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Structural properties of polysaccharide-based microcapsules for soft tissue regeneration

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Abstract Autologous and eterologous cell encapsulation has been extensively studied for clinical application in functional organs substitution, recombinant cell transplantation in gene therapy or in muscle and cartilage regeneration to treat degenerative pathologies. In this work, calcium alginate, calcium alginate/chitosan, calcium alginate/gelatin and pectin/chitosan microcapsules were prepared to be used as innovative injectable scaffolds for soft tissue regeneration by a simple extrusion method from aqueous solutions. Prepared microcapsules had spherical morphology, whereas their size was deeply influenced by the polymeric composition. When incubated in a *physiological-like* environment up to 30 days, they underwent an initial swelling, followed by weight loss at different rates, depending on the microcapsules formulation. The encapsulation of mouse myoblast cells (C2C12 cell line) was obtained in calcium alginate, calcium alginate/chitosan, calcium alginate/gelatin microcapsules. Cells were alive throughout the encapsulation procedure, and were recovered by a mechanical rupture of the microcapsules. After 7 days, fractured microcapsules led cells to migrate gradually out.

1 Introduction

Eterologous cell encapsulation is obtained by developing a stable capsule with sufficient permeability to allow the income of nutrients and oxygen, that are supposed to penetrate into the capsule to maintain cell viability, and the release of the metabolic cellular products into the bloodstream or to adjacent tissues. At the same time, the microcapsule protects the encapsulated cells against the immune cells and antibodies from the patient that would cause rejection and destroy the implant [1].

Several polymeric systems, both synthetic and natural, are currently in use or under development to obtain capsules for eterologous cell encapsulation [2, 3].

Degradable microcapsules can be employed for tissue regeneration [4]; in this case, only autologous cells are encapsulated, thus avoiding the use of immunosuppressors, which are often debilitating for the patient. Once cells are immobilized, the microcapsules can be injected in vivo for cell growth and proliferation [5]. The capsule is supposed to degrade in a short time, leaving only the regenerated tissue without foreign material.

Autologous cell encapsulation preferentially involves natural polymers [6, 7] because of their degradability, associated with well tolerated degradation products [8], their great affinity to water, good biocompatibility with biological tissues in spite of the versatility and the mechanical resistance of synthetic polymers. In addition, natural polymers are easily available, extensively studied and widely used in pharmaceutical and food industry.

Microcapsule preparation involves the use of extrusion [9], emulsion [10] and photopolymerization techniques [11]. Natural polymer microcapsules are often prepared through extrusion systems, mainly air-jet droplet generators [12], vibrating nozzles [13] and the JetCutter technology [14].

The most investigated materials are natural polyuronic acids, alginate and pectin, which can be converted into the anionic form according to the pH of the solution. Polyuronic acids have been combined with other natural

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polymers, e.g., chitosan, an amino-containing polysaccharide which can be converted in the cationic form (Fig. 1), and gelatine, a protein derived from collagen, mainly composed of glycine, proline, hydroxyproline, glutamic acid, alanine and arginine. Microcapsules of pectin have been recently proposed for drug release [15, 16], but no studies are reported, at our knowledge, for cell encapsulation.

Pectin (Fig. 1) contains 1,4-linked α-D-galacturonic acid, with a variable number of methyl ester groups, rhamnogalacturonan-I and rhamnogalacturonan-II, branched polysaccharides containing blocks of neutral monosaccharidic units, galactose and rhamnose [17]. The degree of esterification (DE) is used to classify pectins in high methoxyl pectins (HM, DE > 50) or low methoxyl pectins (LM, DE < 50). HM pectins require low pH for gel formation, whereas LM pectins form gels in the presence of divalent cations [18]. Nowadays, the interest for pectin gels as biomaterials for tissue engineering applications [19] or as carriers for drug delivery [15, 16] is raising.

This work reports a comparative study of microcapsules prepared with natural polymers to be used as cell carriers for the regeneration of different soft tissues. The encapsulation of autologous cells, in particular mesenchymal stem cells, is now deeply investigated for the regeneration of different soft tissues [20–22], as an example for muscle regeneration, in demand in the case of trauma or degenerative pathologies.

Here, microcapsule properties, as evaluated by morphological and structural analyses are discussed, as well as their swelling behaviour and structural stability in phosphate-buffered saline (PBS).

Infrared spectroscopy was employed to evaluate the structural modifications induced by crosslinks or electrostatic interactions after the gel formation of all the considered polysaccharides, according to previous work done for alginate gels [30–32].

2 Materials and methods

2.1 Materials

Sodium alginate from brown algae (molecular weight MW = 76000 Da, mannuronic/guluronic acid ratio = 0.9/1 [23]), anydrous calcium chloride, low viscosity chitosan, gelatin type B (MW ~ 100000 Da) and pectin from apples were all supplied by Sigma–Aldrich and used as received (Fig. 1).

Chitosan molecular weight (6430 Da) was evaluated by viscometry at 25 ± 1°C; chitosan was dissolved in 0.1 M acetic acid containing 0.2 M NaCl. Using Mark-Houwinks equation, the average viscosimetric molecular weight was calculated ($K = 1.81 \times 10^{-3}$, $\alpha = 0.93$) according to literature [24]. Degree of de-acetylation (83.5%) was evaluated from FT-IR spectra [25].

Pectin molecular weight (65000 Da) was evaluated by intrinsic viscosity analysis according to literature [26]; pectin was dissolved in 0.1 M NaCl and the average viscosimetric molecular weight was calculated ($K = 9.55 \times 10^{-4}$, $\alpha = 0.73$). Degree of de-esterification (76%) was evaluated by titration with NaOH [27].

2.2 Preparation of microcapsules

The microcapsules were prepared in different formulations, as described in Table 1, using a simple extrusion method: the polyanionic polymer in solution was extruded dropwise, through a syringe pump, into the gelation solution under gentle stirring. In the case of chitosan/pectin formulation, the amount of chitosan solution was adjusted to get a final weight ratio chitosan to pectin 3:1.

All materials were dissolved in water, except for chitosan that was dissolved in 0.1 M acetic acid. As shown in Fig. 2, the equipment for extrusion [28, 29] consisted in:

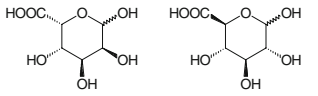
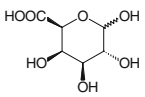
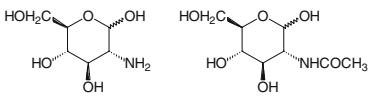
polymer	*main monomeric units*	*structure*
alginate	 L-guluronic acid; D-mannuronic acid	mainly linear blocks of β-(1→4)-linked D-mannuronic acid and α-(1→4)-linked L-guluronic acid residues
pectin	 D-galacturonic acid	mainly linear α-(1→4)-linked, D-galacturonic acid, partially esterified as methyl ester
chitosan	 D-glucosamine; N-acetyl-D-glucosamine	randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine

Fig. 1 Structures of alginate, pectin, chitosan

Table 1 Formulations, acronymous, diameter and eccentricity of the microcapsules

Polyanionic aqueous solution	Polycationic aqueous solution	Acronymous	Diameter	Eccentricity
Alginate 0.2%	$CaCl_2$ 0.15 M	Al(0.2)	398.46 ± 61.78	1.22 ± 0.11
Alginate 1.5%	$CaCl_2$ 0.15 M	Al(1.5)	438.89 ± 21.00	1.04 ± 0.03
Alginate 0.5	$CaCl_2$ 0.15 M Chitosan 0.2%	Al(0.5)/Ch(0.2)	402.92 ± 39.03	1.14 ± 0.12
Alginate 1.5%	$CaCl_2$ 0.15 M Chitosan 0.2%	Al(1.5)/Ch(0.2)	422.35 ± 41.27	1.07 ± 0.06
Alginate 1.5% Gelatin 1%	$CaCl_2$ 0.15 M	Al(1.5)/Gl(1)	461.45 ± 15.82	1.13 ± 0.08
Alginate 1.5% Gelatin 0.2%	$CaCl_2$ 0.15 M	Al(1.5)/Gl(0.2)	415.05 ± 36.51	1.11 ± 0.05
Pectin 0.5%	Chitosan 0.5%	Pc(0.5)/Ch(0.5)		
Pectin 1%	Chitosan 1%	Pc(1)/Ch(1)	–	–
Pectin 3%	Chitosan 0.5%	Pc(3)/Ch(0.5)		

Data regarding pectin were not reported because of their non-reproducible morphology

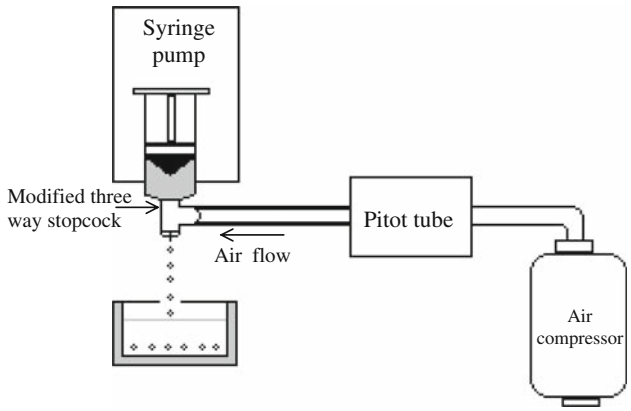


Fig. 2 Scheme of the experimental set up used to prepare microcapsules

- a syringe pump with the syringe containing the anionic polymer and, if the case, the cell suspension;
- a modified Terumo® threeway stopcock;
- an airflow generated by a compressor and controlled by a Pitot tube;
- the gelation bath.

Microcapsules were prepared by varying the syringe pump inflow rate from 10 to 40 ml/h and the coaxial air flow rate from 0.5 to 2 bar, searching for the optimal condition to optimal-sized capsules. The threeway stopcock was modified to let the tip of the syringe needle stay fixed in the center of the conduct and leak out of the stopcock for 2 mm.

The velocity of the air flow was maintained constant by a Pitot tube. The Pitot tube was fabricated in a previous work [29] and consists of two coaxial tubes: the interior tube is perpendicular to the flow and measures the stagnation pressure, while the exterior tube is parallel to the flow and measures the static pressure. A manometer can be used to measure the difference between these two pressures and, using Bernoulli's equation, the flow rate of the fluid can be calculated. The constant flow rate was maintained by regulating a valve on the Pitot tube.

After the preparation, the microcapsules were separated from the gelation solution using a filter with an adequate pore size (200 μm), then washed three times in distilled water for 10 min, to remove any non-reacted reagent.

2.3 Microcapsules characterization

2.3.1 Morphological analysis

Morphological analysis was performed using a stereo microscope (Wild M8). The diameter was estimated as the average of major axis (A) and minor axis (a) of the single microcapsule, while the eccentricity was measured as A/a. If the value of eccentricity is near to 1, the microcapsule can be associated to a sphere, otherwise to an ellipse.

The average of 15 diameters and eccentricity measures gave, respectively, the mean diameter and the eccentricity for each microcapsule formulation.

2.3.2 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Microcapsules were dried to constant weight after 1 h, 3, 7, 14 and 21 days of incubation in phosphate buffered saline. Spectra were acquired at a resolution of 4 cm^{-1} in the region of 4000–700 cm^{-1}, using a FTIR spectrometer (Thermo Electron Corporation, Nicolet 6700), coupled with a single-bounce attenuated total reflectance (ATR) accessory.

2.3.3 Swelling and stability studies

To perform the swelling and stability tests, microcapsules were incubated at 37°C in phosphate buffered saline (PBS, pH = 7.4) containing 0.02% sodium azide as bacteriostatic agent.

Each formulation was investigated at different time points up to 30 days of incubation by calculating the percent weight variation of wet microcapsules, as shown in the following equation:

$$\Delta w = \frac{(w_t - w_0) \cdot 100}{w_0} \quad (1)$$

where w_t is the wet microcapsule weight at time point t and w_0 is the initial weight of the microcapsules, measured immediately after their preparation. The measurements were made in triplicate after gently centrifuging the microcapsules (10 min at 500 rpm).

The microcapsules were also observed after 1 h, 3, 7, 14 and 21 days of incubation by a stereo microscope (Wild M8) to qualitatively evaluate their possible morphological changes.

2.3.4 C2C12 viability assay on pectin

Mouse muscle myoblasts line cells C2C12 (ECACC n. 91031101) were seeded in a multiwell plate at a density of 1.6×10^5 cells/ml in 15 ml 1% pectin solution in DMEM and incubated at 37°C, 5% CO_2. After 3 days of incubation, cells viability was measured by the biochemical assay Alamar Blue (Serotec). Alamar Blue was directly added to the culture medium of each incubated cell sample and to the control (DMEM only). The oxidized form of the Alamar Blue enters the cytosol and is converted to the reduced form, by viable cells. The number of the viable cells is related to the amount of the reduced form of Alamar Blue, by evaluating the absorbance, measured with a Tecan Genius Plus plate reader (test wavelength: 570 nm; reference wavelength: 630 nm). The percentage of the reduced form was calculated from the variation of the concentrations of the oxidized and reduced form, and corrected accounting the negative control.

2.3.5 Cell encapsulation

To obtain cell encapsulation in alginate, alginate/chitosan and alginate/gelatine formulations [Al(1.5), Al(1.5)/Ch(0.2) and Al(1.5)/Gl(0.2)], C2C12 cells were suspended in 15 ml of the different polyanionic solutions at a density of 1.6×10^5 cells/ml. The suspension was dropped into the receiving polycationic solution, [respectively: $CaCl_2$ 0.15 M for Al(1.5) and Al(1.5)/Gl(0.2), and $CaCl_2$ 0.15 M with chitosan 0.2% for Al(1.5)/Ch(0.2)] to obtain the formation of polyelectrolyte complexes with entrapped cells. Microcapsules were incubated at 37°C, 5% CO_2 and observed after 1 h, 3 and 7 days by an inverted microscope.

After 1 and 7 days of incubation, Al(1.5)/Ch(0.2) microcapsules were mechanically broken and a Trypan blue assay was performed on the released cells to check for cell viability.

3 Results

3.1 Microcapsule preparation

Microcapsules, obtained from different materials but having similar size and shape, were prepared.

The parameters of the custom made system were optimized to obtain spherical microcapsules with diameters of 400–500 μm. Optimal results were obtained with a low extrusion rate (20 ml/h). Using a higher flow rate, the formation of aggregates was observed, whereas using a slower flow rate, drop-like microcapsules were obtained.

The distance between the tip of the syringe needle and the gelation bath affected mainly the shape of the drops and, therefore, of the microcapsules. Best results were obtained at a distance of 5 cm. The coaxial air flow, a crucial parameter related to the dimensions of the microcapsules, was conveyed with a pressure of 1 bar.

3.2 Morphological characterization of microcapsules

Morphological analysis showed that alginate-based microcapsules had comparable diameters (in the range of 400–500 μm) and eccentricity measures (Table 1). On the contrary, size and shape of pectin–chitosan microcapsules differed significantly from alginate-based ones (Fig. 3), their diameters being in the wide range of 500–900 μm, roughly approximating those microcapsules with spheres. Few hours after their incubation in PBS at 37°C, pectin/chitosan microcapsules lost their morphology, becoming a shapeless gel.

3.3 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy was performed to qualitatively investigate the variations of the functional groups of the microcapsules prepared with the different polymers.

Alginate, chitosan, gelatin and pectin characteristic bands [30–32], as reported in Table 2, were used to detect the specific interactions occurring in polyelectrolyte complexes during the formation of microcapsules.

FT-IR analysis (Table 3 and Figs. 4 and 5), performed on dried microcapsules, showed that consistent structural modifications occur in the polysaccharides during the formation of ionic crosslinks and polyelectrolyte complexes. These modifications affected mainly the bands related to OH and COOH groups, and resulted in peak shift, broadening of the absorption band or decreasing their intensity.

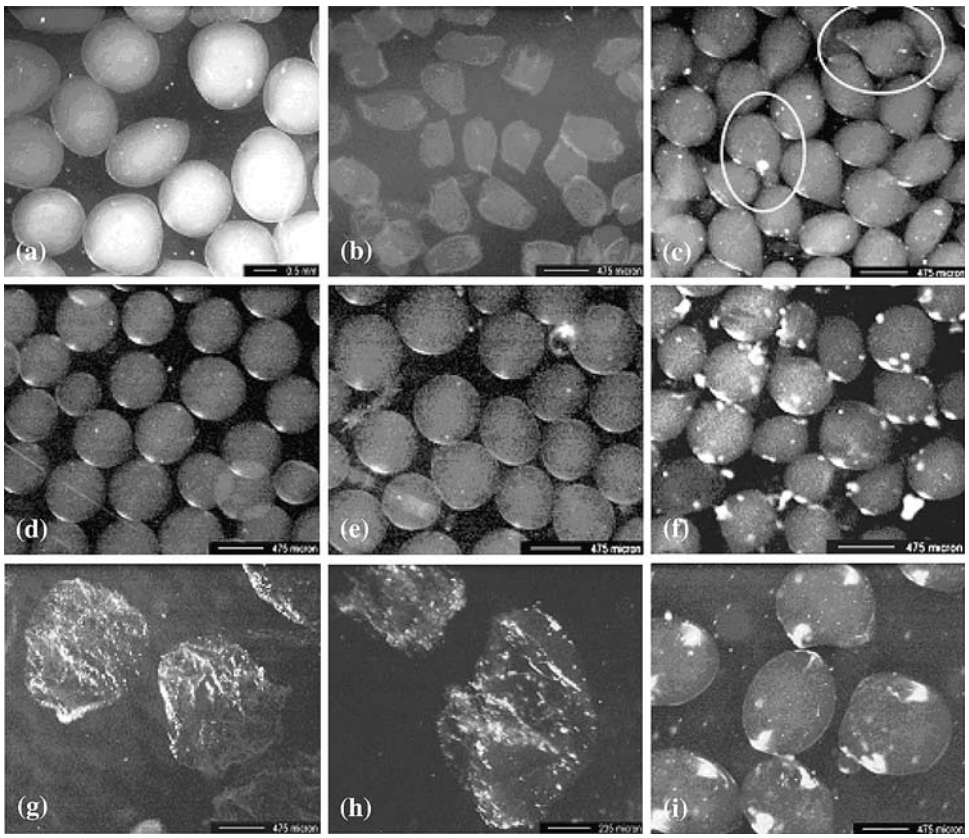


Fig. 3 **a** Al(1.5)/Ch(0.2) microcapsules obtained without the air flow showing not homogeneous shapes (bar = 0.5 mm); **b** elliptic microcapsules formed at low polymer concentrations (Al(0.3)/Ch(0.05)) (bar = 475 μm); **c** Al(1.5)/Gl(1) drop-like microcapsules (*circles*) obtained at high polymer concentrations (bar = 475 μm); alginate-based microcapsules Al(1.5) (**d**), Al(1.5)/Ch(0.2) (**e**), Al(1.5)/Gl(0.2) (**f**) with homogeneous size and shape (bars = 475 μm); pectin-based microcapsules Pc(0.5)/Ch(0.5) (**g**), (bar = 475 μm), Pc(1)/Ch(1) (**h**), (bar = 235 μm), Pc(3)/Ch(0.5) (**i**), (bar = 475 μm) showing not-reproducible morphologies

Disappearance/appearance of some bands were also observed.

3.4 Swelling and stability studies

Stability in PBS was tested to evaluate the solubilization kinetics of the microcapsules, as the aqueous environment is one of the factors involved in the in vivo release kinetics of cells and drugs.

All of the microcapsule formulations showed an initial swelling (Fig. 6) due to water absorption, that expanded the microcapsule and sometimes burst their walls, as shown in Fig. 7a and b. The release of drugs or cells possibly entrapped is driven by the rupture of the microcapsules.

The weight loss of Al(1.5) microcapsules was significant at 3 weeks of incubation (Fig. 6a), as they lost approximately 65% of their initial mass. At longer incubation times, the alginate microcapsules began to physically break down and to produce degradation fragments (Fig. 8).

Al(1.5)/Ch(0.2) and Al(1.5)/Gl(0.2) had a lower water uptake in PBS (Fig. 6b) if compared to Al(1.5).

Pect(3)/Ch(0.5) and Al(1.5)/Gl(0.2) microcapsules (Fig. 6b) lost only the 20% of their initial weight up to 30 days of incubation, showing a greater stability than the control Al(1.5).

3.5 C2C12 viability assay on pectin

Cell viability in the presence of pectin in the culture medium was measured 3 days after C2C12 cell seeding using the biochemical assay Alamar Blue. Alamar Blue was reduced to 65% by the cellular metabolic activity and provided an indirect quantification of the viable cell number. After 7 days the cells, as observed by the inverted microscope, were adherent to the well plate, by reaching confluence.

3.6 Cell encapsulation

Cell encapsulation was performed on Al(1.5), Al(1.5)/Gl(0.2) and Al(1.5)/Ch(0.2) following the procedure above described. One hour after cell encapsulation, no cells can be observed on the well plate (Fig. 7c), thus confirming the efficiency of the encapsulation method.

After 1 and 7 days of encapsulation, cells showed a round morphology because of the non-adhesive properties of alginate [33]. The use of low pH, involved in the preparation of chitosan–based microcapsule, did not affect cell viability.

Many fracture cracks in Al(1.5), Al(1.5)/Ch(0.2), Al(1.5)/Gl(0.2) microcapsules were observable 7 days after

Table 2 FTIR Bands of alginate, chitosan, gelatin and pectin with assignment

Vibration (cm^{-1})	Assignment
Alginate	
3700–3000 (broad)	OH stretching
3000–2850	CH stretching
1594	Antisymmetric COO^- stretching
1405	Symmetric COO^- stretching
1296	Skeletal vibration of CO stretching
1080–1021	Antisymmetric C–O–C stretching
Chitosan	
3290	OH and NH stretching
2870	CH stretching
1649	Amide I, CO and CN stretching
1588	NH bending from amine and amide II
1420	CH_2 bending
1375	CH_3 symmetrical deformation
1152	Antisymmetric C–O–C stretching and CN stretching
1030	Skeletal vibration, CO stretching
Gelatin	
3280	NH stretching
1631	Amide I, CO and CN stretching
1527	Amide II, mainly NH bending and CN stretching
1235	Amide III, mainly NH bending and CN stretching
Pectin	
3700-2500	OH stretching
1735	Ester carbonyl (C=O) absorption
1630	Antisymmetric COO^- stretching

Table 3 Peaks that underwent modifications because of the formation of crosslinks or polyelectrolyte complexes

Vibration (cm^{-1})	Assignment	Modification
A1(1.5) (Fig. 4)		
3400	OH stretching	Increase of intensity, narrowing
3240	OH stretching	Formation of a shoulder
1594	Antisymmetric COO^- stretching	Shift to lower wavenumbers
1080–1021	Antisymmetric C–O–C stretching	Shift to lower wavenumbers
Al(1.5)/Ch(0.2) (Fig. 5a)		
3400	OH stretching	Increase of intensity
1594	Antisymmetric COO^- stretching	Broadening
1080-1021	Antisymmetric C–O–C stretching	Shift to lower wavenumber
Al(1.5)/Gl(0.2) (Fig. 5b)		
1594	Antisymmetric COO^- stretching	Shift to lower wavenumber, broadening
1631	Amide I, CO and CN stretching	Not visible in microcapsules spectra
1527	Amide II, mainly NH bending and CN stretching	Not visible in microcapsules spectra
1235	Amide III, mainly NH bending and CN stretching	Not visible in microcapsules spectra
Pc(3)/Ch(0.5) (Fig 5c)		
1735	Ester carbonyl (C=O) absorption	Shift to higher wavenumbers
1630	Antisymmetric COO^- stretching	Broadening

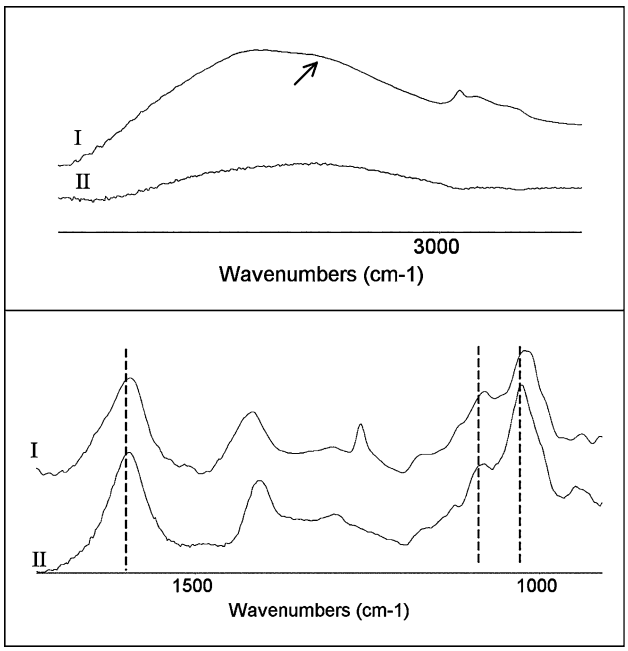


Fig. 4 FT-IR spectra of Al(1.5) microcapsules (I) at day 0 and alginate powder (II)

cell encapsulation. This led the cells to gradually migrate out of the microcapsules, showing an elongated morphology and being adherent to the well plate (Fig. 7d).

4 Discussion

Although the process to obtain microcapsules appears relatively simple, the concentration ratio of the oppositely charged polymers, the pH of the cationic bath and the process parameters (i.e., timing in the extrusion process, pressure of the air flow and extrusion velocity) deeply affect the microcapsules morphology and properties. The custom made extrusion apparatus allowed to easily control the process parameters, so to obtain reproducible microcapsules. The air flow resulted fundamental to set the microcapsules diameters, whereas the distance between the needle tip and the gelation bath affected the microcapsules morphology.

Two different approaches were followed to obtain alginate-based [Al(1.5), Al(1.5)/Ch(0.2), Al(1.5)/Gl(0.2)], and pectin-chitosan microcapsules. All alginate-based microcapsules were prepared through the gelation process involving the cooperative binding of calcium ions between aligned GG blocks of two alginate chains [34].

Pectin-based microcapsules were obtained through a direct ionic complexation involving the positively charged amine groups of chitosan and the negatively charged carboxyl groups of pectin, without the presence of calcium ions and the crosslinking of pectin relied exclusively to the

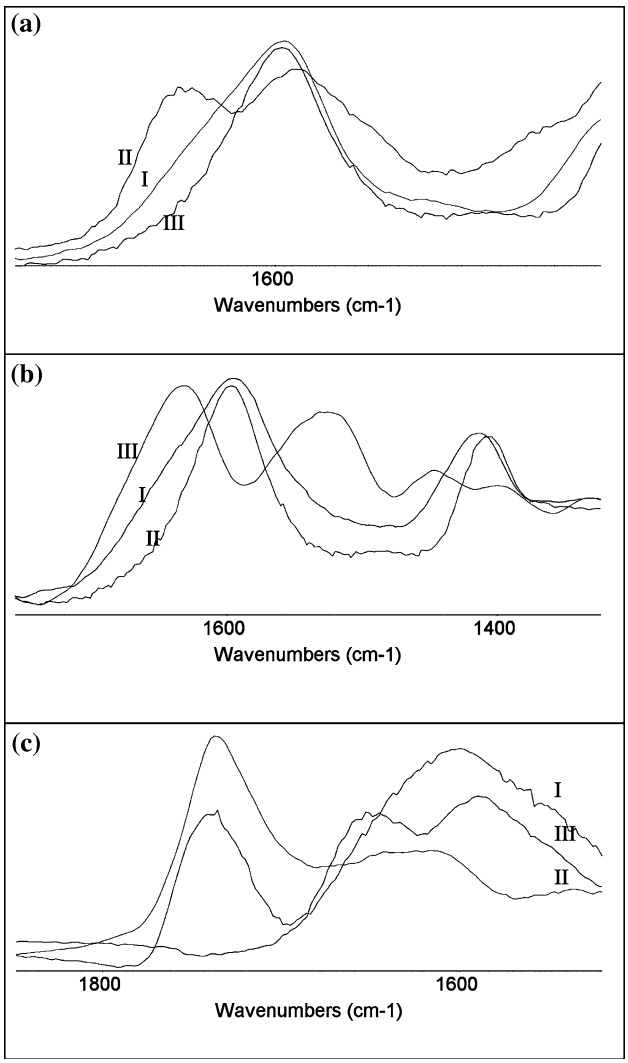


Fig. 5 **a** FT-IR spectra of Al(1.5)/Ch(0.2) microcapsules (I) at day 0, alginate powder (II) and chitosan powder (III). **b** FT-IR spectra of Al(1.5)/Gl(0.2) microcapsules (I) at day 0, alginate powder (II) and gelatin powder (III). **c** FT-IR spectra of Pc(3)/Ch(0.5) microcapsules (I) at day 0, pectin powder (II) and chitosan powder (III)

positively charged chitosan in the coagulation bath. Theoretically, according to their molecular weight and number of charged units, 2.4 mol of chitosan are requested to fully saturate 1 mol of pectin, resulting in a weight ratio 4.2 g of pectin for 1 g of chitosan. For the preparation of the pectin/chitosan microspheres, in this work a large excess of chitosan (about 13 times more than the theoretical value) was employed for two main reasons. The first is that it is a common practice to use a large excess of the cation in the coagulation bath, as not all the dissolved cations in the solution might be involved in the crosslinking process. Moreover, pectin is a branched polysaccharide with the charged units composing the main backbone: lateral neutral chains can hinder the interaction of chitosan, a relatively large polycation compared to calcium ions. Only the

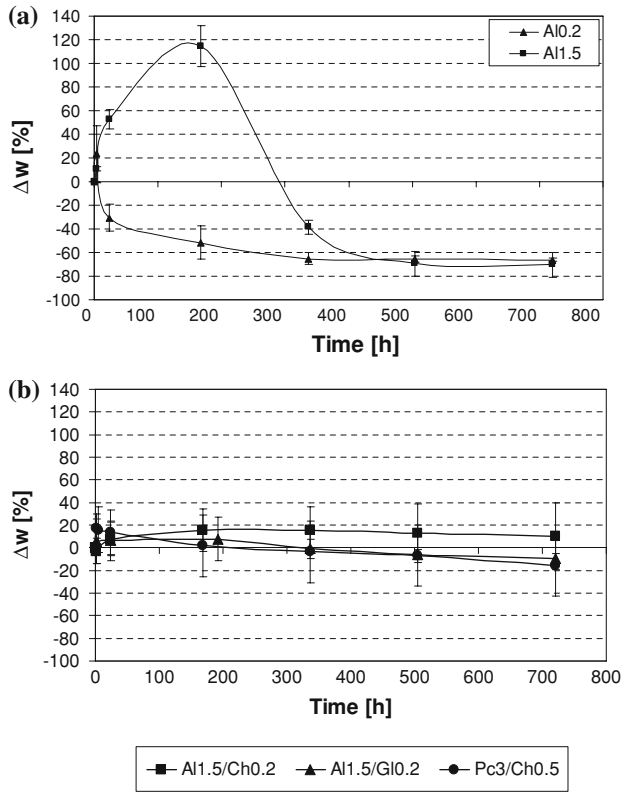


Fig. 6 Weight loss of microcapsules: **a** Al(1.5) and Al(0.2) weight loss, **b** Al(1.5)/Ch(0.2), Al(1.5)/Gl(0.2) and Pc(3)/Ch(0.5) as a function of time of immersion in PBS at 37°C

highest pectin concentration (3%) lead to PBS-stable microcapsules: a possible explanation may be related to the competitive displacement of other counter-ions present in the medium, associated with the low amount of the ionic interactions between the two polymers established in more dilute solutions.

Polymer concentration is a factor deeply influencing microcapsules morphology: lower polymer concentrations led to the formation of elliptic microcapsules, whereas drop-like microcapsules were obtained at higher polymer concentrations. The optimal polymer concentration leading to spherical microcapsules resulted in the range of 0.2–1.5% [w/v] (Fig. 3).

Two factors affect the stability of microcapsules: the formation of more entangled system, partially stabilized by H-bonds, caused by the blending of different high molecular weight polymers, and the presence of polyelectrolyte complexes between them [35]. The entanglement restricts the movement of the polymer chains ad prevent the microcapsules from high swelling, reducing the water uptake and providing the greater stability of the structure.

Microcapsules stability and release rate was related to polymer concentration in the microcapsule preparation, and its effect should be taken into account in order to modulate the release rate of cells or drugs possibly encapsulated. In our work, microcapsules prepared from polymeric solution at higher concentrations loosed weight more slowly than

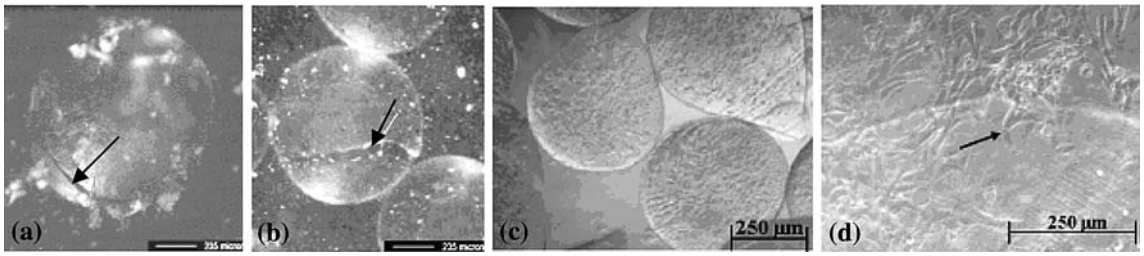


Fig. 7 **a** Al(1.5) (bar = 235 µm) and **b** Al(1.5)/Ch(0.2) (bar = 235 µm) microcapsules after 7 days of incubation. **c** Al(1.5)/Ch(0.2) microcapsules loading C2C12 line cells after 1 h of incubation and **d** after 7 days of incubation. The *arrows* show the fracture crack from which cells can diffuse out of the microcapsules. In the background of **d**, it is possible to observe C2C12 cells adherent on the well plate

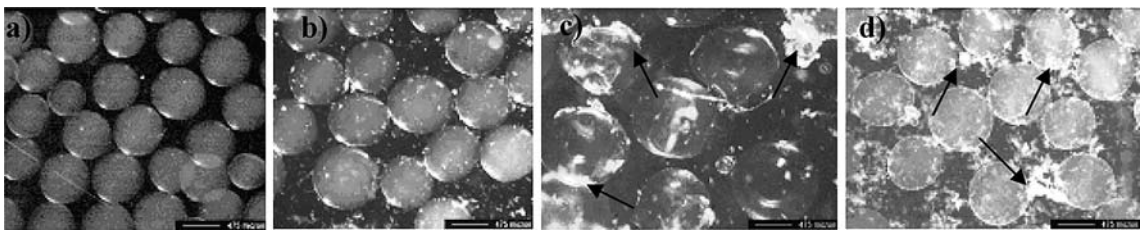


Fig. 8 Images of Al(1.5) microcapsules obtained by the stereomicroscope after **a** 0, **b** 7, **c** 14 and **d** 21 days of incubation in PBS at 37°C. The *arrows* show fragments released from the microcapsules. Bars = 475 µm

the ones prepared with lower polymer concentration (Fig. 6a). As the only difference between Al(1.5) and Al(0.2) is the concentration of the solution during the microcapsules formation process, the higher solution viscosity probably resulted in microcapsules denser and less porous, with higher thickness of their outer wall, thus leading to a slower surface erosion rate. An increase of polymer concentration in solution may also lead to the formation of a more entangled polymer network, thus lowering the water uptake and water-related release processes of the microcapsules.

Alginate gels degradation is usually attributed to loss of stability due to a gradual decrease of the number of calcium alginate bonds, resulting in decreased gel strength and increased permeability of the gel network [36, 37]. Other articles account alginate degradation to the chain scission due to acidic or enzymatic hydrolysis [38, 39] or to the shrinkage of the gel network due to water diffusion outside the microcapsules [40].

In in vitro studies, the stability of alginate-based microcapsules is probably affected by three different phenomena: chain scissions, dissociation of calcium crosslinks and detachment of fragments from the microcapsules. In particular, the dissociation of the crosslinks occurs when calcium ions are displaced from the alginate network and the carboxyl residues of alginate are protonated to form water-insoluble alginic acid. The fragments released from the surface of the microcapsules could be sodium-containing particles [41], that cause the mass loss of the microcapsules.

Degradation appears to begin from the external surface of microcapsules, as can be observed by images acquired at different time points with the stereomicroscope (Fig. 8). The diameters of the microcapsules were decreasing with time, as in the case of surface erosion, and the production of small fragments from the surface of the microcapsules was observed.

The formation of crosslinks or polyelectrolyte complexes during the preparation of the microcapsules modifies the structure of polysaccharides. As reported in previous works [42, 43], the interaction between alginate and calcium ions resulted in the modification of the OH stretching peak (Fig. 4), with either an increase of intensity (+76.48% area increase of the OH stretching at 3400 cm^{-1}, normalized to the area of CH_2 bending at 1420 cm^{-1}) and the narrowing of the band, either in the formation of a OH shoulder at 3240 cm^{-1} (indicated by the arrow in Fig. 4), corresponding, respectively, to the increase of intramolecular and intermolecular bonds.

The COO^- asymmetrical stretching peak of alginate at 1594 cm^{-1}, as well as two peaks associated to C–C and C–O bonds of guluronic blocks (at 1085 and 1035 cm^{-1}) underwent a shift to lower wavenumbers (Fig. 4). This could be indicative of the change induced on the guluronic blocks by the complexation with calcium ions [42].

The IR spectra of chitosan (Fig. 5a) exhibit chitosan characteristic bands at 1649 cm^{-1} (amide I), 1587 cm^{-1} (amide II) and 1376 cm^{-1} (CH_2 bending). The peak at 1594 cm^{-1} became broader in Al(1.5)/Ch(0.2) spectrum probably because of the contribution of COO^- groups of alginate and of the amide groups of chitosan (Fig. 5a). Based on this evidence, it can be concluded that a certain degree of interaction between alginate and chitosan is present in Al(1.5)/Ch(0.2) microcapsules.

The peak associated to the C–O stretching of the guluronic blocks of alginate at 1085 cm^{-1} shifted to 1077 cm^{-1}, as a result of the formation of the alginate salt induced by the complexation with calcium ions [42]. Moreover, the increase in intensity of the OH stretching (+104%, area increase of the OH stretching at 3400 cm^{-1}, normalized to the area of the CH_2 bending at 1420 cm^{-1}) could be associated with the formation of intermolecular bonds between sodium alginate and chitosan.

In the case of Al(1.5)/Gl(0.2) (Fig. 5b), the IR spectrum appears to be related to the concentrations of the polymeric solutions used to prepare the microcapsules: the typical bands of alginate are easily recognised in the IR spectra. The characteristic bands of gelatine at 1631 cm^{-1} (Amide I), 1527 cm^{-1} (Amide II) and 1235 cm^{-1} (Amide III) are not visible in Al(1.5)/Gl(0.2) spectrum, probably because of the more concentrate alginate solution that resulted in microcapsules probably containing a higher amount of alginate than gelatine. However, the strong absorption assigned to the asymmetric stretching of COO^- groups at 1594 cm^{-1}, shifted to a lower wavenumber and became broader, as a result of the contribution of alginate (1604 cm^{-1}) and gelatine (1630 cm^{-1}).

The IR spectra of Pect(3)/Ch(0.5) microcapsules, pectin powder and chitosan powder are shown in Fig. 5c. In the spectrum of pectin powder, the stretching of C=O methyl ester groups can be observed at 1735 cm^{-1}, while the stretching of C=O in the carboxylate group at 1630 cm^{-1}. The main changes are in the range of 1800–1600 cm^{-1}, evidence of the interactions of the amino and carboxyl groups. The broad peak at 1600 cm^{-1} derives from a change in the environment of amine group of chitosan through its interaction with pectin [44], indicating that the complexes were stabilized by the electrostatic interactions developed between the positively charged groups of chitosan (NH_3^+) and negatively charged groups of pectin (COO^-) during the formation of the microcapsules.

On the overall, structural changes on all the microspheres were detectable according to the ionic crosslinking and the electrostatic interactions between oppositely charged polymers.

In regenerative medicine, cells injection in the defect site may be problematic as cells could not directly adhere to the tissue and could be flushed into the surrounding milieu. For this reason, injectable materials are a very attractive alternative as cell carriers for tissue engineering, thanks to the reduced surgical invasivity and the easier adaptation to anatomical sites. Nevertheless, in a 3D gel matrix, cells in the inner zone are likely to suffer or die due to limited diffusion and the consequent lack of metabolites and oxygen. To overcome this problem, the possibility of delivering cells encapsulated in microspheres prepared with gelable materials was explored.

In this work, natural polymer microcapsules are proposed as vehicles for controlled cell delivery and release: as shown in the encapsulation studies, cells survived to the encapsulation process and remained alive after 7 days of incubation. By changing the composition of the gel forming material, complete degradation of the microspheres can be adjusted between few hours and more than 1 month, while showing good cell viability after encapsulation [28]. With this approach, by adjusting material composition, the degradation of microspheres can be programmed, in order to achieve a better control on cell release in vivo.

The rupture of microcapsules, observed 7 days after incubation onwards, and the viability of the released cells, evidenced that the investigated polymeric systems are suitable for autologous cell encapsulation for tissue regeneration (i.e., skeletal muscle, articular cartilage, myocardial tissue). In particular, when the muscle injury is caused by trauma or by degenerative pathologies, the natural regeneration of the tissue follows two steps [45]: the inflammatory process, which occurs between 24 and 48 h, and the regenerative phase. The use of microcapsules as cell carriers would provide protection for the encapsulated cells in the early inflammatory response of the tissue, followed by the gradual release of cells during the regenerative phase, when cell proliferation and differentiation are promoted by growth factors.

On the other side, the inflammatory response could also affect the injected microcapsules degradation, due to the host reaction to tissue lesions provided by injection, or to the microcapsules materials. The accumulation of inflammatory cells around microcapsules and the possible too fast degradation should be avoided, to prevent a quick release of cells into the damaged site.

5 Conclusions

In this study, we evaluated some of the most important in vitro variables adequate to characterize microcapsules: size and shape, stability and toxicity.

The obtained results are encouraging for the development of injectable cell carriers based on natural polymers. The investigated microcapsules could be clinically employed for the treatments of different soft tissues, with particular regard to skeletal muscle and myocardial tissue regeneration, adipose tissue augmentation in plastic and reconstructive surgery, and for the treatments of cartilage diseases.

However, to assess safety and functionality of the described microcapsules, an in vivo model should be considered. In this case, there are many other variables involved, including the type of the tissue, the choice of the cell phenotype, the possible death or de-differentiation of the cells after the release, and polymers susceptibility to enzymatic degradation.

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