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Effect of the alginate composition on the biocompatibility of alginate–polylysine microcapsules

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Alginate–polylysine (PLL) capsules are commonly applied for immunoprotection of endocrine tissues. Alginate is composed of mannuronic acid (M) and guluronic acid (G). Different types of alginate have different ratios of G to M, but little is known of the influence of these differences on biocompatibility. Therefore, we have investigated *in vivo* the effect of the G-content of the alginate on the biocompatibility of the capsules. Capsules prepared of commercially available alginates with either a high or an intermediate G-content were implanted in the peritoneal cavity of rats and retrieved one month later for histological evaluation. The fibrotic reaction was more severe against high-G alginate capsules than to intermediate-G alginate capsules. The majority of the high-G capsules proved to be overgrown and adherent to the abdominal organs whereas with intermediate-G alginate most capsules were found freely floating in the peritoneal cavity and free of any adhesion of cells. This was not caused by the alginate as such but rather by inadequate binding of high-G alginate to PLL since in the absence of PLL, i.e. with beads instead of capsules, no fibrotic reaction was observed. As high-G alginates have beneficial effects for islet encapsulation, efforts should be made to apply polycations which more effectively interact with high-G alginate than PLL.

Keywords: Microencapsulation, alginate, poly-L-lysine, biocompatibility, peritoneal cavity

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Microencapsulation of islets involves enclosing of individual islets in a semipermeable membrane to protect islets against cytotoxic effects of the host immune system. One method of microencapsulation applies an alginate–polylysine(PLL)–alginate capsule as described in 1980 by Lim and Sun[1]. As this procedure does not interfere with the function of the islet[2] and allows for easy modulation of the molecular cutoff of the capsule[3], it is still preferred over other more recently introduced encapsulation techniques such as microencapsulation in agarose[4,5] or AN69[6,7].

Alginate, the main component of the capsule, is composed of chains of guluronic acid (G) and mannuronic acid (M). Commercially, alginates are available with various ratios of the content of G-chains and M-chains (G/M ratio). Alginates with an intermediate-G content are usually applied for encapsulation of islets. Recently, however, we have shown that capsules prepared of high-G alginates have some advantage over capsules prepared of alginates with an intermediate-G content, since they contain much lower numbers of incompletely and therefore inadequately encapsulated islets[8]. Also, they have a higher mechanical stability which provides an advantage in terms of durability[9]. However, up to now these advantages have only been demonstrated *in vitro*, and the influence of the G content of capsules on their biocompatibility have never been studied *in vivo*.

Therefore, the present study was undertaken to assess and compare the recipient responses against capsules composed of different commercially available alginates with varying G/M-ratios.

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MATERIALS AND METHODS

Design of the study

Capsules prepared of Ca-alginates with varying G-content were implanted in the peritoneal cavity, i.e. the usual transplantation site for an encapsulated islet graft, of AO-rats. We used empty capsules so that the effect of the capsule composition on the response was not confused with that of an eventual immune response against the islets. Only highly purified alginates were applied in order to exclude that contaminating components were the cause of an inflammatory response. Before implantation, the capsules were inspected microscopically in order to confirm that the majority of the capsules was intact. Instead of capsules, beads were implanted to determine whether the alginate as such or the PLL had provoked the response. We used Ba beads rather than Ca beads for the higher mechanical stability of Ba alginate.

Alginates

Keltone LV (medium-G) and Manugel (high-G) sodium alginates were obtained from Kelco International (London, UK). These alginates were purified with our own procedure as described elsewhere[10]. Another high-G alginate, Pronova MV, which is a commercially available purified alginate, was obtained from Protean (Pronova UP, MVG, high G, Pronova Biopolymer, Drammen, Norway). Alginates were dissolved at 4°C in Krebs-Ringer-Hepes (KRH) with an appropriate osmolarity and subsequently sterilized by 0.2 μm filtration. As sterile filtration is only possible up to a certain viscosity and since alginate solutions with a high-G content have higher viscosities, we were obliged to lower the alginate concentrations with high-G alginates. We maintained, however, the alginate solutions at the highest alginate concentration feasible, as a higher viscosity of the alginate solution is associated with a better quality of the capsule[11]. Alginates were solved and filtrated in the following concentrations: 3% Keltone LV (medium-G), 2% Manugel (high-G), 1.7% Pronova MV (high-G).

Animals

In a previous report we demonstrated that the response against encapsulated islet grafts is not influenced by the glycemic state of the recipient[12]. Therefore, normoglycemic male inbred Albino Oxford (AO/G) rats served as recipients and were obtained from the Central Animal Laboratory of Groningen. Their body weights ranged from 300 to 350 g.

Formation of capsules and Ba beads

A volume of 1.6–2.0 ml of an alginate solution was converted into droplets using an air-driven droplet generator as previously described[11]. Alginate–polylysine–alginate encapsulation was performed as described elsewhere[12]. Briefly, the alginate droplets were transformed to alginate beads by gelling in a 100 mM $CaCl_2$ (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^{-1} $CaCl_2$. The 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 Ca^{2+}-free KRH containing 135 mM NaCl. The outer alginate layer was subsequently applied by 5 min incubation in a diluted alginate solution. In order to liquify their inner core, the microcapsules were suspended in 1 mM EGTA in Ca^{2+}-free KRH for 10 min, and finally washed three times with KRH containing 2.5 mM $CaCl_2$.

Ba beads were formed by collection of alginate droplets in a 10 mM $BaCl_2$ (10 mM HEPES, 2 mM KCl) solution. The beads were gelled for at least 5 min and subsequently washed three times with saline.

All procedures were performed under sterile conditions. 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. Both the capsules and the Ba beads had a diameter of 600–700 μm. The same microscope was used for inspection of the capsules prior to implantation.

Implantation and explantation of capsules and Ba beads

Capsules or Ba beads were injected into the peritoneal cavity with a 16 G cannula via a small (3 mm) midline incision. The total volume of the capsules implanted varied between 1.2 and 2.0 ml depending upon the type of alginate applied, as the consequence of differences in swelling properties.

Microcapsules and Ba beads were retrieved four weeks after implantation by peritoneal lavage. Microcapsules and Ba beads were either freely floating and non-adherent, or adherent to the surface of abdominal organs. First, non-adherent microcapsules were retrieved by peritoneal lavage, performed by infusing 20 ml KRH through a 3 cm midline incision into the peritoneal lavage, performed by infusing 20 ml 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. From this tube, the microcapsules or Ba beads were brought into a 10 ml measure-cylinder (for a volume of 2.0–2.5 ml) or a 1 ml syringe (for volumes smaller than 1 ml) to assess the retrieved volumes. Subsequently, the microcapsules of Ba beads adherent to the surface of abdominal organs, were excised and processed for histology.

All surgical procedures were performed under ether anaesthesia.

Histology and assessment of capsular overgrowth

Samples of adherent capsules or Ba beads recovered by excision and non-adherent capsules or Ba beads were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05 M phosphate, and processed for glycol methacrylate (GMA) embedding[13]. Sections were prepared at 2 μm and stained with Romanovsky-Giemsa stain and applied for determining the number of capsules or Ba beads with and without overgrowth. The degree of capsular overgrowth was quantified by expressing the number of recovered capsules or Ba beads with overgrowth as the percentage of the total number of recovered capsules or Ba beads for each individual animal.

In order to identify the cell-types present in the overgrowth, portions of adherent and non-adherent capsules were frozen in precooled 2-methyl butane, sectioned at 5 μm, and processed for immunohistochemical staining as previously described[12]. The monoclonal antibodies used were: ED1 and ED2 against monocytes and macrophages[14], HIS-40 against IgM bearing B-lymphocytes[15], and R73 against $CD3^+$ bearing T-lymphocytes[16]. In control sections we used PBS instead of the first stage monoclonal antibody.

In all GMA-embedded histological sections, macrophages and fibroblasts were distinguished on the basis of their completely different morphological appearance.

Statistical analysis

Results are expressed as mean ± s.e.m. Statistical comparisons were made with the Mann Whitney U

test. A P-value < 0.05 was considered statistically significant.

RESULTS

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 (*Figure 1*) while the remaining capsules were either single or in clumps adherent to the surface of the abdominal organs. The portion of freely floating capsules, as quantified by the retrieval rates of the capsules, was larger with intermediate-G alginate capsules than with high-G alginate capsules (*Table 1*). Furthermore, the percentage of freely floating capsules with overgrowth was much smaller with intermediate-G alginate than with the high-G alginates. Not only the number of overgrown capsules but also the cellular composition of the overgrowth was different with intermediate-G and high-G capsules. The overgrowth on capsules recovered by peritoneal lavage was composed of fibroblasts in case of intermediate-G alginate (*Figure 2*), but it was mainly composed of macrophages instead of fibroblasts when high-G alginate had been applied (*Figure 3*). This observation was made in GMA-embedded sections and it was confirmed by applying immunocytochemistry on samples of capsules retrieved from two recipients of intermediate-G capsules and three recipients of Manugel high-G capsules. As in previous studies with empty capsules[12], we found only macrophages and cells with the morphological appearance of fibroblast in the overgrowth and no other immune-cell elements such as B- or T-lymphocytes.

With the exception of two recipients of high-G Manugel capsules, all adherent capsules were overgrown by fibrotic tissue. In these two recipients of high-G alginates we found clumps of capsules which were fibroencapsulated but contained within the clump some capsules which were free of any cellular adhesion.

With microscopical inspection prior to implantation, we observed all batches of microcapsules to contain some broken capsules (*Figure 4*). The number of broken capsules represented not more than approximately 5% of the total number of capsules both in batches of intermediate-G alginate and high-G alginate capsules. Consequently, the severe response against the high-G alginate capsules cannot be explained by the presence of broken capsules as such, and other factors not related to the physical integrity of the high-G alginate capsules must be considered as the cause of bioincompatibility.

In order to investigate whether the overgrowth is initiated by the alginate as such or by the presence of PLL, we have implanted Ba-alginate beads into the peritoneal cavity. These experiments were restricted to the implantation of Ba-beads prepared of the two types

Figure 1 The abdominal cavity of a normoglycemic AO-rat one month after implantation of capsules prepared of Keltone IV alginate. Note the free floating capsules.

Table 1 Recovery rates and percentage of alginate-PLL capsules with overgrowth, one month after implantation in the peritoneal cavity of AO-rats. Capsules are prepared of alginates with intermediate-G or high-G content

Type of alginate	n	% Recovery	% Overgrowth
Intermediate-G (Keltone LV)	4	88.8 ± 4.3	1.5 ± 0.5
High-G (Manugel)	8	40.9 ± 4.5	33.0 ± 11.0
High-G (Pronova MV UP)	12	19.2 ± 7.1	70.4 ± 6.7

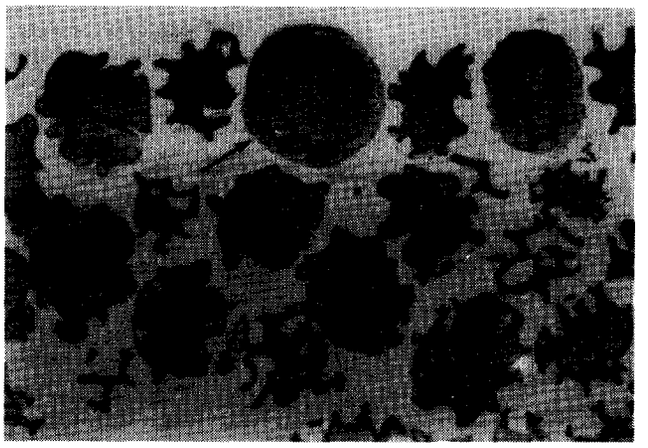

Figure 2 Alginate-PLL capsules prepared of Keltone LV, one month after implantation into the peritoneal cavity of normoglycemic AO-rats. The arrow indicates a capsule covered by several layers of fibroblasts. (GMA-embedded histological section, Syrius Red staining, original magnification × 50.)

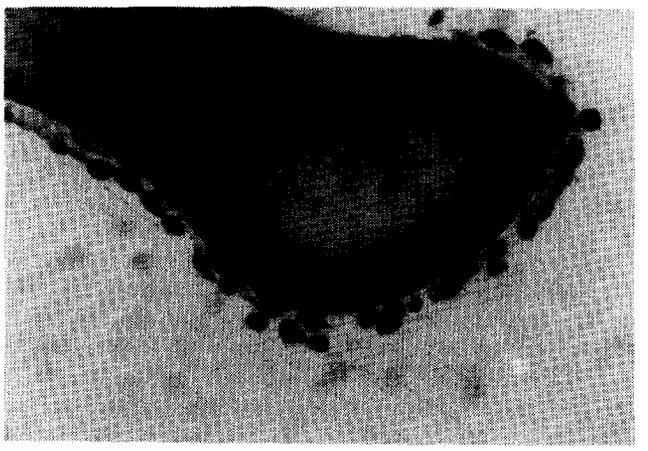

Figure 3 Alginate-PLL capsule prepared of Manugel, one month after implantation into the peritoneal cavity of normoglycemic AO-rats. The capsule is covered by macrophages. (GMA-embedded histological section, Romanowsky-Giemsa stain, original magnification × 800.)

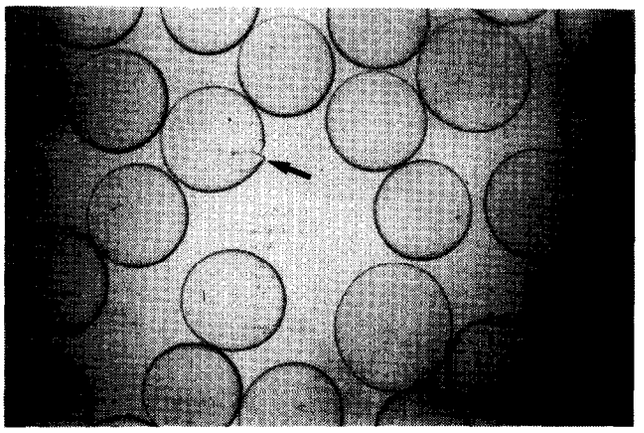

Figure 4 A batch of freshly prepared Keltone IV capsules. The arrow indicates the presence of a capsule with a broken PLL membrane. (Original magnification × 50.)

Table 2 Recovery rates and overgrowth of Ba beads prepared of high-G alginates, one month after implantation in the peritoneal cavity of AO rats

Type of alginate	*n*	% Recovery	% Overgrowth
High-G (Manugel)	4	78.5 ± 2.0	1.3 ± 0.8
High-G (Pronova MV UP)	4	87.3 ± 5.4	2.0 ± 0.0

of high-G alginate, in view of the very modest response to the intermediate-G alginate capsules. As illustrated in *Table 2*, there was only a modest response towards Ba beads composed of high-G alginates. By peritoneal lavage, the vast majority of both the Manugel Ba beads (78.5 ± 2.0%) and the Pronova Ba beads (87.3 ± 5.4%) could be retrieved. Of these beads, only 1.3 ± 0.8% and 1.5 ± 0.5%, respectively, was overgrown. During the subsequent inspection of the abdominal cavity and its contents, the remaining 20% of the beads was not retrieved nor found to be adherent to the peritoneum or the abdominal organs with the exception of one recipient with a clump of beads adherent to the liver capsule.

We hypothesized that the response against high-G capsules was due to inadequate binding of high-G alginates to the positively charged PLL-chains. To test this hypothesis, we have replaced the second high-G alginate incubation by an incubation with the intermediate-G alginate, which experiment was only performed with the high-G alginate Pronova. Thus, we have prepared Pronova MV UP-PLL-Keltone LV capsules, which were subsequently implanted in the peritoneal cavity of normoglycemic AO-rats. These high-G alginate-PLL-intermediate-G alginate capsules provoked a severe fibrotic response similar to the response against capsules completely composed of the high-G alginate Pronova. Only 8.8 ± 4.6% of the capsules could be retrieved by peritoneal lavage of which 70 ± 17% was found to be overgrown. All other capsules were observed to be overgrown by fibrotic tissue and adherent to the abdominal organs.

DISCUSSION

The biocompatibility of alginate-PLL capsules strongly depends on the G-content of the alginate applied. Capsules composed of high-G alginates proved to provoke a severe inflammatory response while the majority of capsules prepared of alginates with an intermediate-G content remained free of overgrowth.

Our findings regarding the bioincompatibility of alginate-PLL capsules prepared of high-G alginates are in conflict with the findings of Soon-Shiong *et al.*[17] and Otterlei *et al.*[18] but confirm the results of Clayton *et al.*[19]. Otterlei *et al.*[18] found that not G but M residues in alginate are responsible for inducing cytokine production by monocytes and, therefore, for initiating an immune response. These results are supported by Soon-Shiong *et al.*[17,20] who found that high-G alginate capsules were less prone to overgrowth than high-M alginate capsules. As both authors applied non-purified alginates, it is well possible that a response against contaminations of the alginate was mistakenly interpreted as a response against M-residues in the alginate. This explanation of the discrepancies between our observations and those of Otterlei[18] and Soon-Shiong *et al.*[17,20] is confirmed by the observations of Zimmermann *et al.*[21] who found the response against low-G and high-G alginate Ba beads to be similar when purified alginates were applied.

We have clearly demonstrated that the severe fibrotic response against high-G alginate capsules is associated with the presence of PLL around the beads. This response could not be prohibited by an incubation with intermediate-G alginate, which indicates that the initiation of the response is associated with the inappropriate binding of PLL onto the high-G alginate core. This is probably the consequence of the conformation of G which in contrast to M does not allow for complete interaction with polylysine[22,23] and induces disordered arrangements and interactions when complexation between PLL and G occurs[22–24].

Some high-G capsules, however, were observed to escape the fibrotic response. These capsules may have been liberated during peritoneal lavage from clumps of capsules caught in fibrosis[25]. However, additional factors should be considered to explain the presence of non-overgrown capsules, since we often observed translucent capsules without overgrowth and freely floating in the peritoneal cavity. One possible explanation is the following. In spite of the inappropriate binding of high-G alginate to PLL, it is quite plausible that, initially, all high-G capsules contain some covering of the PLL-membrane, as high-G alginates contain not only G but also some M. However, this covering of PLL may either be incomplete from the beginning owing to a disordered interaction[21–24] or, alternatively, it may gradually become incomplete, as time continues, owing to a temporary but not persisting interaction between PLL and high-G alginate. This suggestion of temporary attachment of alginate onto PLL is supported by Thu *et al.*[26] who found, up to two months after encapsulation, alginate molecules began to detach from the capsules surface. It also explains for the presence of non-overgrown high-G alginate capsules since in view of this reasoning we may suggest that, at four weeks after implantation, their PLL-membrane was still sufficiently covered by alginate.

For optimal kinetics of diffusion of nutrients, glucose and insulin, the capsule diameter should preferably be as small as possible. In a previous study[27] we firstly

demonstrated with an *in vitro* assay that application of small capsules is associated with inadequate encapsulation of a significant portion the islets and, secondly, that this portion of inadequately encapsulated islets induces *in vivo* a reaction which is associated with failure of the graft. In a subsequent *in vitro* study[8], we showed that the portion of inadequate capsules can be reduced by application of high-G alginates. Unfortunately, however, the present *in vivo* study shows that such high-G alginate capsule provoke a severe fibrotic response, which response is associated with necrosis of the encapsulated islets[9,28] and failure of the islet graft[12,27–29].

There are several approaches to overcome the problem of bioincompatibility of high-G alginate capsules. Firstly, a positive effect has been reported of treatment of the capsules with tolylene 2,4-diisocyanate[30], with poly(ethylene oxide)[31], with poly(ethylene glycol)[32], or with poly(vinyl alcohol)[33]. Alternatively, one could search for polycations which bind to high-G alginates more effectively than PLL such as, for instance, poly(Lys-Ala-Ala)[23] in combination with similarly effective immunoprotective properties.

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