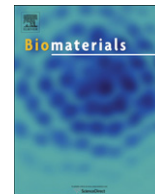


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The association between *in vivo* physicochemical changes and inflammatory responses against alginate based microcapsules

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ABSTRACT

Application of alginate-polylysine (PLL) capsules for immunoisolation of living cells are suffering from a varying degree of success and large lab-to-lab variations. In this study we show that these differences in success rates can be attributed to alginate dependent essential physicochemical changes of the properties of capsules *in vivo* that will render the capsules more susceptible to inflammatory responses. Capsule properties were studied before and after implantation by XPS, by immunocytochemistry, and by measuring zeta potentials. We studied a capsule type which provokes for unknown reasons a strong inflammatory response, i.e. high-guluronic (G) alginate capsules and a capsule type with near identical physicochemical properties but which evokes a minimal inflammatory response, i.e. intermediate-G alginate capsules. The cause of the difference in response was a decrease in nitrogen content on high-G capsules due to detachment of PLL *in vivo* and an increase of the zeta-potential. Our data illustrate an important overlooked phenomena; the physicochemical properties are not necessarily the properties after exposure to the *in vivo* microenvironment and might induce undesired inflammatory responses and failure of encapsulated cellular grafts.

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1. Introduction

The most commonly applied procedure for immunoprotection is microencapsulation of tissues in alginate-based capsules as originally described by Lim and Sun [1]. Alginate, the main component of the capsule, is composed of chains of guluronic acid (G) and mannuronic acid (M) and can have varying quantities of these two acids. The alginate binds polyaminoacids at the surface of the capsules which provides semi-permeable properties. During recent years, important advances have been made with this technology. Human trials have been started and shows temporary but persistent survival of human microencapsulated tissue after allotransplantation of encapsulated parathyroid cells [2] and islets of Langerhans [3–5]. Also, microencapsulation has been shown to allow for prolonged survival of xenotransplanted islet grafts in both chemically induced and autoimmune diabetic rodents [6], dogs [7,8], and monkeys [9]. Although this illustrates the principle

applicability of the alginate-encapsulation technique, a fundamental barrier has to be overcome since graft survival varies considerably from several days to months [10]. This variation in success rate is usually attributed to differences in the tissue responses (i.e. biocompatibility) against the applied capsules.

Many report different causes for the tissue responses against alginate-based capsules. Purity of the alginate [11–14], the interaction of the alginate with the polyamino acid [13,15–18], and the mechanical stability [12,19] are all considered to be crucial factors in the responses against the capsules. Also the alginate-type has been subject of studies focusing on identification of factors influencing biocompatibility, but conclusions on which type of alginate is most adequate can still not be drawn [18]. This is caused by the fact that in most of these studies alginate-effects are overshadowed by variations in experimental design which makes comparison and sound interpretation difficult if not impossible. Many studies [18,20] compare alginates with different guluronic acid content in capsules with a different or poorly documented stability. Also the applied alginates lack details on purity or have a documented different degree of purity [12,18,21]. In addition to capsule properties also other factors such as limitations of the transplantation site such as the low oxygen tension in the peritoneal cavity may contribute to failure and enhancement of host responses [22].

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In the present study we investigated the role of the *in vivo* microenvironment on physicochemical properties of capsules and the role of potential changes in the physicochemical properties on the inflammatory responses against the capsules. To this end we compared the responses and the surface properties of capsules that provoke a strong inflammatory response, i.e. high-G alginate capsules with that of capsules with a known high degree of biocompatibility, i.e. intermediate-G alginate capsules. We meticulously selected alginates with a different guluronic acid content but an identical purity degree to prevent overshadowing responses associated with contaminations such as endotoxins [12,18,21]. Also we constructed capsules with a near identical mechanical stability. Subsequently these capsules were implanted in the peritoneal cavity of AO-rats to compare the responses and the physical chemical surface characteristics of these capsules at day 1, 5, and 7 after implantation.

2. Materials and methods

2.1. Graft recipients

Male inbred Albino Oxford (AO/G) rats served as recipients of capsules and were obtained from the Central Animal Laboratory of Groningen. Their body weights ranged from 300 to 350 g. NIH guidelines for the care and use of laboratory animals have been observed.

2.2. Alginates and purification procedure

Alginates of different composition were obtained from ISP Alginates Ltd UK. Intermediate-G (43% G-content) and high-G (greater than 53% G-content). Crude sodium alginate was dissolved at 4 °C in a 1 mM sodium EGTA solution to a 1% solution for intermediate-G alginate and to a 0.25% solution for high-G alginate under constant stirring. Subsequently the solutions were filtered over successively, 5.0, 1.2, 0.8, and 0.45 µm filters (Schleicher & Schüll, Germany). During this filtration step, all visible aggregates were removed.

Next, the pH of the solution was lowered to 3.5 by addition of 2 N HCl + 20 mM NaCl. The solution was kept on ice to prevent hydrolysis of alginate. The next step was slow lowering of the pH from 3.5 to 1.5 and was associated with gradual precipitation of alginate as alginate acid [23]. Routinely, the solutions were brought at a pH of 2.0 and subsequently filtered over a Buchner funnel (pore size 1.5 mm) to washout non-precipitated contaminants. To extend the washout of non-precipitated contaminants, the precipitate was brought in 0.01 N HCl + 20 mM NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times.

Then, proteins were removed by extraction with chloroform/butanol [24]. The alginate acid was suspended in 100 ml of 0.01 N HCl + 20 mM NaCl and supplemented with 20 ml chloroform and 5 ml 1-butanol. The mixture was vigorously shaken for 30 min and filtered over the Buchner funnel. This chloroform/butanol extraction was performed three times. Next, the alginate acid was brought in water and slowly dissolved by gradually raising the pH to 7.0 by slow addition of 0.5 N NaOH + 20 mM NaCl over a period of at least 1 h. The alginate solution obtained was subjected to a chloroform/butanol extraction to remove those proteins which can only be dissolved in chloroform/butanol at neutral pH [24]. The solution was vigorously shaken in a mixture of chloroform (20 ml at each 100 ml alginate solution) and 1-butanol (5 ml at each 100 ml alginate solution) for 30 min. The mixture was centrifuged for 3–5 min at 3000 rpm, which induced the formation of a separate chloroform/butanol phase which was removed by aspiration. The extraction was repeated once.

The last step was precipitation of the alginate with ethanol [25,26]. To each 100 ml of alginate solution we added 200 ml absolute ethanol. After an incubation period of 10 min all alginate had precipitated. The alginate was filtered over the Buchner funnel and washed two times with absolute ethanol. Subsequently, the alginate was washed three times with ethylether. Finally, the alginate was freeze-dried overnight. In order to include alginates with a similar degree of purity we only included alginates which after the purification had an endotoxin content which was always <0.006 ng/mg as measured by LAL.

2.3. Encapsulation

Purified alginates were dissolved at 4 °C in Krebs-Ringer-Hepes (KRH) with an appropriate osmolarity. In order to produce capsules with a similar mechanical stability we tested intermediate-G capsules and high-G capsules from alginate solution with a different concentration. This increases the intracapsular polymer network and reinforced the stability. For high-G capsules we could not apply solutions higher than 2% as this produces solutions with a viscosity above 4 cps which is above the 0.2 µm filtration. This latter filtration step is required for sterilization of alginate.

In order to qualify for inclusion in this study, the alginate-polylysine solution should be able to withstand the following challenge for mechanical stability [15,27]. First, we incubated samples of 100 capsules *in vitro* for 24 h in a hypotonic solution of ultrapure water in a waterbath shaking at a frequency of 60 rounds per minute (i.e. the so-called explosion assay) [28,29]. The diameter of 10 microcapsules and the number of broken microcapsules were measured after 24 h. Only capsules that did not swell more than 10% over a period of 48 h were included.

We tested the mechanical stability of capsules prepared of high-G alginate and compared it with capsules prepared of solutions with graded loads of intermediate-G alginates. Only intermediate-G solution with a concentration of 2.8% qualified.

Before application the solutions were sterilized by 0.2 µm filtration. The alginate solution was converted into droplets using an air-driven droplet generator as previously described [30]. Polylysine-alginate encapsulation was performed as described elsewhere [31]. Briefly, the alginate droplets were transformed to alginate beads by gelling in a 100 mM CaCl₂ (10 mM HEPES, 2 mM KCl) solution for at least 5 min. Subsequently, the Ca-alginate beads were suspended for 1 min in Krebs-Ringer Hepes buffer containing 2.5 mmol/l CaCl₂. A poly-L-lysine (PLL) membrane was formed by suspending the alginate beads in 0.1% PLL solution for 10 min (poly-L-lysine-HCl, Mw: 22,000, Sigma). Non-bound PLL was removed by three successive washings during 3 min with Ca²⁺-free KRH containing 135 mM NaCl. The outer alginate-layer was subsequently applied by 5 min incubation in ten times diluted alginate solution. The diameters of capsules and beads were measured with a dissection microscope (Bausch and Lomb BVB-125, and 31–33–66) equipped with an ocular micrometer with an accuracy of 25 µm. The capsules had a diameter of 600–700 µm. The same microscope was used for inspection of the capsules prior to implantation.

2.4. Implantation and explantation of empty capsules

Capsules were injected into the peritoneal cavity with a 16 G cannula via a small incision (3 mm) in the linea alba. The abdomen was closed with a two-layer suture. The implanted volume was always 2.0 ml as assessed in a syringe with appropriate measure. The transplants contained at least 4000 capsules.

For studies to the tissue response in the first week we did subject the animals to a repeated laparotomy. The microcapsules were retrieved at day 1, 5, and 7 days after implantation by peritoneal lavage (at least $n = 4$ per time point). Peritoneal lavage at day 1 and 5 was performed by infusing 5 ml KRH through a 3 mm midline incision into the peritoneal cavity and subsequent aspiration of the KRH containing the capsules.

On day 7 the animals were subjected to laparotomy to inspect the peritoneal cavity. Microcapsules were either freely floating and non-adherent, or adherent to the surface of abdominal organs. First, non-adherent microcapsules were retrieved by peritoneal lavage, and brought into a syringe with appropriate measures for quantification of the retrieval rate [32]. Subsequently, the microcapsules adherent to the surface of abdominal organs, were excised and processed for histology.

All surgical procedures were performed under halothane anesthesia.

2.5. X-ray photoelectron spectroscopy (XPS)

For measuring protein adsorption, samples of fresh capsules and capsules retrieved by lavage at day 1, 5, and 7 after implantation were three times washed with ultrapure water and gradually lyophilized (Leybold Herecus, Combitron CMI). Since the XPS-spectroscopy only identifies elements at the surface of the capsules, it is a prerequisite that the membranes of the capsules are intact and not broken. Therefore, before applying XPS, we confirmed the integrity of the surfaces and membranes by scanning electron microscopy [33–35].

Samples of lyophilized beads or capsules with intact capsule membranes were fixed on a sample holder. The sample holder was inserted into the chamber of an X-ray photoelectron spectrometer (Surface Science Instruments, S-probe, Mountain View, CA). An aluminium anode was used for generation of X-rays (10 kV, 22 mA) at a spot size of 250 × 1000 µm. During the measurements, the pressure in the spectrometer was approximately 10⁻⁷ Pa. First, scans were collected over the binding energy range of 1–1100 eV at low resolution (150 eV pass energy). Next, we recorded at high resolution (50 eV pass energy) C_{1s}, N_{1s}, and O_{1s} peaks over a 20 eV binding energy range. The protein content of the capsule's surface was expressed as a percentage of the total C, N, and O content of the membrane. Pure lyophilized PLL was measured after bringing it on the sample holder in the same fashion as described above.

Experiments were repeated four times to exclude variations between different encapsulation sessions.

2.6. Electrophoretic mobility

The zeta potential was measured using the Electro Kinetic Analyzer (EKA, Anton Paar GmbH, Austria). The EKA operates according to the principles of streaming potential and includes a powder measuring cell, the electrolyte circuit, and a pair of Ag/AgCl electrodes. The electrolyte (10⁻³ M KCl solution) is forced through the measuring cell containing the sample. A pressure drop (ΔP) depending upon the flow resistance of the sample is detected across the measuring cell. The circulation of

electrolyte through the cell results in a flow of ions (streaming current). The resulting potential difference (streaming potential, U_p) is detected by electrodes placed at each end of the cell. During a measurement, ΔP and U_p are recorded.

The zeta potential (ζ) was calculated using the equation as follows [36]:

$$\zeta = \frac{U_p \cdot \eta \cdot \alpha}{\Delta P \cdot \epsilon \cdot \epsilon_0}$$

In this equation η is the dynamic viscosity of the electrolyte solution, α is its electrical conductivity, ϵ is the liquid permittivity, and ϵ_0 is the permittivity of free space.

If not otherwise mentioned, the pH of the electrolyte solution was kept at 7.0 since this is the physiological pH to which capsules are exposed *in vivo*. During the assessment of the streaming potential, the temperature was kept at 25 °C.

2.7. Microscopy

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 the composition and degree of overgrowth after implantation, samples of adherent capsules recovered by excision and samples of 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 [37]. Sections were prepared at 2 μ m and stained with Romanovsky–Giemsa stain and applied for detecting imperfections in the capsule membrane and for quantifying the composition of the overgrowth and determining the number of capsules with and without 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, erythrocytes, and multinucleated giant cells. To confirm the adequacy of this approach, portions of adherent and non-adherent capsules were frozen in precooled iso-propane, sectioned at 5 μ m, and processed for immuno-histochemical staining and quantification of the different cell types as previously described [38]. The monoclonal antibodies used were: ED1 and ED2 against monocytes and macrophages [39], HIS-40 against IgM bearing B-lymphocytes [40], and R73 against CD3⁺ bearing T-lymphocytes [41]. In control sections we used PBS instead of the first stage monoclonal antibody. Quantification of these cell types after immunocytochemistry were compared with the assessments on the basis of morphological markers and always gave similar results.

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.

2.8. Statistical analysis

Results are expressed as mean \pm SEM. Statistical comparisons were made with the Mann Whitney *U* test. A *P*-value <0.05 was considered statistically significant.

3. Results

A major pitfall in the studies comparing alginates with different composition has been the application of capsules that not only vary in the type of alginate but also have a different purity or mechanical stability [11–18]. This has interfered with sound interpretation of the effect of alginate types due to overshadowing effects of purity issues and responses to broken capsules. Therefore, in this study we selected alginates with an endotoxin content <0.006 ng/mg. Endotoxin content is a crucial factor in provoking purity-related inflammatory responses [18]. A concentration of <0.006 ng/mg is considered to be far below the level that could induce an inflammatory response [4,18] and below the endotoxin content of commercially available alginates. Also, we constructed capsules with an identical mechanical stability.

3.1. Surface properties before implantation

Before implantation we compared the surface properties of the constructed capsules by applying XPS. In previous studies we showed that especially variations in poly-L-lysine content, i.e. N-content, can be held responsible for inflammatory responses [34,42]. In the present study we therefore engineered capsules with a similar N-content (Table 1). Also we measured the zeta-potential of the capsules as a measure for the amount of unbound poly-lysine

Table 1

Elemental surface composition of alginate-PLL capsules ($n = 4$) prepared of intermediate-G and high-G alginate before implantation.

	C (%)	N (%)	O (%)	Others (Na, K, Si) (%)
Day 0				
Inter-G	56.2 \pm 0.6	5.8 \pm 1.2	33.5 \pm 1.8	4.5 \pm 0.8
High-G	56.0 \pm 0.9	5.9 \pm 0.5	33.9 \pm 0.8	4.2 \pm 0.6

on the capsules surface. Free positive charges on high-G capsules are considered to be responsible for inflammatory responses. Before implantation, the applied high-capsule grafts had a zeta-potential of -3.6 ± 0.2 mV which value was similar if not identical to the zeta-potential of intermediate-G capsule grafts (-3.5 ± 0.2 mV).

3.2. Tissue response in the immediate post-transplant period

In spite of the similarities in purity, mechanical stability, and the physicochemical properties of the capsules we observed different responses against the capsules after implantation in the peritoneal cavity of AO-rats. The majority of capsules prepared of intermediate-G alginate capsules were freely floating in the peritoneal cavity while the majority of the high-G alginates capsules were found to be adherent to the surface of the abdominal organs as illustrated by a high retrieval rate of the intermediate-G alginate capsules and a low retrieval rate of the high-G alginate capsules. With intermediate-G alginate, we washed out 20–40% of the capsule graft from the rat peritoneal cavity on day 1, 5, and 7 after implantation while it was only 10–30% with high-G alginate. After the three lavages, the total retrieval rate of the capsules was $92 \pm 8\%$ for intermediate-G alginate which was significantly higher than the $45 \pm 6\%$ for high-G alginate ($P < 0.01$). The majority of the capsules prepared of high-G alginate was found to be adherent to the surface of abdominal organs. These adherent capsules were all overgrown.

The tissue responses against intermediate-G alginate capsules were always less severe than against high-G alginate capsules. The percentage of capsules with cellular overgrowth with intermediate-G alginate increased during prolonged stay in the peritoneal cavity from $0.6 \pm 0.4\%$ on day 1 to $2.8 \pm 1.4\%$ on day 7 while with high-G alginate it was $1.5 \pm 0.7\%$ on day 1– $85.3 \pm 9.4\%$ on day 7 (Fig. 1).

3.3. Differences in the composition of the cellular overgrowth

With both types of alginate we found the overgrowth on capsules recovered by peritoneal lavage to be composed of monocytes/macrophages, granulocytes, fibroblasts, erythrocytes,

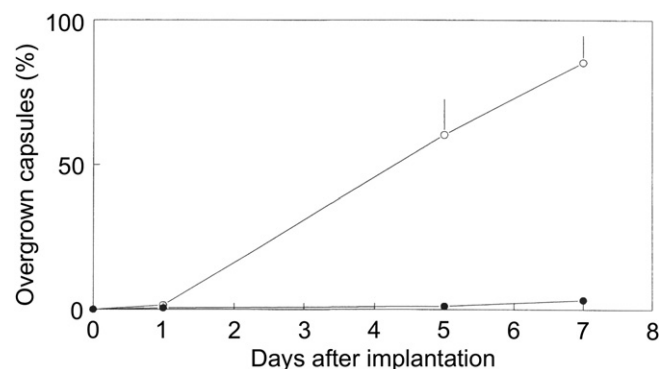


Fig. 1. Percentage of freely floating alginate-PLL capsules prepared of intermediate-G alginate (closed circles) or high-G alginate (open circles) with cellular overgrowth at 1, 5, and 7 days after implantation in the peritoneal cavity of AO-rats. Values represent mean \pm SEM of four experiments.

multinucleated giant cells, and basophils. This observation was done in GMA-embedded sections and it was confirmed by applying immunocytochemistry on frozen sections. As in previous studies with empty capsules [43], we found no elements of the specific immune system such as B- or T-lymphocytes.

During the tissue response against microcapsules we observed an immediate influx and adherence to the capsules of monocytes, macrophages, granulocytes, and some basophils. These inflammatory cells gradually disappear from the capsule surface during prolonged stay in the peritoneal cavity (Fig. 2). When the inflammatory cells disappear from the capsule surface, we observed a fibroproliferative response characterized by an influx of fibroblasts and the development of fibroconnective tissue around the capsules.

Although the same cell-types were involved in the tissue response against intermediate-G and high-G alginate, we found at day 5 after implantation more monocytes/macrophages ($P < 0.001$) and less fibroblasts ($P < 0.05$) on the overgrown intermediate-G capsules than on the overgrown high-G capsules. This illustrates a faster initiation of the fibroproliferative response against high-G capsules than against intermediate-G capsules.

With both intermediate-G and high-G alginate we found some erythrocytes, multinucleated giant cells but these were present in too low numbers to allow for reliable quantification. Erythrocytes were found on capsules at day 1 but not on later days suggesting that the erythrocytes have been introduced during or after trauma associated with the surgical implantation of the capsules. Multinucleated giant cells were absent in slices of capsules retrieved at day 1 and present in low numbers in slices of capsules retrieved at day 5 and 7. Multinucleated giant cells were mainly observed in and around capsules containing localized defects suggesting that these cells are involved in breakdown of imperfect capsules.

3.4. Tissue response at week 2, 4, and 8 weeks after implantation

It has been assumed that the reaction against capsules is complete within seven days after implantation [35,44,45]. This assumption is based on quantification of the number of capsules that is affected by cellular overgrowth and not on analyses of the composition of the overgrowth. Therefore, we repeated the implantation studies with intermediate-G alginate and high-G alginate and retrieved the capsules at 2 weeks ($n = 4$), 4 weeks ($n = 5$), and 8 ($n = 4$) weeks after implantation for analyses of the number of overgrown capsules and composition of the infiltrate. In these experiments, the animals were not subjected to repeated peritoneal lavages but sacrificed for peritoneal lavage and excision of the capsules from the abdominal organs.

With intermediate-G alginate, we washed out more than 85% of the capsule graft from the rat peritoneal cavity on week 2, 4, and 8 after implantation. The retrieval rate was much lower with high-G alginate. At 2 weeks after implantation we retrieved less than 20% of the initially implanted capsules. This further decreased to a retrieval rate of zero at 4 and 8 weeks after implantation. At these time points all capsules prepared of high-G alginate were found to be adherent and integrated into the abdominal organs, overgrown, and covered by fibroproliferative tissue (Table 2).

The percentage of overgrown capsules in grafts composed of intermediate-G alginate was $4.2 \pm 2.1\%$ at two weeks after implantation and not statistically significantly different from the portion overgrown capsules at day 7 after implantation. However, at 4 weeks and 8 weeks post-implant we found respectively, $7.8 \pm 1.1\%$ and $7.2 \pm 0.8\%$ of the retrieved capsules to be affected by cellular overgrowth which were statistically significantly higher percentages than that found at two weeks and 7 days after implantation (P always < 0.05).

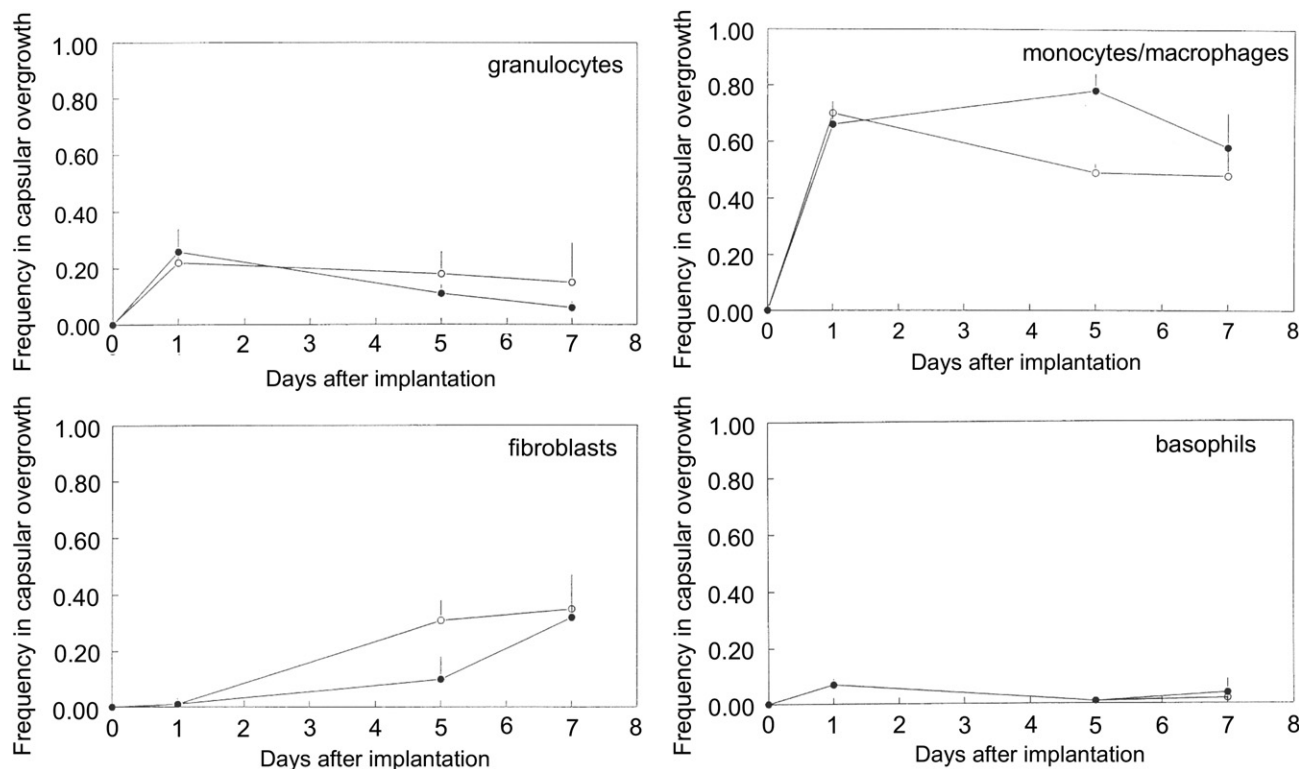


Fig. 2. Frequency of granulocytes (top left), macrophages (top right), fibroblasts (bottom left), and basophils (bottom right) in the cellular overgrowth on alginate-PLL capsules prepared of intermediate-G alginate (closed circles) or high-G alginate (open circles) on day 1, 5, and 7 after implantation. Values represent mean \pm SEM of four experiments.

Table 2

Composition of cellular overgrowth on alginate-PLL capsules at 2, 4, and 8 weeks after implantation in the peritoneal cavity of AO-rats.

Weeks after implantation	n	Intermediate-G alginate		High-G alginate	
		Monocytes/macrophage	Fibroblasts	Monocytes/macrophages	Fibroblasts
2	4	40.4 ± 8.3	59.2 ± 6.8	18.8 ± 2.6	81.1 ± 8.2
4	5	22.5 ± 4.1	77.1 ± 10.4	–	100%
8	4	2.2 ± 0.8	97.6 ± 8.6	–	100%

The composition of the tissue response was also subject to change after the first 7 days of transplantation. As of week two, we found predominantly monocytes, macrophages, fibroblasts, and occasionally multinucleated giant cells. The granulocytes and basophils we observed in the first week after implantation had disappeared from the graft. Monocytes/macrophages will stay somewhat longer in the graft but also disappear in due time. The rate of disappearance is alginate-type dependent. With intermediate-G alginate we found that at four weeks postimplant a substantial portion of the cells to be composed of monocytes/macrophages while with high-G alginate virtually all monocytes/macrophages had been deleted from the grafts. It took till week 8 with intermediate-G alginate before virtually all monocytes/macrophages had disappeared from the graft.

3.5. Surface properties after implantation

From the foregoing follows that the differences in the responses against the microcapsules prepared of intermediate-G and high-G alginate already occurs in the first days after implantation. As we implanted capsules with identical physico-chemical and mechanical properties we questioned whether the capsules might undergo changes *in vivo* that might be hold responsible for the differences in responses. As we found in a recent *in vitro* study [46] that the properties of the capsules surface may change after exposure to human peritoneal fluid we questioned whether there might be a difference in properties of the intermediate and high G capsules after implantation. To this end we implanted in a separate series of experiments capsules in the peritoneal cavity of rats which were retrieved by peritoneal lavage at 1, 3, and 7 days after implantation. First we compared the protein adsorption at the capsule surface after implantation. This was done by XPS (Table 3). After implantation of intermediate-G capsules, the nitrogen signal gradually increases from 5.8% but to 8.6% at day 7 ($P < 0.05$) as the consequence of protein release in the vicinity of capsules and adsorption of those proteins by the capsule's surface. With high-G capsules we observed a somewhat different profile. Initially we observed a statistical significant decrease ($P < 0.05$) of the N-signal from 6.0 ± 0.5% on fresh capsule to 5.5 ± 0.3% at day 1 after implantation. Thereafter we found an increase from 5.5 ± 0.3% on

Table 3Elemental surface composition of alginate-PLL capsules ($n = 4$).

	C (%)	N (%)	O (%)	Others (Na, K, Si) (%)
Day 0				
Inter-G	57.2 ± 0.8	5.8 ± 0.6	34.3 ± 2.1	2.7 ± 0.8
High-G	55.3 ± 0.7	6.0 ± 0.5	33.9 ± 0.8	4.8 ± 0.6
Day 1				
Inter-G	57.8 ± 3.6	5.8 ± 1.0	30.0 ± 3.9	6.4 ± 1.0
High-G	56.1 ± 1.0	5.5 ± 0.3	34.2 ± 1.6	4.2 ± 0.6
Day 5				
Inter-G	58.9 ± 1.7	7.2 ± 0.9	30.1 ± 1.2	3.8 ± 0.4
High-G	64.5 ± 4.1	7.4 ± 0.8	23.2 ± 2.3	4.9 ± 0.8
Day 7				
Inter-G	61.6 ± 2.6	8.6 ± 0.8	26.3 ± 2.7	3.5 ± 0.5
High-G	62.3 ± 2.4	7.5 ± 0.6	27.1 ± 2.9	3.1 ± 0.3

day 1 to 7.5 ± 0.6 at day 7 after implantation. This decrease in N signal reflects a loss of PLL at the surface of high G capsules and exposure of positive charges. Free positive charges on high-G capsules are considered to be responsible for inflammatory responses and are considered to be causative for inflammatory responses to alginate-PLL capsules [34,47]. To measure possible positive charges on capsules we quantified the zeta-potential of implanted capsules at day 1 after implantation. Although the zeta-potential of intermediate and high-G capsules was equal at the day of implantation, we found that the zeta potential of high-G capsules increased to -0.5 ± 0.7 mV at day one after implantation ($P < 0.01$). This was not observed with intermediate-G capsules.

4. Discussion

In the present study we show that in spite of similar mechanical and physicochemical surface properties high-alginates capsules provoke a stronger inflammatory response than capsules prepared of intermediate-G alginate. We show that this is caused by undesired changes on the surface of the high-G capsules with an increase of the zeta-potential as a consequence. This increase reflects an elevation of the number of positive charges at the surface. This is a well-known factor in cell adhesion and inflammatory responses against microcapsules [36,47]. On the basis of our results we propose the following mechanism (Fig. 3). The high-G and intermediate-G capsules have similar physicochemical properties before implantation. This changes rapidly after implantation. After implantation, a non-specific immune response is activated with influx of cells and pH changes in the implantation site as a consequence [38,48–54]. This together with adsorption of proteins from the peritoneal fluid [46] may result in structural surface changes. Alginate-PLL capsules prepared of intermediate-G alginate seem to be resistant to the changing microenvironment in the implantation site. This however is different with the capsules prepared of high-G alginate which loose, as shown in the present study, some poly-L-lysine immediately after implantation and contains at day 1 after implantation many positive charges from poly-L-lysine molecules that are not bound to alginate at the surface. This results in cellular adhesion and fibrosis of the high-G microcapsules.

It might be suggested that different results may be obtained if other polycations than poly-L-lysine would have been applied. However, we selected poly-L-lysine for a reason. In a previous study [15] we investigated the effect of poly-L-lysine (PLL), poly-D-lysine (PDL) and poly-L-ornithine (PLO) and found that only PLL was associated with minimal inflammatory responses. PLL requires G-M blocks to interact with the alginate matrix [55]. It is unknown what ligand is required for other polycations such as PDL and PLO for optimal interaction with alginate matrices.

Our study illustrates an important and overlooked item in encapsulation research, i.e. the physicochemical properties *in vitro* are not necessarily the properties after exposure to the *in vivo* microenvironment. Upon exposure to body fluids microcapsules will adsorb bioactive components which will change the properties of the capsule surface and the responses against the capsules

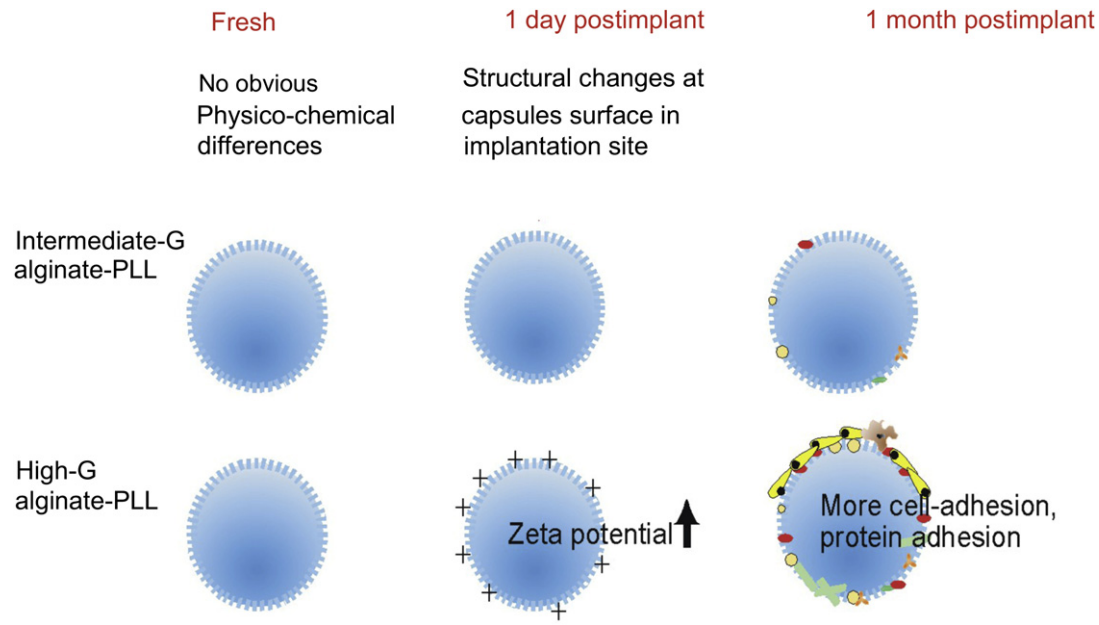


Fig. 3. Fresh alginate-PLL capsules can have similar physicochemical properties. This will change after implantation in an alginate type dependent way. After implantation, a non-specific immune response occurs with influx of cells and pH changes in the implantation site as a consequence. This change in the microenvironment of the capsules results on high-G alginate PLL capsules in structural changes such as an elevation of the positive charges at the surface with cell adhesion and inflammatory responses against the high-G microcapsules as a consequence. Intermediate-G alginate PLL capsules are not sensitive for the changes in the microenvironment and remain free from cellular adhesion.

[16,46]. In recent *in vitro* studies we showed that fluctuations in pH in body fluids changes the properties and charge density of capsules making capsules more susceptible for adsorption of body proteins and inflammatory responses [36]. We now confirm that this also occurs *in vivo* with alginate-poly-L-lysine capsule prepared of high-G alginate while capsules of intermediate-G alginate can resist the *in vivo* microenvironment. Our data suggest that for predicting biocompatibility of alginate-based capsules we should not only focus on the properties of the freshly prepared capsules but also on the properties of the capsule surface after exposure to body fluids. Our recently published system in which capsules are first incubated to peritoneal fluid and subsequently exposed to human peripheral blood mononuclear cells to quantify inflammatory responses may serve as a fast *in vitro* assay to predict pro-inflammatory structural surface changes on alginate-based capsules after exposure to body fluids [46].

Our study was not only performed to identify the cause for the difference in responses against capsules prepared of different types of alginate but also to gain more insight in the composition of the inflammatory responses against alginate-based microcapsules. At present many groups focus on the design of means to reduce inflammatory responses in the immediate period post-transplant as this is associated with a significant loss of tissue in the capsules [56]. In order to design adequate intervention strategies we require more detailed insight in the cellular composition of the responses, its dynamics and whether it is alginate dependent. We show in the present study that this inflammatory response is a dynamic rather than a static process involving many different cell types in a time dependent fashion. We show that in the first week after implantation the capsules attract many cell types such as monocytes/macrophages, granulocytes, fibroblasts, erythrocytes, multinucleated giant cells, and basophiles which population of cells is reduced to monocytes/macrophages and fibroblasts in the second week. After the second week we observe a gradual disappearance of the monocytes/macrophages which were substituted by fibroblasts. Although, we found with intermediate-G and high-G alginate no difference in the type of cells involved the tissue response, we

found an earlier decrease in number of inflammatory cells and a faster fibroproliferative response with high-G than with intermediate-G capsules.

Inflammatory cells such as monocytes and macrophages have been shown to produce cytotoxic components with deleterious effects for enveloped tissue such as pancreatic islets [57–60]. Losses of up to 60% of the insulin producing islet grafts has been reported [44,56]. An inflammatory response cannot be avoided since it starts already with the mandatory surgery to implant the capsules and since it cannot be avoided that some capsules contain imperfection [21,35,42,56] and provoke a response. Therefore, the current strategy of many research groups is to apply anti-inflammatory drugs to inhibit the activation of monocytes and macrophages in the vicinity of the capsules [44,45]. In most cases these drugs are applied in the immediate posttransplant period up to two weeks. Our data show however that activated macrophages and monocytes can be found up to 8 weeks after implantation. This was even found on grafts that elicit not more than a minimal response to not more than 7% of the capsules. We therefore conclude that it might be advisable for prevention of loss of the enveloped tissue to apply anti-inflammatory drugs for longer periods of up to 8 weeks after implantation.

The high-G capsules membranes have a lower protein content at day 1 after implantation than before implantation was an unexpected observation. This indicates loss of PLL from the capsule membrane immediately after implantation. It may be suggested that it corroborates a previous suggestion that high-G alginate takes up excessive amounts of PLL during the coating procedure with the polyamino-acid since high-G only has limited numbers of binding sites for PLL [12,55]. In our experimental set up however we washed the capsules excessively and did zeta-potential measurements before implantation to confirm that the capsules did not contain any unbound poly-L-lysine. Rather our data corroborate our recent observations that high-G alginate-PLL complexes are less stable and more susceptible for structural changes in body fluid than intermediate-G-PLL complexes [36,46]. This could be explained by the fact that intermediate-G alginate

contains more G-M block polymer than high-G alginates. These G-M blocks are responsible for PLL binding and the formation of stable α -helix, antiparallel β -sheet and random coil conformation [55]. Our data therefore suggest to avoid the application of high-G alginates for the preparation of alginate-PLL capsules.

During the past decade many procedures to fabricate alginate-based capsules have been described. Unfortunately, most of these procedures lack an adequate documentation of the characterization of the microcapsules [18]. As a result many procedures show an extreme lab-to-lab variation and cannot be adequately reproduced. Our present results illustrate that adequate documentation of the polymers and standardization of testing procedures can no longer be neglected as we show that a minor difference in G-content of the applied alginate of not more than 10% has a profound effect on the responses against the capsules. In most studies the alginate type is poorly or not documented at all [18]. Our data suggest that this is an important factor contributing to the extreme lab-to-lab variations of the technology [18,21].

5. Conclusion

We show that alginate-based capsules may undergo undesired physicochemical changes *in vivo*. These changes are dependent on the type of alginate applied. As a consequence of these *in vivo* changes, high-G capsules provoke an inflammatory response which is different in composition, in numbers of inflammatory cells, and in duration when compared to intermediate-G capsules. The detachment of PLL *in vivo* and an increase in positive charges on the capsule surface should be held responsible for these strong responses against high-G capsules. We feel we may conclude on the basis of our results that our study illustrate an important overlooked issue; the physicochemical properties *in vitro* are not necessarily the properties after exposure to the *in vivo* microenvironment. The changes *in vivo* should gain more attention in future engineering efforts.

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