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Biomaterials 27 (2006) 4831–4839
Biomaterials
www.elsevier.com/locate/biomaterials

# Chemistry and the biological response against immunoisolating alginate–polycation capsules of different composition

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Received 21 February 2006; accepted 6 May 2006

**Abstract**

Implantation of microencapsulated cells has been proposed as a therapy for a wide variety of diseases. An absolute requirement is that the applied microcapsules have an optimal biocompatibility. The alginate-poly-L-lysine system is the most commonly applied system but is still suffering from tissue responses provoked by the capsule materials. In the present study, we investigate the biocompatibility of microcapsules elaborated with two commonly applied alginates, i.e. an intermediate-G alginate and a high-G alginate. These alginates were coated with poly-L-lysine (PLL), poly-D-lysine (PDL) and poly-L-ornithine (PLO). The main objective of this study is to determine the interaction of each alginate matrix with the different polycations and the potential impact of these interactions in the modulation of the host's immune response. To address these issues the different types of microcapsules were implanted into the peritoneal cavity of rats for 1 month. After this period the microcapsules were recovered and they were evaluated by different techniques. Monochromatised X-ray photoelectron spectroscopy (XPS) was performance and the degree of capsular recovery, overgrowth on each capsule, and the cellular composition of the overgrowth were evaluated by histology. Our results illustrate that the different observed immune responses are the consequence of the variations in the interactions between the polycations and alginates rather than to the alginates themselves. Our results suggest that PLL is the best option available and that we should avoid using PLO and PDL in its present form since it is our goals to produce capsules that lack overgrowth and do not induce an immunological response as such.


*Keywords:* Alginate; Biocompatibility; Immune response; Microcapsule; Surface modification; X-ray photoelectron spectroscopy (XPS)

## 1. Introduction

Transplantation of microencapsulated cells aims on long-term "de novo" production of therapeutic molecules in recipients with different types of disorders. The system allows for transplantation of cells in the absence of undesired immunosuppression. Because of these possibilities microencapsulation of cells has been proposed as a therapeutic option for a wide variety of diseases such as hypothyroidism [1], hypoparathyroidism [2], dwarfism [3], hemophilia B [4], liver and renal failure [5,6], central nervous system insufficiencies [7,8], and diabetes mellitus [9,10].

The classical and most commonly applied capsule-type is the alginate-poly-L-lysine system as designed by Lim and Sun [11]. This technology is based on the formation of a polycationic membrane composed of poly-L-lysine (PLL) around a polyanionic core formed by calcium alginate. PLL is necessary to form the semipermeable membrane of controlled porosity and to provide strength to the microcapsules [12,13].

Alginate is considered the most suitable polymer for microcapsules fabrication [14] since it is not deleterious for the enveloped tissue and because of its ability to form rigid gels under physiological conditions [15]. However, the

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doi:10.1016/j.biomaterials.2006.05.014

biocompatibility of alginate-PLL based microcapsules remains a subject of debate. A major factor causing bioincompatibility is insufficient binding of positively charged PLL at the capsule surface [16] which has been shown to induce foreign body reactions by attracting macrophages and fibroblasts [17]. Moreover, it has been shown that the capacity of neutralization of this positive charge depends on the alginate applied [18].

Alginates with intermediate guluronic acid (G) content have been shown to bind to the positively charged PLL more adequately than high-G alginates [18]. However, these findings should not be interpreted as a suggestion to avoid the application of high-G alginates since the latter has been shown to provide a better formation of the capsules, with a substantial reduction in the percentage of inadequate elaborated capsules than those prepared using intermediate-G alginates [19]. Furthermore, in order to obtain more biocompatible microcapsules, different types of alginate-coating polycations such as poly-D-lysine (PDL) and poly-L-ornithine (PLO) have been studied [20]. Recent data suggest that PLO coating can reduce swelling and increase the mechanical strength of alginate microcapsules when compared to PLL coating [21]. In addition, PLO coating restricts more effectively higher molecular weight components than PLL.

In the present study we have compared the biocompatibility in rats of microcapsules composed of two different alginates, i.e. intermediate-G alginate and high-G alginate. PDL and PLO were tested as possible substitutes for PLL since these molecules have been shown to have a high affinity for alginate [18,22]. Only highly purified alginates were applied to avoid inflammatory reactions against contaminants in crude alginate. The surface chemical characterization of all capsules after explantation from rats was performed using X-ray photoelectron spectroscopy (XPS). Furthermore, the degree of capsular recovery, the overgrowth rate of each capsule, and the cellular composition of the overgrowth is evaluated and discussed.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate, Keltone LV, with an intermediate content in guluronic acid, and sodium alginate, Manugel, with a high content in guluronic acid were obtained from ISP alginates, UK). PLL hydrobromide, PDL hydrobromide and PLO hydrochloride were purchased from Sigma Chemical (St. Louis, MO).

### 2.2. Purification of the alginates

Two types of alginates, an intermediate G content (40% G) or a high G-content (>50% G) were used to elaborate the capsules. These alginates were purified following the procedure described by De Vos et al. [23]. Briefly, crude sodium alginate was dissolved at 4 °C in 2 L of 1 mM sodium EGTA solution to a 1.25% of intermediate-G alginate and to reach a 0.625% solution of high-G alginate under constant stirring. Subsequently the solutions were filtered over 5.0, 1.2, 0.8, 0.45 μm filters (Schleicher& Scuell, Dassel, Germany) removing all the aggregates after the filtration step. After this, the alginate solution is acidified by the addition of 2 N HCl with 20 mM NaCl. During this step the alginate must be kept on ice to prevent hydrolysis of alginate. The gradual descent of the pH permits the precipitation of the alginate as alginic acid. Then, the proteins were removed by a chloroform/butanol extraction. For this, 100 mL of alginic acid was suspended in 20 mL of chloroform and 5 mL of 1-butanol. The mixture was maintained on ice and was vigorously shaken at some intervals of time. After 30 min the mixture was centrifugated at 1200 rpm for 4 min, and the proteins were removed. This chloroform/butanol extraction was performed three times. Subsequently, the alginate was dissolved in chloroform/butanol at neutral pH. This was done gradually by raising the pH to 7 by slowly adding 0.5 N of NaOH with 20 mM of NaCl. The alginate solution obtained was subjected to a chloroform/butanol extraction as described above for 30 min. After this, the mixture was centrifugated at 1600 rpm for 4 min which induced the formation of a separate chloroform/butanol phase which was removed by aspiration. The extraction was repeated two times to remove remnants of proteins.

Finally, the alginate was precipitated by the addition to the alginate solution of a double volume of absolute ethanol for 10 min. After this, the precipitated alginate was washed three times with ethylether and freeze-dried overnight. The purification run was started with 25 g of intermediate-G alginate and 12.5 g of high-G alginate of which 6–7 g of purified alginate was remained at the end of the procedure.

### 2.3. Encapsulation

Microcapsules were produced according to the three-step procedure as described previously [23]. Since an adequate viscosity of the alginate solution is required for the production of spherical droplets without any tails or other imperfections two different percentage, 3.4% of an intermediate-G alginate and 1.9% of a high-G alginate were used in order to have the same viscosity (4cps). First, the alginate solution is converted into droplets using an air-driven droplet generator. Subsequently, these droplets were collected in 100 mM calcium chloride solution (10 mM Hepes, 2 mM KCl) to form alginate beads. Next, the calcium alginate beads were suspended for 1 min in Krebs–Ringer–Hepes (KRH) buffer containing 2.5 mmol/L of calcium chloride. In order to form a semipermeable membrane with the corresponding polycation the alginate beads were suspended in a 0.1% of the corresponding polycation solution for 10 min (PLL, PDL, PLO). Non-bound polycation was removed by three successive washings during 3 min with calcium free KRH containing 135 mM NaCl. Finally, the capsules were suspended for 5 min in the same alginate used to elaborate the core but 10-times diluted. After the procedure the microcapsules had the following diameters; elaborated with intermediate-G alginate: PLL (635,93±18,74 μm), PDL (660,65±58,87 μm), PLO (678,43±74,48 μm) and elaborated with high-G alginate: PLL (639,75±9,52 μm), PDL (647,12±37,34 μm), PLO (666,56±16,27 μm).

In a few experiments we applied Barium alginate beads, to allow implantation of alginate in the absence of a crosslinking agent. Barium alginate beads have a higher mechanical stability than Ca-beads. Here, the alginate solution (3.4% of an intermediate-G alginate and 1.9% of a high-G alginate) is also converted into droplets using an air-driven droplet generator. Subsequently, these droplets are collected in 10 mM barium chloride solution (10 mM Hepes, 2 mM KCl) to form Barium-alginate beads.

### 2.4. Stability of the elaborated microcapsules

Transplantation of capsules requires an adequate physical integrity of the majority of the transplanted capsules. The physical integrity of capsules was tested in our laboratory as follows. Firstly, we incubated samples of 100 capsules in vitro for 24 h in a hypoosmotic solution of ultrapure water in a waterbath shaking at a frequency of 60 rpm (i.e. the so-called explosion assay) [18]. The diameter of 10 microcapsules and the number of broken microcapsules were measured after 24 h.

### 2.5. Implantation and explantation of microcapsules

Albino Oxford (AO/G) rats served as recipients of the capsules and NIH guidelines for the care and use of laboratory animals were observed. Under anaesthesia, the capsules were injected into the peritoneal cavity with 16G cannula via a small (3 mm) midline incision in the linea alba. The abdomen was closed with a two-layer suture. The implanted capsule volume varied between 1.4 and 2 mL. The total implanted volume, including the injected KRH varied between 2.5 and 3 mL.

The microcapsules were retrieved 1 month after implantation. Microcapsules can either be freely floating and non-adherent, or adherent to the surface of abdominal organs. The freely floating microcapsules were retrieved by peritoneal lavage with approximately 20 mL of KRH through a 3 cm midline incision into the peritoneal cavity and subsequent flushing with additional KRH for two or three times above a 50 mL centrifuge tube. The microcapsules were brought into a 2 mL measure-cylinder to quantify the retrieved microcapsules volumes. Subsequently, the microcapsules adherent to the surface of abdominal organs, were excised and processed for histology. The number adherent capsules were not quantified with only excised to study the composition of the overgrowth.

All the surgical procedures were performed under anaesthesia.

### 2.6. X-ray photoelectron spectroscopy

The samples consisted of 10–15 fresh microcapsules before implantation and 10–15 retrieved freely floating microcapsules at day 30 post-implantation. XPS analysis was performed using an imaging Kratos Axis Ultra (UK) X-ray photoelectron spectrometer equipped with a conventional hemispherical analyzer. The X-ray source employed was a monochromatized Al K$\alpha$ (1486.6 eV) operated at 150 W, to avoid X-ray degradation. Spectral acquisition is performed under ultra-high vacuum (UHV; $10^{-7}$ Pa) conditions. Analysis was performed on a 0.21 $mm^2$ (300 $\mu m \times 700\ \mu m$) sample area using a take-off angle of 90° relative to the substrate surface. The pass energies were 80 and 20 eV for wide-scan and high-resolution elemental scans, respectively. These pass energies correspond to energy resolutions of 1.6 and 0.4 eV, respectively. Charge compensation was performed with a self-compensating device using field emitted low energy electrons ($\leqslant$10 eV) to adjust the main hydrocarbon (C–C, C–H) component to 285 eV. The data reduction (atomic concentration, shift, curve fitting, etc.) was performed with CasaXPS Version 2.2.93 software. The operating software, Vision v2, corrects for the transmission function. The sensitivity factors were 0.328, 0.891, 0.278, 5.987, 0.78, 2.957, and 1.685 for Si 2p, Cl 2p, O 1s, Ag 3d, O 1s, Fe 2p, and Na 1s, respectively. Spectra were fitted after linear background subtraction assuming a Gaussian–Lorentzian (70/30) peak shape. Prior to XPS analysis, all samples were outgassed overnight, under UHV conditions, to maintain an appropriate pressure in the analysis chamber.

### 2.7. Histology and assessment of capsular overgrowth

To assess the integrity of capsules before implantation, samples of capsules were meticulously inspected for the presence of irregularities or broken parts in the capsule membranes by using a dissection microscope. To detect physical imperfections and to assess overgrowth after implantation, samples of adherent capsules recovered by excision or non-adherent capsules were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05 M phosphate in saline (pH 7.4), and processed for glycol methacrylate (GMA) embedding [24]. Sections were prepared at 2 μm and stained with toluidin stain and applied for detecting imperfections in the capsule membrane and for determining the number of capsules with and without overgrowth as well as for quantifying the composition of the overgrowth. Different cell types in the overgrowth were assessed by identifying cells in the capsular overgrowth with the morphological characteristics of monocytes/macrophages, lymphocytes, granulocytes, fibroblasts, basophiles, and multinucleated giant cells. At least 800 cells were counted.

Moreover, the degree of capsular overgrowth was quantified by expressing the number of recovered capsules with overgrowth as the percentage of the total number of recovered capsules for each individual animal. At least 1000 capsules were counted.

### 2.8. Statistical analysis

Results are expressed as mean ± standard error. Statistical comparisons were made with the Mann Whitney $U$ test. A $P$-value $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Characterization of the microcapsules

The biocompatibility of the microcapsules depends not only on the material employed in their elaboration [25] but also on their morphology. A perfect spherical, smooth shape and a membrane without irregularities [26] are required to prevent undesired host reactions. Therefore, in the present study, we always applied microscopy to determine the adequacy of the morphology of the capsules, confirming that the capsules employed in this study were totally spherical, with a smooth shape and uniform size (Fig. 1).

### 3.2. Stability of the microcapsules

Another important issue in the application of biocompatible capsules is the long-term structural stability of the capsules [27]. To confirm that the implanted capsules were structurally stable we applied the so-called explosion assay which allows us to predict the survival rates of capsules when exposed to shear forces after and during implantation [15]. In this assay we measured the diameter increase of the capsules under hypoosmotic conditions. The lower the increase in diameter, the higher the structural stability. Capsules batches should not explode to be considered suitable for transplantation. As shown in Fig. 2, in the majority of cases high-G capsules experienced a more severe increase in diameter than capsules prepared of intermediate-G alginate. These differences however only reached statistical significance ($P<0.05$) with application of PLL as the polycation. Since severe swelling causes rupture of capsules with severe tissue responses as a consequence [28], the percentage of rupture was measured. However, rupture (Fig. 3) was a rare phenomenon with the polycation concentration applied in the present study.

### 3.3. Biological response against the microcapsules

With all capsule implants, we observed at the time of peritoneal lavage a portion of the capsules to be freely floating in the peritoneal cavity while the remaining capsules were either single or in clumps adherent to the surface of the abdominal organs. The percentage of capsules retrieved by peritoneal lavage depended on both the alginate composition and type of polycation applied.

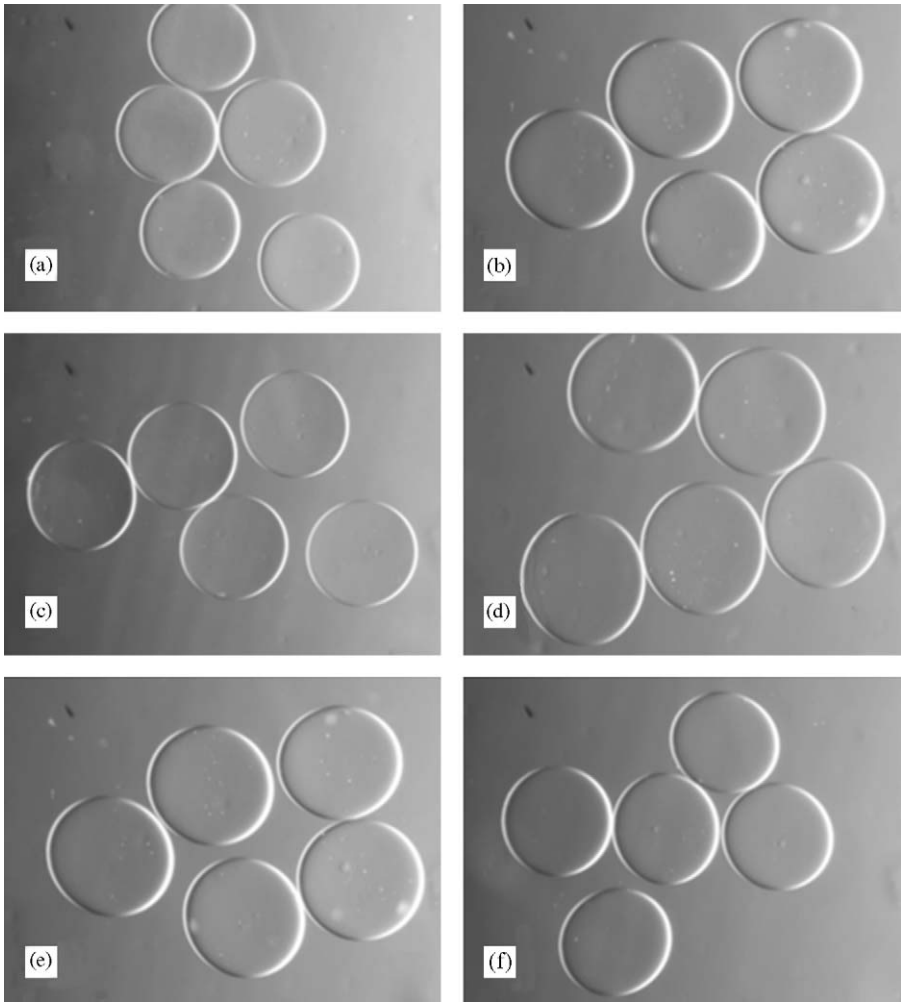


Fig. 1. Microcapsules elaborated with the different content in guluronic acid alginates and different polycation: (a) alginate G-intermediate –PLL, (b) alginate G-high –PLL, (c) alginate G-high –PLO, and (d) alginate G-intermediate –PLO. (Original magnification × 10.)

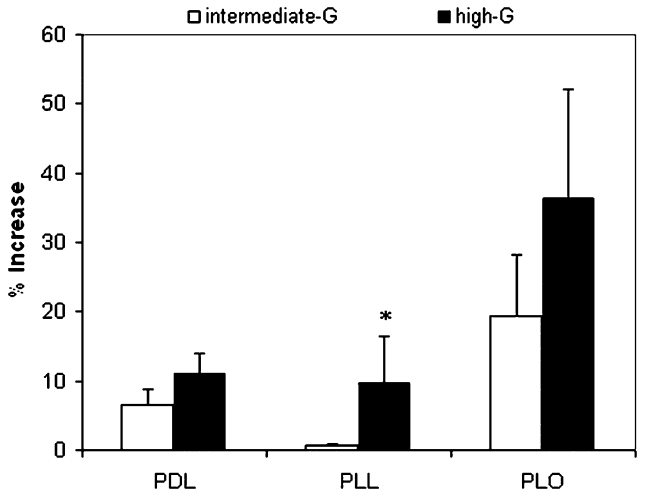


Fig. 2. Percentage of increase after the explosion assay of microcapsules elaborated with an intermediate-G alginate and a high-G core coated with different types of polycations. *$P<0.05$ between the different content in guluronic acid microcapsules for PLL.

When we implanted alginate beads without a polycation layer we found the majority of the beads to be freely floating in the peritoneal cavity with virtually no overgrowth. There were no differences between intermediate and high-G alginate beads (Table 1). The results were quite different when polycations were applied as crosslinking agent. When PLL was applied we found the majority of the capsules to be freely floating in the peritoneal cavity as illustrated by a retrieval rate of 85% of capsules with intermediate-G and 60% of capsules with high-G alginate capsules. Of the retrieved capsules a portion of 8% was affected by overgrowth when intermediate-G PLL capsules were applied while it was 30% when high-G PLL capsules were implanted.

These retrieval and overgrowth rates changed with application of the other types of polycations. When PDL was applied as crosslinking agent we were able to retrieve approximately 92.5% of capsules with intermediate-G and 13% of capsules with high-G alginate, illustrating that the fast majority of intermediate-G capsules were freely

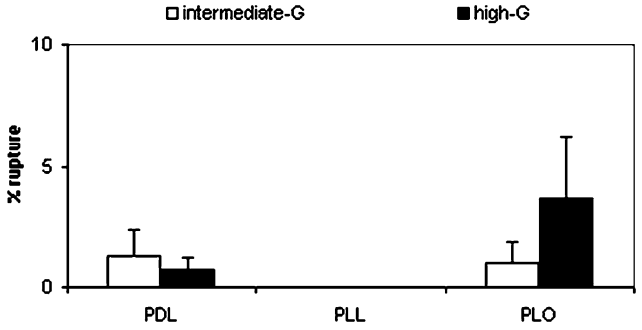


Fig. 3. Percentage of rupture after the explosion assay of microcapsules elaborated with an intermediate-G alginate and a high-G alginate core and coated with different types of polycations.

Table 1
Recovery rates and percentage of alginate beads with overgrowth, 1 month after implantation in the peritoneal cavity of AO rats

| Type of alginate | *n* | % Recovery | % Overgrowth |
|---|---|---|---|
| Intermediate-G content | 6 | 91.2±6.3 | 0.2±0.2 |
| High-G content | 4 | 88.5±8.6 | 0.4±0.2 |

Beads are prepared of alginates with intermediate-G, or high-G content. The alginate is crosslinked with Ba. There were no statistically significant differences in the recovery rates and percentage of alginate between the intermediate and high-G alginate.

floating and not adherent to the abdominal organs. The magnitude of the response was more severe against high-G capsules than against intermediate-G capsules since 24% was overgrown with intermediate alginate while it was 98% with high-G alginate ($P<0.05$). When PLO instead of PLL was applied we were able to retrieve 39% of intermediate-G alginate capsules and 87% of high-G alginate capsules. With PLO we found no differences in the number of overgrown capsules between high-G and intermediate-G capsules. In both cases it was 52%. The overgrown capsules was far higher that with alginate-PLL capsules since it was a 8% for PLL with intermediate-G alginate and with 30% high-G alginate (Table 2).

Not only the number of capsules affected by overgrowth but also the type of cells in the overgrowth varied with the different applied alginates and polycations. In general, the overgrowth on capsules retrieved by peritoneal lavage was composed by two different cells, i.e. fibroblasts and macrophages (Fig. 4) whereas all adherent capsules were overgrowth with fibroblasts (Fig. 5). While intermediate-G capsules with overgrowth contained predominantly fibroblasts, it were mainly macrophages in the case of high-G capsules. Capsules containing PLO or PDL had a different composition of overgrowth. With application of PDL or PLO we always found some lymphocyte infiltration in the capsular overgrowth. Moreover, giant cells were only observed in the vicinity of PDL and PLO intermediate-G alginate composed capsules. In some slices we found some highly granulated basophils (Table 3).

Table 2
Recovery rates and percentage of alginate microcapsules with overgrowth, 1 month after implantation in the peritoneal cavity of AO rats

| *Type of alginate and polycation* | *n* | % Recovery | % Overgrowth |
|---|---|---|---|
| *Intermediate-G content* | | | |
| PLL | 4 | 85.3±2.69 | 8.36±2.46* |
| PDL | 4 | 92.59±6.41 | 24.14±4.07* |
| PLO | 4 | 38.88±19.77 | 52.40±4.60 |
| *High-G content* | | | |
| PLL | 4 | 60.6±11.5 | 30.82±8.77 |
| PDL | 4 | 12.96±13.12 | 98.87±1.59 |
| PLO | 4 | 87.32±6.76 | 52.48±4.76 |

Microcapsules are prepared of alginates with intermediate-G, or high-G content and covered by different types of polycations (PLL, PDL, PLO). *$P<0.05$ in the percentage of overgrowth between the different content in guluronic acid microcapsules for PLL and PDL.

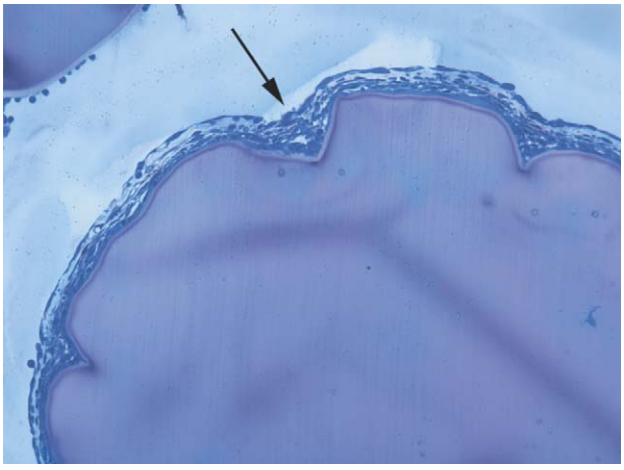

Fig. 4. The arrow indicates the immune response against microcapsules retrieved by peritoneal lavage is formed basically by macrophages and fibroblasts. This photograph corresponds to the overgrowth around high-G alginate microcapsules crosslinking with PLO (original magnification × 40).

### 3.4. The surface chemical composition of biocompatible capsules

It has been shown that the implantation of biomaterials is associated with the release of a large number of bioactive proteins that can be absorbed on their surface and facilitate the tissue response against them [25]. To investigate whether bioactive proteins are released and adsorbed after implantation of the different types of capsules, we applied XPS on the capsule's surface. Table 4 shows the elementary composition of the different microcapsules before and after implantation and the composition of the different polycations. In theory a molecule of sodium alginate should have an atomic composition of 46% C, 46% O and 8% Na [29]. This conformation changed when polycations were crosslinked with the alginate. Before implantation, the surface of the different microcapsules was composed primarily of

C, O, N, and Na. The polycation content in the membrane can be calculated by dividing the N/C ratio of the corresponding polycation through the N/C ratio of the capsule membrane [30]. From this calculation it follows that the high-G alginate microcapsules membranes contain more polycation (38.70% PLL, 104.31% PDL, 20.63% PLO) than intermediate-G alginate membranes (22.62% PLL, 35.56% PDL and 15.72% PLO).

For all types of capsules we found an increase in N-signal after implantation. This increase can be related with the immune response since this increase is an indicative of the adsorption of proteins in the capsule's surface. It seems to dependent on both the type of alginate and polycation applied. The highest increase was observed for high-G alginates capsules. This result is according with the high overgrowth rates present around this type of capsules. Moreover, the higher increase was observed for PLO microcapsules. Concomitant with the N-increase we observed an increase in the C-signal and a decrease in the O-signal.

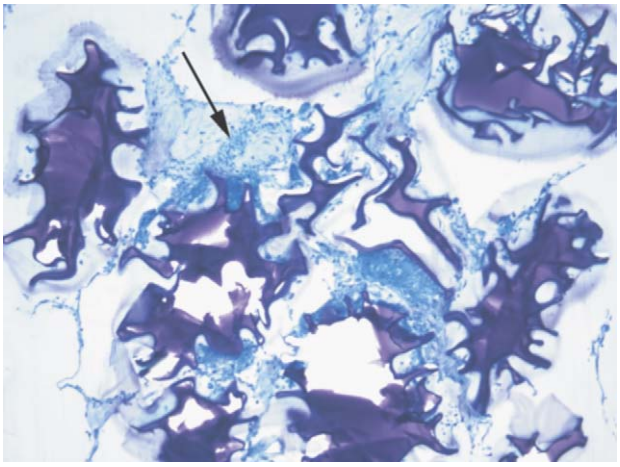

Fig. 5. The arrow indicates the overgrowth around microcapsules recovered in form of clumps were formed by an extend fibrotic tissue. This photograph corresponds to the overgrowth around high-G alginate microcapsules crosslinking with PDL (original magnification × 10).

## 4. Discussion

An adequate encapsulation system requires a gel entrapment system that is chemically and mechanically stable as well as biocompatible both for the host and the entrapped cells [31]. Many report advantages of new polycations and alginate-formulations over the classical alginate-PLL system [32,33]. Most of these studies show benefits by application of in vitro assays. Up to now, a comparative in vivo study is lacking.

In this study we have mainly evaluated the host response against the capsules which should not interfere with the function of the cells in the capsules. It has been shown that an inflammatory response resulting in overgrowth induces necrosis of the encapsulated cells with failure of the graft as a consequence. There are several factors influencing the host immune response against capsules. First the capsules should be able to withstand the osmotic pressures in vivo and should not explode due to osmolarity changes due to diffusion or mechanical frictions. Also, the capsules should be perfectly smooth and intact in order to prevent adherence of inflammatory cells on irregularities on the capsule surface. Finally, it is well recognized that the in vivo responses against microcapsules depend on a mutual relationship between the alginate and polycation combination.

The explosion assay was applied not only to serve as a predictive value for the stability of capsules in vivo but also as a measure for adequacy of binding of the polycation to the capsule network. A higher swelling illustrates a less efficacious binding between the polycation and the alginate network. PLL-capsules suffered from a statistically significant higher swelling in the case of application of high-G capsules. No statistical differences were detected between the swelling behaviour of high-G and intermediate-G capsules coated both by PDL and PLO. It has often been argued that differences in the responses against capsules is to a large extend caused by differences in capsules integrity [33] rather than by chemical variations. This argumentation was excluded in our study since all batches of elaborated microcapsules were checked for the presence of imperfections in the membrane. As a consequence all

Table 3
Frequency of different types of cells i.e fibroblasts, macrophages in the cellular overgrowth such as on free floating samples as on adherent microcapsules on 30 days after implantation

| Alginate/polycation | Fibroblasts (%) | Macrophages (%) | Lymphocytes (%) | Giant cells (%) | Granulocytes (%) | Mastocytes/basophiles (%) |
|---|---|---|---|---|---|---|
| *Intermediate-G content* | | | | | | |
| PLL | 92.25±7.63 | 7.75±7.63 | | | | |
| PDL | 84.66±5.03 | 10.66±3.78 | 3.33±2.88 | 0.33±0.57 | 0.33±0.57 | 1±1 |
| PLO | 33.5±10.47 | 63.5±13.1 | 2.5±2.08 | 0.25±0.5 | 2.5±3.31 | 0.25±0.5 |
| *High-G content* | | | | | | |
| PLL | 4.25±1.5 | 95.75±1.5 | | | | |
| PDL | 1.75±2.21 | 93±6.68 | 3±2.44 | | 2±2.44 | 0.25±0.5 |
| PLO | 32±20 | 62±18.018 | 3±1.825 | | 3±1.825 | |

Table 4
Elemental surface composition of the different microcapsules before and after implantation ($n = 1$) and analysis of pure PLL, PDL, PLO

| Before implantation | C (%) | N (%) | O (%) | Ca (%) | Na (%) | Cl (%) | S (%) |
|---|---|---|---|---|---|---|---|
| *Intermediate-G* | | | | | | | |
| PLL | 53.70 | 2.98 | 37.93 | 0.70 | 4.33 | 0.21 | 0.59 |
| PDL | 65.24 | 2.26 | 25.07 | 0.62 | 1.43 | 0.59 | 0.50 |
| PLO | 77.55 | 2.98 | 18.26 | 0.66 | 0.54 | 0 | 0 |
| *High-G* | | | | | | | |
| PLL | 60.20 | 5.71 | 28.54 | 0.26 | 2.99 | 1.35 | 0.52 |
| PDL | 55.36 | 5.62 | 27.76 | 0.47 | 3.35 | 1.94 | 2.78 |
| PLO | 63.33 | 2.42 | 28.47 | 0.57 | 1.61 | 0.41 | 0.23 |
| After implantation | C (%) | N (%) | O (%) | Ca (%) | Na (%) | Cl (%) | S (%) |
| *Intermediate-G* | | | | | | | |
| PLL | 63.55 | 5.49 | 27.55 | 0.65 | 0.36 | 0.12 | 0.14 |
| PDL | 69.66 | 7.07 | 20.94 | 0.28 | 1.15 | 0.20 | 0.07 |
| PLO | 64.77 | 8.05 | 19.39 | 0.10 | 2.22 | 2.01 | 1.38 |
| *High-G* | | | | | | | |
| PLL | 66.84 | 8.64 | 21.33 | 0.43 | 1.66 | 0 | 0.02 |
| PDL | 72.79 | 7.61 | 18.96 | 0 | 0.33 | 0.31 | 0 |
| PLO | 67.26 | 12.36 | 18.53 | 0.03 | 0.61 | 0.61 | 0.17 |

| Composition of the polycations | C (%) | N (%) | O (%) | Others (%) |
|---|---|---|---|---|
| PLL | 66.02 | 16.17 | 10.60 | 0 |
| PDL | 75.11 | 7.31 | 14.49 | 0.81 |
| PLO | 70.12 | 12.98 | 10.53 | 0.11 |

types of microcapsules showed a perfect spherical and smooth shape without imperfections.

By applying capsules with similar mechanically stability and integrity we were able to study the influence of chemical variations on the host response. We first excluded that it is the alginate itselve that provokes a response. Therefore, we studied the in vivo response against alginate beads without an additional polycation layer. No obvious host response was found against the alginate beads, a conclusion previously reported by our group [34].

The host response against crosslinked alginate capsules was always higher than against naked alginate beads. Also there was a variation in the response depending on the type of polycation applied. PLL and PDL showed a higher degree of biocompatibility with intermediate-G alginates than high-G alginates. It illustrates two phenomena. First it shows a higher capacity of intermediate-G alginates to neutralize the proinflammatory positive charges than high-G alginate. This is plausibly due to a difference in interaction between the polycation and the alginate [18]. Secondly, it is the consequence of the higher affinity of the polycations for GM residues which is more abundantly present in the intermediate G than in the high-G alginates [18]. Moreover, PDL provoked a higher immune response than PLL for both alginates. Considering this, PDL is probably not an advisable alternative for PLL since for both types of alginate we found a higher response against the PDL-crosslinked capsules than against the conventional PLL-capsules ($P < 0.05$).

PLO shows a similar biocompatibility with both intermediate and high-G alginates which might be due to its higher charge density [35] but also because of the fact that amino acid monomers of PLO is shorter in structure than PLL. This difference in structure allows PLO to bind more efficiently to the alginate membrane [21]. These results were corroborated by the XPS results. We found more polycation in the high-G alginates than in intermediate-G alginate. A large amount of polycations are diffused into the high-G alginate microcapsules. However, the number of binding sites is limited. Therefore not all the polycations molecules or only parts of the long polycations molecules will have interaction with the high-G alginate [36]. Since there is a high quantity of polycations in high-G alginate it is quite conceivable that more polycation diffuses out of the capsules after implantation and induces an inflammatory response.

Our study shows for the first time that the composition of the overgrowth of the varying types capsules is different, which suggest that the type of immunological response against capsules depends on the type of alginate and polycation applied. By now it is more accepted that immune responses against "foreign materials" is far more complicated than initially assumed and composed of different separate immunological responses. The response already starts with the mandatory surgery to implant the "foreign material". This mandatory surgery induces an inflammatory response due to rupture of bloodvessels which is associated with influx of inflammatory cells and

release of bioactive factors such as cytokines and fibronectin [20,37]. It depends on the material's properties whether this results in adsorption of proteins and subsequently cell adherences onto the surface. Next, cells can take up components of the foreign material and initiate a specific immune response characterized by the presence of lymphocytes in the vicinity of the materials [38,39]. The observation that capsules produced with the polycation PDL and PLO have lymphocytes in their vicinity suggests that these capsules loose components that produce a specific immunological response. These results suggest that we should avoid using these polycations in its present form in the capsule construction since it is our goals to produce capsules that lack overgrowth and do not induce an immunological response as such.

As explained above, the magnitude of the immunological response depends on the chemical composition of the surface. The type of polycation applied and its interaction with the alginate-core is therefore essential. It is mainly responsible for the protein adsorption which is the first step towards overgrowth. Consequently, it is important and, unfortunately still largely unrecognized, to quantify and study protein adsorption on biomaterials. Since the variation of the N content in the capsules surface is associated with the adsorption of proteins in the capsule's surface [30] we can estimate the protein absorption in the capsules surface.

Our results show that high-G alginate microcapsules present a higher absorption of proteins in the capsules surface than intermediate-G capsules. After the adsorption we observed adherence of typical cells of the immune response as fibroblast, macrophages, basophiles, mastocytes. However, the composition of the cellular overgrowth varies in function of the used alginates. In fact, a higher number of fibroblasts is present around the intermediate-G alginate while around high-G alginates microcapsules a higher number of macrophages is observed. Moreover, our results show that the chemical variations in the capsules have a large influence on the quantitative adsorption of protein and the consequent inflammatory reaction.

A significant number of lymphocytes, macrophages, basophiles, mastocytes and granulocytes are observed in the vinicity of PLO and PDL capsules illustrating an ongoing process of inflammation [40,41]. This could suggest that around these type of microcapsules an active immune response exists even one month after transplantation. These results indicate that the tissue response against capsules is not a static process but a rather dynamic event with involvement of varying cell types in function of the alginate and polycation applied.

The protein adsorption is not only an indicative measure for the magnitude of the provoked host response but also a measure for the expected changes in the physicochemical properties of capsules. Protein adsorption implies a change in diffusion properties inside and outside the capsules. Therefore, a failure of the transplant may be expected in the case of microcapsules affected by a high protein adsorption. Since the PLO microcapsules are the most affected by the protein adsorption we can expect the failure of the graft after implantation of this type of microcapsules containing cells.

## 5. Conclusions

Our results illustrate that the different observed immune responses are the consequence of the variations in the interactions between the polycations and alginates rather than to the alginates themselves. Our results suggest that PLL is the best option available and that we should avoid using PLO and PDL in its present form since it is our goals to produce capsules that lack overgrowth and do not induce an immunological response as such.

## Acknowledgements

We thank to the Ministry of Education, Culture and Sport of Spain for the research Grant (APA2000-2337) given to Sara Ponce. This project was partially supported by Ministry of Education and Science (BIO2005-02659).

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