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Barium alginate capsules for 3D immobilisation of living cells: morphology, membrane properties and permeability

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Encapsulation in a barium alginate membrane is a promising strategy to obtain a three dimensional culture of living cells: membrane properties are crucial for a realistic clinical application. A one-step encapsulation technique, recently developed for controlled release of boar semen, was employed to prepare barium alginate and protamine-alginate membranes: permeability to two model molecules (haemoglobin and glucose) was evaluated. Capsules were evaluated for technological properties and scanning electron microscopy was used to examine the external morphology of the capsules and the 3D distribution of the cells within the core. The results indicate that 3D arrangement and cell shape are maintained, capsule dimensions and mechanical properties can be modulated, as well as their permeability to model molecules such as haemoglobin and glucose.

Key words: Cell encapsulation – Alginate – Membrane permeability – SEM – 3D cell arrangement.

Cell encapsulation is a strategy in which three-dimensional (3D) cell cultures are obtained by entrapping a pool of live cells within a semi-permeable membrane [1, 2]. The optimal degree of selectivity and permeability of the membrane to allow both diffusion of metabolites, oxygen and nutrients and cell life is the primary challenge in capsule development [3]. Other challenges include obtaining the desired mechanical properties of the membrane in terms of resistance to rupture, elasticity, particle size distribution, surface properties and morphology. All these characteristics combine to determine the ease of handling and the possible applications of the capsules [4].

In vitro cultures of mammalian cells isolated from tissues have a broad range of applications, including reproductive strategies [4]; nevertheless, standard monolayer *in vitro* cell cultures cannot adequately simulate the *in vivo* development of tissues which require both a 3D arrangement of the cells and a physiological extracellular matrix (ECM). There have been several successful attempts to use polymeric matrices, or scaffolds to culture isolated cells in 3D space [5, 6]. Hydrogels, and in particular alginate, mimic ECM well, as reviewed by Gombotz and Fong Wee [7].

A one-step encapsulation technique in barium alginate, described by Klein *et al.* [8], was recently developed for controlled release of semen for the artificial insemination of swine [9-14] and for 3D culture of ovarian follicular cells [15]. The barium alginate capsules are formed of a core containing the live cells surrounded by a gel membrane which may, if desired, be coated by an external layer of protamine. The advantage of this method is its versatility: core properties can be modified, and depending on cell type, different suitable polymers can be employed to mimic artificial ECM. Alternatively, cells in the core can be maintained into their physiological matrix. Another advantage of this method is the minimal effect of concentration of gellifying cation on cell functions: ions diffuse very quickly from the core leading to the alginate membrane growth. Membrane properties can also be modulated in terms of gel thickness, mechanical properties [12] and release kinetics [11] by changing the ion type, its concentration in the core, and by cross-linking of the external surface with polyamines, as for example, protamine. Also capsules dimensions can be adapted to the use, changing the extrusor needle diameter.

The aim of this work was to evaluate the permeability of the membrane to two model molecules (haemoglobin (Hb) and glucose), as well as to characterize the physical and mechanical properties of the capsules (weight, total diameter, core diameter, gel thickness and capsule strength). Bovine erythrocytes were encapsulated as a source of haemoglobin and as model cells for encapsulation process: in fact, these kinds of cells have fragile membranes and are easily damaged in non-physiological environment.

Capsule membranes were modified by superficial treatment with protamine sulfate (PS) at different concentrations and for different time intervals in order to modulate morphological and functional properties. Furthermore, the capsules were examined by scanning electron microscopy (SEM) to investigate their external morphology and the 3D distribution of the cells within the core.

I. MATERIALS AND METHODS

In order to study the permeability of the capsules to the chosen molecules, a protein (haemoglobin, MW 64,000) and a small non-ionic carbohydrate (glucose, MW 180), and to examine the 3D arrangement of encapsulated live cells (bovine erythrocytes), two sets of capsules were produced. One set contained bovine red blood cells (RBC) in the form of an erythrocyte-rich fraction, the other set contained physiological saline in order to evaluate the reduction in glucose concentration in the supernatant after incubation.

1. Preparation of the erythrocyte-rich fraction

Blood samples were collected into evacuated heparin-containing glass tubes (Vacutainer) from six adult Friesian Holstein cows 30 min after milking; at least 30 ml of blood/sample was centrifuged (1500 x g, 10 min) to produce an erythrocyte-rich fraction (ERF). The ERF was separated from the plasma and buffy coat, resuspended, washed with NaCl 0.9% at 4°C and centrifuged three times. The haematocrit of the final ERF ranged between 80 to 85% (microhaematocrit, Sanyo micro Centaur).

2. Encapsulation

The encapsulation was performed using a method previously described for swine spermatozoa [10].

For the haemoglobin diffusion study, a solution of xanthan gum 0.25% w/v (Satiaxane CX2, SKW Biosystems, I) in NaCl 0.9% w/v was added to the ERF (ERF/xanthan solution volume ratio 1:1) and then a saturated $BaCl_2$ solution was added to obtain a final Ba^{++} ion concentration of 20 mM. The resulting suspension was dropped (through a 26Gx1/2" needle) into a 0.5 % w/v sodium alginate solution (sodium alginate medium viscosity, Sigma-Aldrich, Germany) where barium alginate capsules were obtained. These capsules, called gel capsules, were collected by filtration, rinsed twice in NaCl 0.9% solution and resuspended in the same medium. A second kind of capsule was prepared by treating the external surface of the gel capsules with PS to modulate the membrane properties. Different PS concentrations and cross-linking times were assessed: 0.5% w/v PS solution for 3 min (0.5% 3'); 0.5% w/v PS solution for 30 min (0.5% 30'); 1% w/v PS solution for 3 min (1% 3') and 1% w/v PS solution for 30 min (1% 30').

For the glucose permeability study, the capsules were prepared as above, but with physiological solution (NaCl 0.9%) instead of ERF.

3. Scanning electron microscopy (SEM)

Membrane and core structure were investigated by scanning electron microscopy (SEM): the capsules were dehydrated with serial ethanol solutions from 50 to 100%, critical point dried, gold sputtered (purity degree 99.9%) with an Edwards S 150A sputter coater (Boc Edwards Italia SpA, Milan, Italy) and then observed by a scanning electron microscope (Cambridge Stereoscan 250, Cambridge Instruments Ltd., Cambridge, United Kingdom), operating at 20 kV.

4. Technological characterization of capsules

Capsules were macroscopically photographed by a digital video camera connected to an image analyser (CV 9000 Ver. 4.0 Image Analyzer, FKV Srl, Sorisole, BG, Italy). The whole capsule diameter, the core diameter and gel capsule thickness were measured and the weight of the capsules was determined.

The mechanical properties of the capsule membranes were evaluated by means of a texture analyser (Stable MicroSystems, model TA-XT2, UK) equipped with a 3 cm diameter plexiglass probe. During the test, the probe descends at the rate of 0.1 mm/s until a force resistance of 50 g is detected, and then maintains this position for 30 s. The force versus time plot characterizes the consistency of the capsule and the force value (in grams) detected after 30 s can be considered as the strength index (F30) of the capsule. All measurements were repeated on five randomised capsules per batch.

5. Haemoglobin diffusion measurements

Three sample capsules were incubated at 25°C in a hypo-osmotic sodium chloride solution (500 μl, 0.45% w/v, pH 7.1, equivalent to the isoelectric point of Hb, 25 °C) in an Eppendorf tube. The osmotic shock caused the red blood cells to rupture and the haemoglobin to leak out. Diffusion of Hb through membrane was assessed by measuring light absorbance in the supernatant (100 μl of extracapsular medium + 100 μl of Drabkin reagent to convert Hb into cyanometaHb). Optical density (OD) readings were performed by a microplate reader (Labsystems Multiskan EX), with filter at λ = 540 nm. The percentage of Hb diffused as a function of time was estimated as the increase in Hb concentration in the supernatant, with respect to the Hb concentration after the mechanical membrane disruption of a standard sample, taking into account the mean volume of capsules for each batch.

The following formula was applied:

$$\text{Hb \% increase} = [500/(500 + nV_c)]*100\ (ODt/ODd)$$

where n is the number of capsules added in each tube, V_c the mean capsule volume for each batch, ODt the optical density at each sampling time, and ODd the optical density after membrane disruption.

6. Glucose permeability measurements

Three sample capsules were incubated at 25°C in 500 μl of an aqueous glucose solution (180 mg glucose/100 ml) in an Eppendorf tube. The concentration gradient leads to the diffusion of glucose into the capsule, thus decreasing the concentration of glucose in the external solution. At fixed times, the reduction in glucose concentrations was determined by an automated haematochemistry analyser (Kone mod. Specific-selective, Kone Instrument Corp., Evry, France) with a specific glucose kit (hexokinase method), based on end-point reduction of NAD to NADH at a λ = 340 nm, linearity 600 mg/100 ml, and incubation temperature 37°C (Dasit Italia, Cornaredo, Milan, Italy).

Results were calculated as the decrease in glucose concentration in the supernatant, with respect to the initial concentration, taking into account the mean volume of capsules for each batch, using the following formula:

$$\text{glucose \% decrease} = [500/(500 + nV_c)]*100\ (C_t/C_0)$$

where n is the number of capsules added in each tube, V_c the mean capsule volume for each batch, C_t the glucose concentration at each sampling time, and C_0 the glucose concentration at time zero.

The complement to 100 is reported in the results.

7. Statistical analysis

Results concerning the technological properties of the capsules (weight, total diameter, core diameter, gel thickness

and capsule strength) for each formulation were analysed by a one-way ANOVA procedure, followed by a least significant difference (LSD) test. Results were reported as mean value, standard deviation (SD) and sample size (n). For each molecule model, concentration reduction profiles were analysed using a two-way ANOVA, with capsule formulation and time of sampling as factors, as described by O'Hara *et al.* [16] and Yuksel *et al.* [17]. Differences between formulations were assessed by the LSD test.

The level of statistical significance was set at an alpha of 0.05. Differences between groups for each variable are represented with different superscripts.

II. RESULTS AND DISCUSSION

With the aid of a digital camera connected to an image analyser, capsules were examined macroscopically and photographed. The differences between gel capsules and PS-treated capsules are evident: gel capsules showed a clear core of erythrocytes, characterized by the brilliant red colour of packed red blood cells (Koeppe's criterion), well separated from the surrounding barium alginate membrane. This membrane appeared hyaline, smooth and spherical (*Figure 1a*). Following the osmotic shock caused by the hypotonic solution, the gel capsules appeared swollen and lengthened. The hypoxic state influenced the colour of the capsule, which changed from brilliant red to dark red, and no longer allowed the core within the membrane to be appreciated (*Figure 1b*). Protamine capsules (*Figure 1c*) had a completely different aspect: they were smaller and markedly spherical. Their external surface was rough, and the coating produced an opaque membrane, making examination of the core difficult. After osmotic shock, the PS-coated capsules also appeared ellipsoidal, with an irregular surface. The reddish core was not distinguishable (*Figure 1d*).

SEM images of RBC-loaded protamine capsules are presented in *Figures 2 to 4*. The RBCs are visible within the core of the capsule, and maintain their typical bi-concave shape (*Figure 2*). Small particles are visible on the surface of the RBCs; these are probably dehydrated xanthan gum. The red cells appear to be arranged in a 3D structure and lie on the alginate membrane, which is visible as a smooth, homogeneous surface (*Figures 2 and 3*).

Microscopically, the outer surfaces of the gel and PS-coated capsules appear different (*Figure 4*): gel capsules have a granular, irregular surface (*Figure 4a*), but protamine-treated capsules show even more marked surface irregularity, with a heterogeneous appearance (*Figure 4b*).

These SEM images of the external surface of the capsules are similar to those published by Chandy *et al.* [18], who photographed alginate-chitosan microcapsules including polyethylene glycol and cross-linkers (carbodiimide and glutaraldehyde) and by Lee and Min [19] where the capsules were dehydrated at room temperature. The SEM images do, however, differ from those recorded in a previous study [13], in which the alginate network appears spongiform: these differences are presumably related to the dehydration method used to treat the sample before the SEM examination.

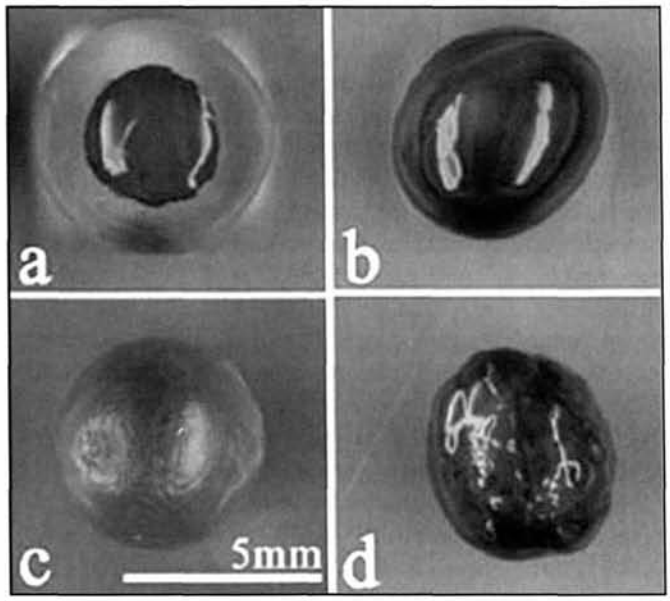


Figure 1 - Macroscopic morphology of erythrocyte-loaded capsules: a) gel capsule before osmotic shock; b) gel capsule 48 h after osmotic shock; c) protamine capsule (1% 3') before osmotic shock; d) protamine capsule (1% 3') 48 h after osmotic shock.

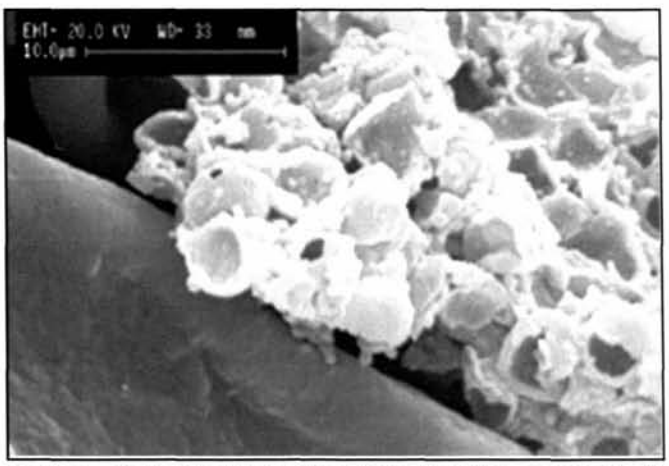


Figure 2 - SEM of the inner surface (smooth surface on the left) of a PS1% 30' capsule with a clump of RBC (X 2800).

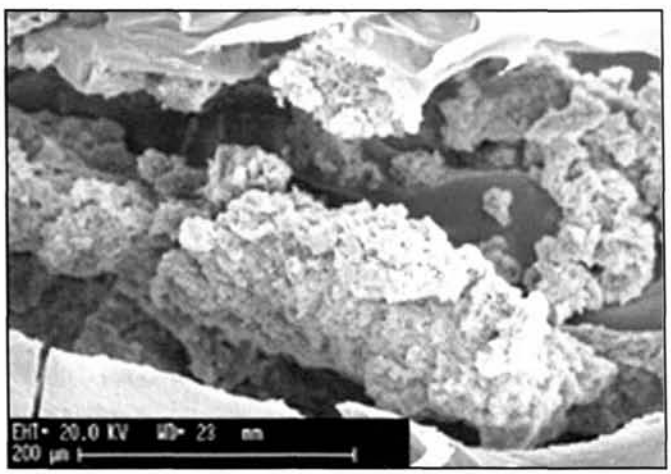


Figure 3 - SEM of a protamine capsule (0.5% 30') crack. Note the erythrocyte aggregates in the core of the capsule (X 180).

The technological properties of the capsules are altered dramatically by treatment with PS: capsule weight, total diameter and core diameter all decreased significantly as the PS concentration and cross-linking time increased (*Table I*). The same effects on weight and total diameter were recently observed by Orive *et al.* [20], and could be due to the shrinkage of the alginate matrix during protamine treatment. It should be stressed that gel thickness does not seem to depend on PS concentration, but rather on the time allowed for the coating to take place (*Table I*). The gel thickness also indicates a superficial reaction, confirmed by SEM (*Figure 4*). PS treatment of capsule membrane (F30) led to an increase of mechanical resistance (*Table I*); similar results due to the use of cross-linkers (carbodiimide and glutaraldehyde) were reported by Chandy *et al.*, [18]: mild treatment with glutaraldehyde greatly enhances the mechanical stability of microcapsules.

Figure 5 shows the profile of Hb release and two-way ANOVA results. These highlight the effect of treatment ($P < 0.001$) and release time ($P < 0.001$) on Hb diffusion, with there being a significant interaction effect ($P < 0.05$). In other words, the treatment effect on Hb diffusion differs depending on the different sampling times. This can be explained by the different permeability properties of heterogeneous membranes.

PS cross-linking markedly influenced Hb diffusion through the membrane (*Figure 5*): in fact, the gel capsule profile differs from the others starting from 18 h of incubation ($P < 0.05$). Brief treatment with PS (3') causes an intermediate diffusion behaviour, which is not statistically distinguishable when 0.5% and 1% PS were compared. Longer treatment with PS (30') led to the formation of a membrane totally impermeable to Hb (profile not reported in *Figure 5*) for PS 1%, whilst an irregular release was observed for PS 0.5%. The sudden leakage of Hb detected after 120 h of incubation was due to the abrupt loss of integrity of the capsule.

Our results are in accordance with those of Chandy *et al.* [18] for alginate microspheres: surface treatment significantly modifies Hb release from the gel core microbead structure. Huguet and Dellacherie [21] studied the permeability properties of chitosan-coated calcium alginate beads, and remarked that the release of molecules depends not only on the characteristics of the membrane, but also on the nature of the encapsulated molecule (flexible or rigid), net charge, molecular weight, ionisation and specific conformation.

Glucose concentration reduction in the supernatant following the incubation of the five types of capsules and two-way ANOVA results are reported in *Figure 6*. The reduction was very fast, even through the cross-linked capsules. ANOVA pointed out a significant difference for both sampling time ($P < 0.0001$) and treatment ($P < 0.001$). The *post-hoc* test (LSD) showed a difference ($P < 0.01$) between gel capsules – with the higher mean value – and the PS-treated ones, with the exception for 1%-3' PS capsules, which did not differ from the gel type ($P = 0.06$). The greatest differences between gel capsules PS-treated ones were mainly appreciable during the first 10-20 min of incubation, whilst after 30 min, the differences between treatments were reduced.

Dembczynski and Jankowski [22] characterized the diffusion of small molecules in hydrogel membrane liquid-core capsules: the transport of a solute in a liquid core capsule is more complex than that in a whole gel bead, and the diffusion of a solute in a capsule membrane can differ from that in the inner core. They calculated diffusion coefficients for several

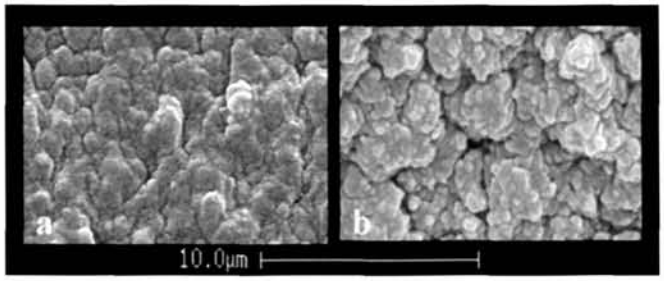


Figure 4 - SEM of: (a) gel capsule; (b) protamine capsule (0.5% 30'). External surface (X 2400).

Table I - Technological properties of gel and protamine capsules. Different letters (a, b, c) indicate a $P<0.05$ difference between groups (LSD post-ANOVA test).

		Gel	0.5% 3'	0.5% 30'	1% 3'	1% 30'
Weight (mg)	Mean	90.7^{a}	91.8^{a}	85.7^{b}	85.0^{b}	84.1^{b}
	SD	12.7	11.1	13.4	9.8	10.8
	n	160	60	60	100	80
Total diameter (mm)	Mean	5.85^{a}	5.60^{b}	5.53^{b}	5.62^{b}	5.62^{b}
	SD	0.33	0.23	0.26	0.32	0.34
	n	160	60	60	100	80
Core diameter (mm)	Mean	3.45^{a}	3.09^{b}	3.47^{a}	3.26^{c}	3.47^{a}
	SD	0.35	0.23	0.34	0.36	0.38
	n	160	60	60	100	80
Gel thickness (mm)	Mean	1.20^{a}	1.21^{a}	1.06^{b}	1.22^{a}	1.28^{c}
	SD	0.17	0.17	0.18	0.14	0.28
	n	160	60	60	100	80
F30 (g)	Mean	9.44^{a}	11.64^{b}	12.19^{b}	11.44^{b}	10.30^{ab}
	SD	2.89	3.72	3.35	3.55	3.13
	n	40	15	15	25	20

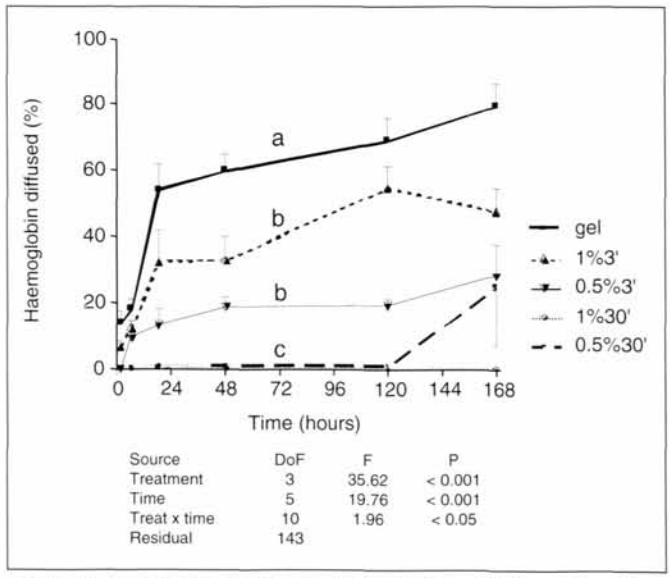
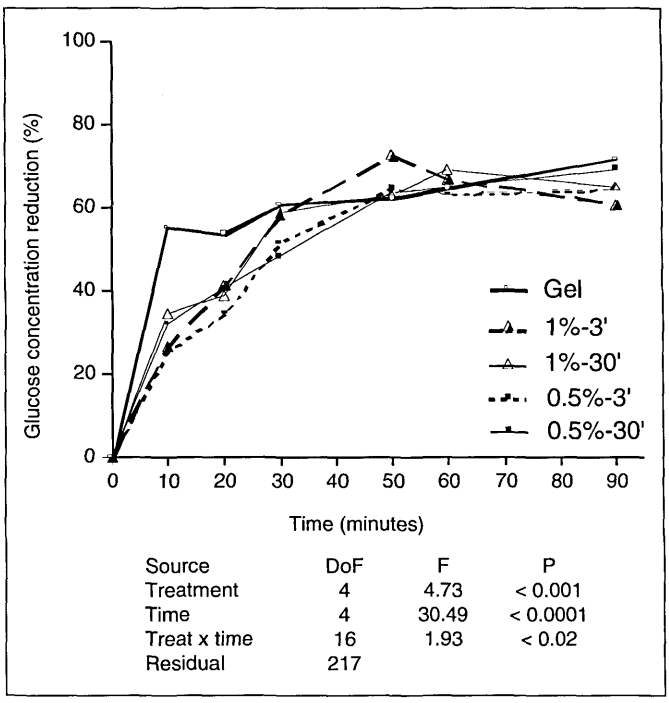


Figure 5 - Haemoglobin diffusion profiles (mean ± SEM on at least four replicates at each time point; different letters indicate significant differences between groups) and ANOVA results (two factors and interaction) for the five types of capsule.



Source	DoF	F	P
Treatment	4	4.73	< 0.001
Time	4	30.49	< 0.0001
Treat x time	16	1.93	< 0.02
Residual	217		

Figure 6 - Profiles of glucose concentration reduction (%) and ANOVA results (two factors and interaction) for the five formulations. Mean values of at least four replicates.

low molecular weight solutes (including glucose) through the whole capsule, and pointed out that mass transport through the membrane is the main factor controlling diffusion in this kind of capsule. Supported by these findings, the glucose, as well as other small molecules, can distribute homogeneously in the inner core, assuring optimal nutrient supply and metabolite exchange between encapsulated cells.

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Barium alginate capsules for 3D immobilization of living cells have been developed; in this paper, morphology, membrane properties and permeability were investigated. Scanning microscopy images show that 3D arrangement and shape of cells is maintained. Capsule dimensions and their mechanical properties can be modulated by superficial treatment with protamine. The results of this study indicate that protamine treatment acts as a cross-linking agent to obtain a semipermeable membrane; in this way, it is possible to modulate the mechanical properties of the capsules as well as their permeability to a model protein such as haemoglobin or a small neutral molecule such as glucose and oxygen. This simple, cheap and versatile technology seems to be suitable for the encapsulation of living cells, since the membrane properties can be easily modulated to meet the needs and purposes of their intended application.

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MANUSCRIPT

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