23*(15), 3227 3234.
19. Baruch, L., & Machluf, M. (2006). Alginate chitosan complex coacervation for cell encapsulation: Effect on mechanical prop erties and on long term viability. *Biopolymers, 82*(6), 570 579.
20. Devi, M. P. (2012). A novel wound dressing material fibrin chitosan sodium alginate composite sheet. *Bulletin of Materials Science, 35*(7), 1157 1163.

21. Lim, S. M., et al. (2008). In vitro and in vivo degradation behavior of acetylated chitosan porous beads. *Journal of Bio materials Science, Polymer Edition, 19*(4), 453 466.
22. Chellat, F., et al. (2000). In vitro and in vivo biocompatibility of chitosan xanthan polyionic complex. *Journal of Biomedical Materials Research, 51*(1), 107 116.
23. Yan, X. L., Khor, E., & Lim, L. Y. (2001). Chitosan alginate films prepared with chitosans of different molecular weights. *Journal of Biomedical Materials Research, 58*(4), 358 365.
24. Denuziere, A., et al. (1998). Chitosan chondroitin sulfate and chitosan hyaluronate polyelectrolyte complexes: Biological properties. *Biomaterials, 19*(14), 1275 1285.
25. Chen, H., et al. (2006). Genipin cross linked alginate chitosan microcapsules: Membrane characterization and optimization of cross linking reaction. *Biomacromolecules, 7*(7), 2091 2098.
26. Shigemasa, Y., et al. (1996). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules, 18*(3), 237 242.
27. Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive & Functional Polymers, 46,* 1 27.
28. Mancini, M., Moresi, M., & Sappino, F. (1996). Rheological behaviour of aqueous solutions and dispersions of algal sodium alginates. *Journal of Food Engineering, 28,* 283 295.
29. Grasdalen, H. (1983). High field, 1H n.m.r. spectroscopy of alginate: Sequential structure and linkage conformations. *Car bohydrate Research, 118,* 255 260.
30. Rinaudo, M., Pavlov, G., & Desbrieres, J. (1999). Influence of acetic acid concentration on the solubilization of chitosan. *Polymer, 40*(25), 7029 7032.
31. Becheran Maron, L., Peniche, C., & Arguelles Monal, W. (2004). Study of the interpolyelectrolyte reaction between chitosan and alginate: Influence of alginate composition and chitosan molec ular weight. *International Journal of Biological Macromolecules, 34*(1 2), 127 133.
32. Chen, F., et al. (2012). Preparation and characterization of oxi dized alginate covalently cross linked galactosylated chitosan scaffold for liver tissue engineering. *Materials Science & Engi neering C Materials for Biological Applications, 32*(2), 310 320.
33. Liu, X., et al. (2015). Tunable tissue scaffolds fabricated by in situ crosslink in phase separation system. *RSC Advances, 5*(27), 100824 100833.
34. Liu, X., et al. (2015). Novel biodegradable poly(propylene fumarate) co poly(l lactic acid) porous scaffolds fabricated by phase separation for tissue engineering applications. *RSC Advances, 5*(27), 21301 21309.
35. Watkins, J. D., et al. (2010). Liquid liquid biphasic electro chemistry in ultra turrax dispersed acetonitrile aqueous elec trolyte systems. *Electrochimica Acta, 55*(28), 8808 8814.
36. Carmo, Danieli M., Oliveira, Marcia G., & Soares, B. G. (2014). Effect of the dispersive method in the preparation of the poly urethane/hydrotalcite nanocomposites by in situ polymerization. *Applied Clay Science, 101,* 128 135.
37. Lee, K. Y., Park, W. H., & Ha, W. S. (1997). Polyelectrolyte complexes of sodium alginate with chitosan or its derivatives for microcapsules. *Journal of Applied Polymer Science, 63*(4), 425 432.
38. Peniche, C., & Arguelles Monal, W. (2001). Chitosan based polyelectrolyte complexes. *Macromolecular Symposia, 168*(1), 103 116.
39. Rinaudo, M., Pavlov, G., & Desbrieres, J. (1999). Solubilization of chitosan in strong acid medium. *International Journal of Polymer Analysis and Characterization, 5*(3), 267 276.
40. Mancini, M., Moresi, M., & Rancini, R. (1999). Mechanical properties of alginate gels: Empirical characterisation. *Journal of Food Engineering, 39*(4), 369 378.
41. Lee, K. Y., & Mooney, D. J. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science, 37*(1), 106 126.
42. Hill, R. (1952). The elastic behavior of a crystalline aggregate. *Proceedings Physical Society, 65,* 349 354.
43. Willemse, R. C., et al. (1999). Tensile moduli of co continuous polymer blends. *Polymer, 40*(24), 6645 6650.
44. Araki, J., Yamanaka, Y., & Ohkawa, K. (2012). Chitin chitosan nanocomposite gels: Reinforcement of chitosan hydrogels with rod like chitin nanowhiskers. *Polymer Journal, 44,* 713 717.
45. Di Renzo, F., et al. (2005). Hierarchical macroporosity induced by constrained syneresis in core shell polysaccharide composites. *Chemistry of Materials, American Chemical Society, 17,* 4693 4699.
46. Meng, X., et al. (2010). Chitosan and alginate polyelectrolyte complex membranes and their properties for wound dressing application. *Journal of Materials Science Materials in Medicine, 21*(5), 1751 1759.
47. Norajit, K., Kim, K. M., & Ryu, G. H. (2010). Comparative studies on the characterization and antioxidant properties of biodegradable alginate films containing ginseng extract. *Journal of Food Engineering, 98*(3), 377 384.
48. Arzate Vázquez, I., et al. (2012). Microstructural characterization of chitosan and alginate films by microscopy techniques and texture image analysis. *Carbohydrate Polymers, 87*(1), 289 299.
49. Quong, D., Yeo, J. N., & Neufeld, R. J. (1999). Stability of chitosan and poly L lysine membranes coating DNA alginate beads when exposed to hydrolytic enzymes. *Journal of Microencapsulation, 16*(1), 73 82.
50. Lee, J. H., et al. (1997). Interaction of cells on chargeable functional group gradient surfaces. *Biomaterials, 18*(4), 351 358.
51. Choksakulnimitr, S., et al. (1995). In vitro cytotoxicity of macromolecules in different cell culture systems. *Journal of Controlled Release, 34*(3), 233 241.
52. Suzuki, T., & Mizushima, Y. (1997). Characteristics of silica chitosan complex membrane and their relationships to the char acteristics of growth and adhesiveness of L 929 cells cultured on the biomembrane. *Journal of Fermentation and Bioengineering, 84*(2), 128 132.
53. Kasemo, B. (2002). Biological surface science. *Surface Science, 500*(1 3), 656 677.
54. Ma, Z., Mao, Z., & Gao, C. (2007). Surface modification and property analysis of biomedical polymers used for tissue engi neering. *Colloids Surfaces B Biointerfaces, 60*(2), 137 157.