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Published in:
Biomaterials

DOI:
[10.1016/S0142-9612\(02\)00319-8](https://doi.org/10.1016/S0142-9612(02)00319-8)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Vos, P., van Hoogmoed, CG., van Zanten, J., Netter, S., Strubbe, JH., & Busscher, HJ. (2003). Long-term biocompatibility, chemistry, and function of microencapsulated pancreatic islets. *Biomaterials*, 24(2), 305-312. [PII S0142-9612(02)00319-8]. [https://doi.org/10.1016/S0142-9612\(02\)00319-8](https://doi.org/10.1016/S0142-9612(02)00319-8)

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Long-term biocompatibility, chemistry, and function of microencapsulated pancreatic islets

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Received 15 May 2002; accepted 23 July 2002

Abstract

Transplantation of encapsulated living cells is a promising approach for the treatment of a wide variety of diseases. Large-scale application of the technique, however, is hampered by insufficient biocompatibility of the capsules. In the present study, we have implemented new as well as previously reported technologies to test biocompatibility issues of immunoisolating microcapsules on the long term (i.e. 2 years) instead of usually reported short time periods. When transplanted empty, the capsules proved to be highly biocompatible not only for short periods (i.e. 1 month) but also on the long term as evidenced by the absence of any significant biological response up to 2 years after implantation in rats. The immunoprotective properties of the capsules were confirmed by prolonged survival of encapsulated islet allografts up to 200 days. The surface of the applied capsule was analyzed and provides new insight in the chemical structure of true biocompatible and immunoprotective capsules applicable for transplantation of encapsulated islets in type I diabetes.

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Keywords: Alginate; Microencapsulation; Encapsulation; Islets; Insulin; Biocompatibility

1. Introduction

The recent advances in clinical islet transplantation [1] have increased the interest in transplantation of an endogenous insulin source for control of the diabetic blood sugar. A major obstacle to overcome the large-scale application is the requirement to apply effective immunosuppression, which is associated with serious side effects, especially when applied for a life-long period. Immunoisolation by microencapsulation does not require the use of immunosuppression and has therefore the potential to be developed into a widely applicable therapy for patients with insulin dependent diabetes.

A commonly used procedure for immunoprotection is microencapsulation of tissues in alginate-based capsules as originally described by Lim and Sun [2]. During

recent years, important advances have been made with this technology. Human trials have been started and showed temporary but pertinent survival of human microencapsulated tissue after allotransplantation of encapsulated parathyroid cells [3] and islets [4]. Also, microencapsulation has been shown to allow for prolonged survival of xenotransplanted islet grafts in both chemically induced and autoimmune diabetic rodents [5], dogs [4], and monkeys [6]. 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 [7]. This variation in success rate is usually attributed to differences in the biological responses (i.e. biocompatibility) against the applied capsules. In order to improve the capsule properties, many groups have introduced their own technical modifications. As a consequence there are many different procedures with specific chemical characteristics which, regrettably, are never documented.

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In the foregoing years we have concentrated on the identification and deletion of factors inducing biological responses against encapsulated islets. Alginates are composed of mannuronic acid (M) and guluronic acid (G) and can be contaminated with inflammatory substances. We found that the biological response against capsules can be reduced by applying alginates with a G-content of 40–45% instead of more than 50% and by introducing alginates with a high purity degree [5,8–11]. Also, an inadequate mechanical integrity of the capsules [9,12] can cause immunological reactions in the recipient. Finally, we found that grafts can fail as the consequence of incomplete encapsulation of islets [13,14].

In the present study, we changed the capsule production process by applying modifications which have previously shown to influence the biological response against capsule grafts [5,8–12]. Empty capsules produced according to our modified procedure were implanted in the peritoneal cavity and retrieved after varying time intervals for evaluation of the biological response against the capsules. Next, syngeneic and allogeneic rat islets were encapsulated and transplanted in diabetic rats in order to assess the immunoprotective properties of the capsules. Finally, we analyzed and present the chemical structure of the immunoprotective capsule, which remained free of any biological response up to 2 years after implantation in rats.

2. Materials and methods

2.1. Design of the study

Only highly purified alginates were applied in order to exclude that contaminating components were the cause of an inflammatory response. Capsules were prepared of alginates with an intermediate guluronic acid (G) content (40% G) in order to prevent biological responses associated with a high G-content. High viscosity alginate solutions were applied to produce capsules with an adequate mechanical integrity. The capsules were inspected before and after implantation in order to confirm that the majority of the capsules was intact.

Batches of empty alginate-PLL capsules were divided into six portions. Five samples were implanted in the peritoneal cavity, i.e. the usual transplantation site for an encapsulated islet graft, of five AO-rats. The sixth sample was stored for analyses of the chemical structure of the capsules. At 1, 3, 6, 12, or 24 months after implantation, one of the five implanted rats was killed and subjected to peritoneal lavage. We used five series of five rats for this experiment. Recipients of capsules prepared from unpurified alginates served as controls.

Streptozotocin diabetic AO-rats served as recipients of capsules containing either AO-(isografts) or Lewis-islets (allografts). Non-encapsulated allografts served as

controls. At 4 weeks after the implantation of successful encapsulated islet allografts the rats were provided with cardiac catheters implanted via the right jugular vein for blood sampling in unanaesthetized and freely moving rats [15,16]. Subsequently, the glucose tolerance of encapsulated islet allografts was tested by a meal test. Normal AO-rats served as controls. One or two weeks after the reoccurrence of hyperglycemia (i.e. blood glucose levels >8.4 mmol/l), microcapsules were retrieved from the diabetic recipients by peritoneal lavage.

Empty or islet containing capsules retrieved by peritoneal lavage were always processed for histological examination of the degree of overgrowth of the graft (i.e. a measure for the biological response against the capsules [5,8,9,12]).

2.2. Islet isolation and graft recipients

Male inbred Albino Oxford (AO/G) rats were obtained from the Central Animal Laboratory of Groningen. AO/G-rats weighing 300–350 g served as donors. AO/G-rats weighing 290–320 g were used as recipients of islet grafts. Diabetes was induced in these rats by injection of 75–90 mg/kg of Streptozotocin (Zanosar, Upjohn Co., Kalamazoo, MI) via the tail vein.

Islets were isolated as previously described [17]. Briefly, after surgical from the abdomen, the pancreas was chopped, digested using a two stage incubation of 20 min at 37°C with successively 1.0 and 0.7 mg/ml collagenase (Sigma type XI, Sigma, St. Louis, MO, USA). Islets were separated from exocrine tissue using a discontinuous dextran gradient [18] and further purified by handpicking. The total islet volume obtained by the isolation procedure was determined by measuring the diameters of islets in a 4% aliquot of the islet suspension. In each transplant experiment we collected 3500–4200 islets which corresponds to 10–15 μ l of islet tissue which is approximately equal to the endocrine volume of one normal pancreas.

2.3. Alginates

Sodium alginate was obtained from Kelco International, London, UK. Purification of alginate was performed as described in detail by our group elsewhere [8]. All identified factors influencing biological responses were tested in the present study [5,8–14]. The composition of the alginate was studied after purification by nuclear magnetic resonance analysis [8]. Only alginates with a G-content between 40% and 45% were applied [11]. Selected alginates were dissolved at 4°C in Krebs–Ringer–Hepes (KRH) with an appropriate osmolarity to a solution with a viscosity of 4 cps. This viscosity is necessary for the production of spherical droplets without any tails or other imperfections associated with

bioincompatibility. The viscosity of an alginate solution is determined by the concentration of alginate but different alginates have different viscosities. This implies for the intermediate-G (i.e. low viscosity) solution a concentration of 3% to obtain a viscosity of 4 cps. The solutions were sterilized by 0.2 μ m filtration.

2.4. Encapsulation

Capsules were produced according to a three-step procedure. First, we produced rigid Ca-alginate beads, by converting an alginate solution into droplets using an air-driven droplet generator as previously described [19]. The droplets were collected in a Ca-rich solution to gelify into rigid Ca-beads. Secondly, the Ca-beads were subjected to a procedure to form a semipermeable PLL-membrane. Finally, to cover incompletely bound PLL, the alginate-PLL beads were coated to form a so-called second Ca-alginate layer [20]. Capsules had a diameter of 600–700 μ m. All procedures were performed under sterile conditions.

For producing islet-containing capsules islets were mixed with alginate in a ratio of 2000/ml alginate. This ratio is required to prevent protrusion of islets [21]. The adequacy of encapsulation was controlled before grafting by applying our lectin-binding assay [13,14] for identification of protruding and incompletely encapsulated islets. The percentage of inadequate capsules was always less than 3% of the total amount of capsules.

Capsules were injected into the peritoneal cavity. Upon peritoneal lavage, 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 [12]. 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)

XPS is a technology in which X-ray is applied onto the surface of an object of interest. This X-ray induces the release of kinetic energy from the surface which is collected by a spectroscope. The kinetic energy can be analyzed and is characteristic for the elements present in the surface of the object.

For XPS analyses in the present experiments, Ca-beads (i.e. alginate-droplets gelified in a Ca-rich solution) and capsules were three times washed with ultrapure water and gradually lyophilized (Leybold Herecuis, Combitron CMI). Since the spectroscope 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 both at low magnification (i.e. by light microscopy) and at high magnification (i.e. by scanning electron microscopy).

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 a X-ray photoelectron spectrometer (Surface Science Instruments, S-probe, Mountain View, CA). An aluminum anode was used for generation of X-rays (10 kV, 22 mA) at a spot size of 250 \times 1000 μ m. 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. Experiments with beads and capsules were repeated four times to exclude variations between different encapsulation sessions.

2.6. Microscopy

The biological response against capsules was assessed by quantifying the number of capsules overgrown by macrophages and fibroblasts [5,9]. Therefore, samples of adherent capsules recovered by excision and 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 [22]. Sections were prepared at 2 μ m and stained with Romanovsky–Giemsa stain and applied for detecting imperfections in the capsule membrane and for determining the number of capsules with and without overgrowth. 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 [5,8,9,12].

2.7. Glucose tolerance testing

The meal tests were carried out by offering the animal 2 g rat chow (containing 53% carbohydrates, 20% protein, 5% fat and 22% other constituents (minerals, cellulose, water), mixed with 2 ml water. The animals had been habituated to consume the meal within 5 min. Blood samples were taken at 10 min and immediately before the start of the tests, and at 1, 3, 5, 7, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and 120 min after the start of the tests. Blood lost during sampling was replaced by transfusing blood from normal donor rats after every blood sample. Glucose concentrations were determined in whole blood by a ferricyanide method with a Technicon autoanalyzer.

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

3.1. Biological response against empty capsules

The vast majority of empty alginate-PLL capsules was freely floating in the peritoneal cavity without adhesion to the abdominal organs (Fig. 1A). The free capsules could readily be flushed out of the peritoneal cavity, resulting in an explantation of 80–100% up to 2 years postimplant (Table 1). Of the recovered capsules only a portion of not more than 10% of the capsules contained

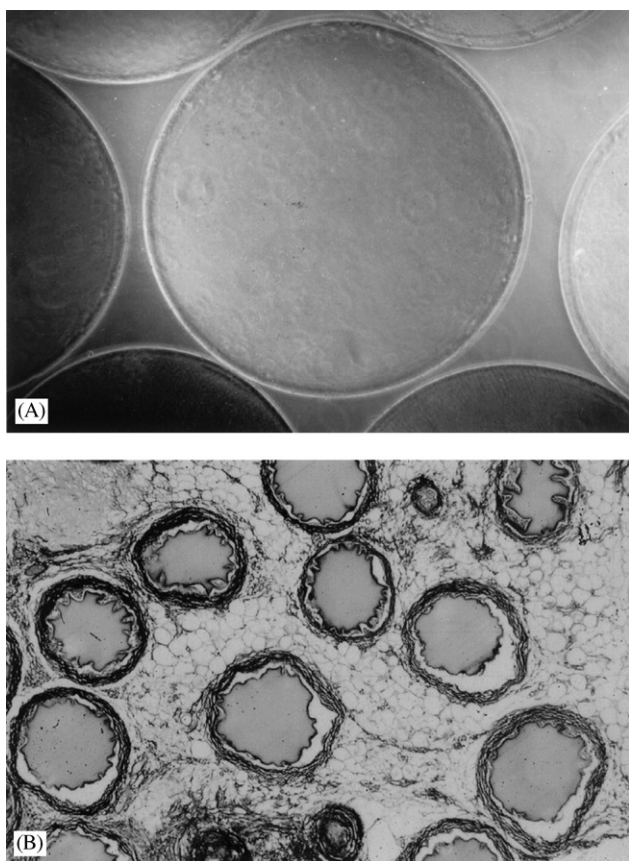


Fig. 1. Explanted alginate-PLL capsules 1 year after implantation in the peritoneal cavity of rats. (A) Capsules were produced according to the procedure in which physical, chemical, and immunological factors influencing the biological response against capsules were successfully controlled (original magnification $\times 70$). Note that the capsule is free of any obvious cell-adhesion. (B) Histological section of capsules were produced from unpurified, crude alginate. All capsules are adherent to omental tissue (GMA-embedded histological section, Syrius Red staining, original magnification $\times 50$).

Table 1

Recovery rates of capsules after lavage and percentage of empty alginate-PLL capsules with overgrowth at 1, 3, 6, 12 and 24 months after implantation in the peritoneal cavity of AO-rats. Values represent mean \pm SEM

Time after implantation (months)	<i>n</i>	% Recovery	% Overgrowth
1	5	94.6 \pm 3.0	6.0 \pm 1.5
3	5	87.4 \pm 7.5	7.9 \pm 2.0
6	5	91.6 \pm 3.5	3.4 \pm 2.0
12	5	94.6 \pm 4.5	5.2 \pm 2.1
24 ^a	2	85.90	9.4

^aThree out of five rats died because of old age before the 24 months time point. The results of the remaining two animals are given individually.

some macrophages and fibroblasts. This small percentage of overgrowth was caused by imperfections on the capsule surface since we recurrently observed that some capsules contained defects which were surrounded by inflammatory tissue or fibroblasts.

Empty capsules prepared of unpurified alginate were all overgrown by fibrotic tissue within 1 month postimplant. The capsules were found to be adherent to omentum tissue (Fig. 1B), liver, and guts.

3.2. The surface chemical composition of biocompatible capsules

Capsules are usually considered to be composed of a Ca-bead covered by a two-layer membrane of alginate-PLL for immunoprotection, and of Ca-alginate to prevent biological reactions associated with non-bound PLL [5,23]. This is an assumption which is based on the theoretical three-step procedure applied for producing capsules and not on data from chemical evaluations of true capsules. To get a full insight on the chemical structure of biocompatible capsules, we evaluated the structure of the surface by applying X-ray photoelectron spectroscopy (XPS). To get a full insight in the structure of the elemental capsule-structure we analyzed both the Ca-beads and the membrane.

As shown in Table 2, the surface of Ca-beads was composed of the elements carbon and oxygen, which originate from the alginate. Also, we observed the elements Ca and Cl which derive from the CaCl_2 applied for crosslinking of the alginate molecules.

The membrane was as expected different in composition. We found nitrogen which derives from the PLL bound by alginate. Surprisingly, we found no calcium in the surface of the membrane. Instead, we found some sodium which is probably bound by carboxyl groups present in alginate.

Table 2

Elemental surface composition of Ca-beads ($n = 4$) and alginate-PLL capsules ($n = 4$) before implantation and an analysis of pure PLL (representative sample is shown)

	C (%)	N (%)	O (%)	Ca (%)	Na (%)	Cl (%)	Others (%) ^a
Ca-beads	55.4±0.8	0	33.3±1.1	5.4±0.4	0	3.7±0.6	2.2
Alginate-PLL capsules	58.6±1.9	6.2±0.5	31.4±0.8	0	2.7±0.4	0	1.1
PLL	67.2	10.7	17.9	0	0	4.2	0

^asuch as S and Si.

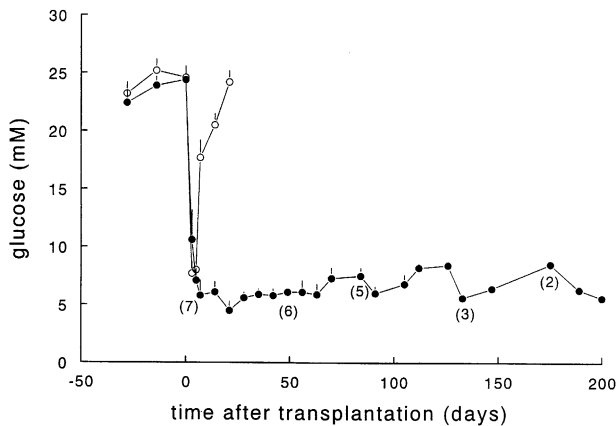


Fig. 2. Non-fasting blood glucose concentrations in streptozotocin diabetic AO-rats after implantation of microencapsulated allografts (closed symbols, $n = 7$) or non-encapsulated, free islet allografts (open symbols, $n = 6$). All the recipients of non-encapsulated, free allogenic islets became normoglycemic for not more than 2 days. The number of cured, normoglycemic recipients of microencapsulated allografts at the different time points is shown in parentheses. Values represent mean±SEM.

3.3. Adequacy of immunoprotection

Transplantation of free, non-encapsulated islets allografts in diabetic AO-rats induced normoglycemia for not more than 2 days after which the islets were fully rejected. In contrast, diabetic recipients of encapsulated islet allografts became normoglycemic within 5 days postimplant and remained normoglycemic for 42 till more than 200 days (Fig. 2).

During meal testing, the blood glucose levels of encapsulated islet allografts were somewhat elevated when compared to normal controls (Fig. 3). However, blood glucose levels never exceeded 8.0 mmol/l which illustrates the adequacy of blood glucose control by the encapsulated grafts.

Isografts showed similar survival times as allografts (Table 3) which suggests that eventual failure of encapsulated islet allografts was not caused by rejection. This argumentation was further substantiated by our observation that we found no inflammatory cells indicating an ongoing process of rejection in the transplantation site. Capsules containing islet isografts or allografts were freely floating in the peritoneal cavity and only a small portion of imperfect capsules of less

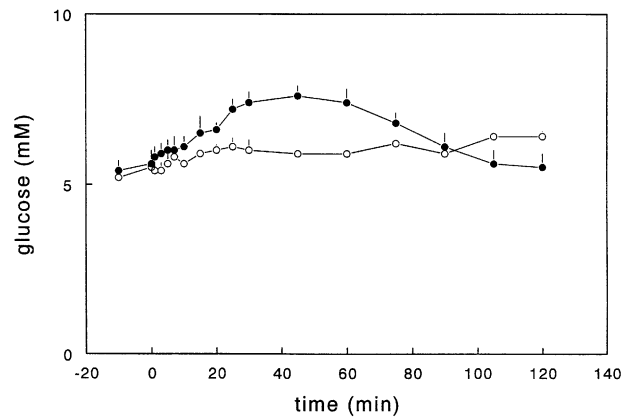


Fig. 3. Blood glucose levels after spontaneous ingestion of a meal ($n = 6$) in AO-recipients of microencapsulated allografts (closed symbols) and in normal controls (open symbols). Values represent mean±SEM.

Table 3

Mean graft survival, recovery rates and percentage of capsules with overgrowth after implantation of microencapsulated rat islet iso- and allografts. Values represent mean±SEM. Note that the survival times of isograft and allografts are similar

Type of encapsulated graft	<i>n</i>	Graft survival (days)	% Recovery	% Overgrowth
Isograft	5	98.4±11.5	83.2±9.1	8.9±2.4
Allograft	7	102.6±9.7	88.5±4.7	7.4±3.5

than 10% was overgrown by macrophages and fibrotic tissue. Some capsules, however, contained some precipitates which were retained by the capsule membrane (Fig. 4A) and in our histological studies we found many islets containing central necrosis (i.e. dead cells in the center of the islets) (Fig. 4B). This indicates that failure is caused by islet-cell death within the capsule [12], which results from either islet toxicity because of deposition of metabolic waste, or from nutrient deficiency.

4. Discussion

Up to now the success rates of transplantation of microencapsulated islets were low [5,24–27]. This is

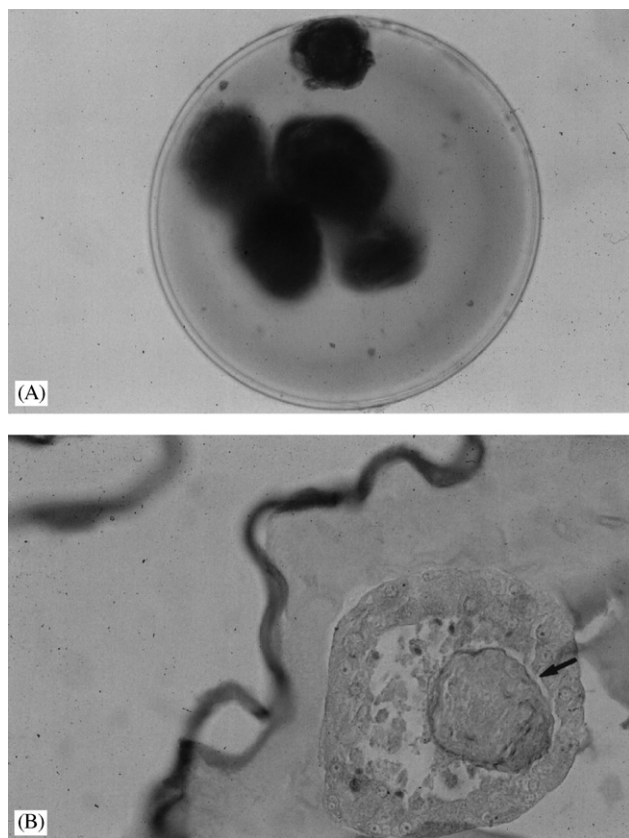


Fig. 4. Causes of failure of microencapsulated islet grafts. (A) An islet-containing capsule retrieved 180 days postimplant. Note the hazy layer underneath the membrane which is a deposition of metabolic waste material that cannot pass freely through the capsule membrane (original magnification $\times 70$). (B) An islet-containing capsule retrieved 100 days postimplant. The arrow indicates the necrotic center of the islet (GMA-embedded histological section, original magnification $\times 150$).

mainly caused by insufficient biocompatibility of the applied capsule materials [5,28–30] which induced a non-specific foreign body reaction against not only a small portion but against the whole capsule graft with fibrosis of the capsules and necrosis of the islets as a consequence. In the present study, we show for the first time that a procedure in which pertinent factors inducing tissue responses against the capsules are deleted, can create a capsule graft which does not elicit any significant biological response up to 2 years of implantation in the rat, i.e. the life-span of a rat.

Unfortunately, the application of biocompatible capsules in islet transplantation did not bring about permanent survival of the graft since graft survival was limited to periods of 42 till 200 days. There are several lines of evidence supporting that this limited duration of graft survival was not caused by insufficient biocompatibility or inadequate immunoprotection. First, only a portion of less than 10% of the capsules was affected by fibrotic overgrowth, the loss of which has been shown not to interfere with graft function [8]. Second, we found

that isografts survived for similar if not identical time periods as allografts which illustrates the adequacy of immunoprotection. Instead, we found clear indications that graft failure was associated with insufficient supply of nutrients and deposition of metabolic waste in the capsules as evidenced by our observation of central necrosis in the islets and depositions of metabolic waste in the capsules.

Our study differs from previous work in several ways. First, we have documented in the present transplantation series in contrast to our previous series all available factors influencing biocompatibility of the capsules [2]. As a consequence the survival times of encapsulated islet grafts was improved with a mean of several months when compared to previous studies [8]. This documentation of factors influencing biocompatibility is pertinent for comparing studies between different groups in the field since many have introduced their own technical modifications without further documentation of the consequences of these changes for the properties of the capsules. We have often observed that these kind of empiric changes have a drawback on essential properties of the capsules. For example, in alginate-PLL capsules the application of high-G alginates to improve the mechanical characteristics of capsules holds the pitfall of a drawback in biocompatibility since high-G alginate interact inadequately with the inflammatory poly-L-lysine [14]. Another difference between previous and the present study is that we have introduced a new technique to document the chemical composition of the capsules. This will contribute to a better understanding of the chemical factors determining success and failure of the capsule graft.

The present study gives new insight in the structure of the membrane of alginate-PLL capsules. Up to now (Fig. 5A), the capsule was assumed to be composed of a core of Ca-alginate which is enveloped by a membrane composed of two layers, i.e. an inner layer of alginate-PLL and an outer layer of Ca-alginate [2,23,31]. The data, which have lead to this model, were almost exclusively obtained by studying the chemical interactions of PLL with solved, non-Ca bound and often individual components of alginate (i.e. guluronic (G)-acid and mannuronic (M)-acid monomers) and not by studying the chemical structure of the capsules as such. In the present study on true capsules, we never observed Ca^{2+} in the membrane of the capsules, which has the following implications for the assumed structure of the capsules. First, the alginate-PLL layer is not composed of the combination of alginate-PLL and alginate- Ca^{2+} but of the alginate-PLL only. The absence of Ca^{2+} also implies that the outer Ca-alginate layer does not exist and, consequently, that the membrane is not composed of two but of one layer only. Finally, we found sodium in the membrane, which is bound by carboxyl groups on consecutive blocks of G- and M-molecules, which

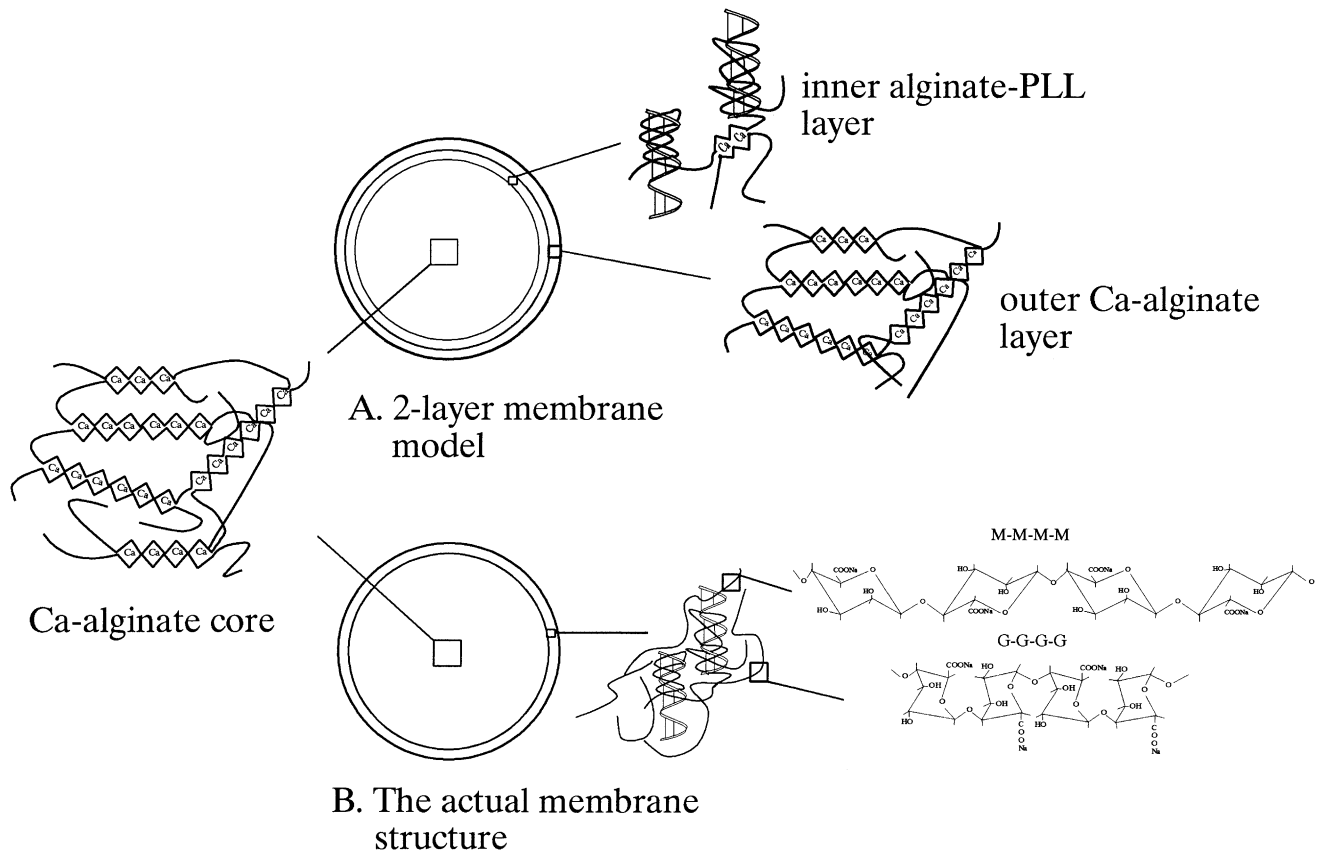


Fig. 5. Schematic presentation of the composition of the alginate-PLL membranes. (A) The two-layer model as originally proposed by Thu et al. [23,32]. The inner layer of alginate-PLL is considered to be formed by two types of interactions. The first type is that of interactions between PLL and mixed sequences of guluronic acid (G) and mannuronic acid (M) in the alginate molecules. This interaction reduces the permeability of the capsules by inducing the formation of complexes at the capsule surface of α -helical PLL surrounded by superhelically orientated polysaccharide molecules [33,34]. The other interaction is that between Ca^{2+} and alginate which is already formed in the gelation step in 100 mM CaCl_2 [35]. In this step, Ca^{2+} has connected alginate molecules by binding consecutive G-blocks located on each of both molecules in a highly cooperative manner [35]. The outer layer, the layer of Ca-alginate, is also composed of connections between Ca^{2+} and alginate. (B) The actual membrane structure as follows from our XPS analysis. We found no Ca^{2+} in the membrane but only alginate, PLL and Na^+ (for further explanation see Section 4).

remain unbound between the complexes of PLL and mixed sequences of G and M. Fig. 5B shows the actual structure of alginate-PLL capsules.

The present study shows that capsules prepared of alginates are biocompatible and stable *in vivo* up to 2 years after implantation and, thereby, deletes the major obstacle in clinical applicability of microencapsulated islets. The capsules do not interfere with islet-function and effectively protects the islets against rejection as illustrated by the prolonged survival of the graft. In our attempts to further improve the survival of the grafts we focus on two approaches. First, we are determining the optimal permeability of the capsules in order to create a porosity, which allow for fast exchange of metabolic waste materials and essential nutrients while components of the immune system, which can interfere with islet function, are not able to pass the membrane. The other approach is to find a transplantation site which is better vascularized than the unmodified peritoneal cavity warranting adequate exchange of nutrients,

metabolic waste, glucose and insulin. Such a site should also hold the advantage of retrievability of the graft in case encapsulated grafts will require repeated replacements. These studies are pertinent for the design of strategies applicable for clinical islet transplantation.

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