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# FACTORS INFLUENCING THE ADEQUACY OF MICROENCAPSULATION OF RAT PANCREATIC ISLETS<sup>1</sup>

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## Abstract

**The observation that only a portion of all alginate-polylysine microcapsules are overgrown after implantation suggests that physical imperfections of individual capsules, rather than the chemical composition of the material applied, are responsible for inducing insufficient biocompatibility and thereby fibrotic overgrowth of those capsules. We recently developed a lectin binding assay that allows for quantifying the portion of inadequately encapsulated islets, and demonstrated that inadequately encapsulated islets induce a fibrotic response associated with graft failure.**

**The present study investigates factors influencing the adequacy of encapsulation of pancreatic islets. We applied our lectin binding assay and found that the number of inadequate, and particularly incomplete, capsules is influenced by the following factors. (1) A capsule diameter of 800 µm is associated with a lower percentage of inadequate capsules than smaller (500µm and 600 µm) or larger (1800 µm) capsules. (2) A high rather than low guluronic acid content of the alginate is associated with a lower percentage of inadequate capsules. This can be explained, at least in part, by smaller ranges of swelling and subsequent shrinkage during the encapsulation procedure. (3) An increase in viscosity caused by applying a higher alginate concentration compensates for a low guluronic acid content. This effect of increased viscosity cannot be explained by a reduced range of swelling and shrinkage during the encapsulation procedure.**

**We conclude that alginates with a high guluronic acid content and a viscosity near the filtration**

**limit are preferable in order to minimize the number of inadequate capsules.**

Graft failure of encapsulated islets is usually interpreted as a consequence of a nonspecific foreign body reaction against the capsules that results in fibrotic overgrowth of the capsules, with ischemia and subsequent necrosis of the islets (1, 2). Some authors have reported complete overgrowth of microcapsules recovered from recipient animals within a few weeks after implantation (3, 4). Others, however, have observed that only a portion of the capsules was overgrown after several months of implantation, while the other capsules remained free of fibrotic overgrowth (2, 5). Should the chemical composition and/or purity of the alginate applied be responsible for insufficient biocompatibility, we would expect all, rather than a portion, of the capsules to be overgrown. The latter observation, therefore, suggests that physical imperfections, such as incomplete and thereby inadequate capsule formation, may be involved in inducing a lack of biocompatibility, since this may conceivably affect some, but not all, capsules.

The importance of inadequate capsule formation has been demonstrated in a previous study (6). We showed that reduction of the diameter of the capsules in order to reduce the volume of the encapsulated islet graft was associated with an increase in the number of capsules containing an inadequately encapsulated islet. The immune response toward the inadequately encapsulated islet interfered with and abrogated graft function.

The present study was undertaken to identify some factors that influence the adequacy of encapsulation of pancreatic islets. Identification of such factors appears mandatory for modifying the alginate-poly-L-lysine (PLL\*) microencapsulation procedure in order to reduce the number of inadequately encapsulated islets.

**MATERIALS AND METHODS**

*Animals and islet isolation.* Islets were isolated from the pancreas of male Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 300-350 g. The donor pancreas was distended by ductal infusion of 10 ml of Krebs-Ringer-Hepes (KRH) buffer containing 10% bovine serum albumin. The pancreas was excised and cut into small pieces. Subsequently, the chopped pancreas was digested using a two-stage collagenase incubation (7) of 20 min at 37 °C with, successively, 1.0 and 0.7 mg/ml collagenase (type XI, Sigma Chemical Co., St. Louis, MO). Islets were separated from the exocrine tissue by centrifugation in a discontinuous dextran density gradient (8). Islets with a diameter between 50 and 250 µm were handpicked, using a dissection microscope with a fluorescent illuminator (Bausch and Lomb BVB-125, and 31-33-66).

*Islet microencapsulation.* Alginates are composed of two acids, guluronic acid and mannuronic acid. The alginates used in this study (Keltone LV and Manugel, both from Kelco International, London, UK) differ in their guluronic acid to mannuronic acid ratio (G:M ratio). Manugel contains more guluronic acid than Keltone LV and therefore has a higher G:M ratio. A suspension of 1000 islets/ml alginate was converted into droplets using our droplet generator, as described previously (9). Polylysine-alginate encapsulation was performed as described elsewhere (10). The alginate droplets were transformed to alginate beads by gelling in a 100 mM  $\text{CaCl}_2$  (10 mM HEPES and 2 mM KCl) solution for at least 5 min. Subsequently, the calcium-alginate beads were suspended for 3 min in 50 ml of  $\text{Ca}^{2+}$ -free KRH buffer containing 135 mM NaCl. A PLL membrane was formed by suspending the alginate beads in 10 ml of 0.1% PLL solution for 10 min (PLL-HCl, m.w. 22,000; Sigma). Nonbound PLL was removed by three successive washings with 10 ml of  $\text{Ca}^{2+}$ -free KRH buffer for 3 min. The outer alginate layer was subsequently applied by 5 min of incubation in 10 ml of 10 $\times$  diluted alginate solution. To liquefy their inner core, the microcapsules were suspended in 50 ml of 1 mM EGTA in  $\text{Ca}^{2+}$ -free KRH buffer for 10 min, and finally washed three times with KRH containing 2.5 mM  $\text{CaCl}_2$ . Microencapsulated islets and control islets were cultured in RPMI (Gibco, no. 22511) for 1 hr at 37 °C in humidified air containing 5%  $\text{CO}_2$ .

*Assessment of diameters of beads and capsules.* The diameter of the beads or capsules was measured using a dissection microscope with a fluorescent illuminator (Bausch and Lomb BVB-125, and 31-33-66), equipped with an ocular micrometer with an accuracy of 25  $\mu\text{m}$ .

*Identification of inadequate capsules.* The adequacy of encapsulation was assessed by identifying the capsules that provide inadequate immunoisolation of the islet, as described previously (6). The adequacy of encapsulation was quantified by expressing the number of inadequate capsules as the percentage of the total number of islet-containing capsules.

Positive fluorescence was assessed by using a fluorescence microscope (Olympus TMT-2) equipped with inverted phase contrast. Microscopic examination was always performed, first to confirm that the fluorescence was caused by labeling of islet cells and not by nonspecific labeling, and second to decide upon the specific nature of inadequate encapsulation. Two different manifestations of inadequate encapsulation were identified. One manifestation is that the islet partly protrudes from the microcapsule (Fig. 1A). We have defined this manifestation as “incomplete encapsulation.” The other manifestation is that the capsule, although completely surrounding the islet, shows a number of localized defects. We have termed this “defective encapsulation” (Fig. 1B). Thus, incomplete and defective encapsulation are different manifestations of inadequate encapsulation.

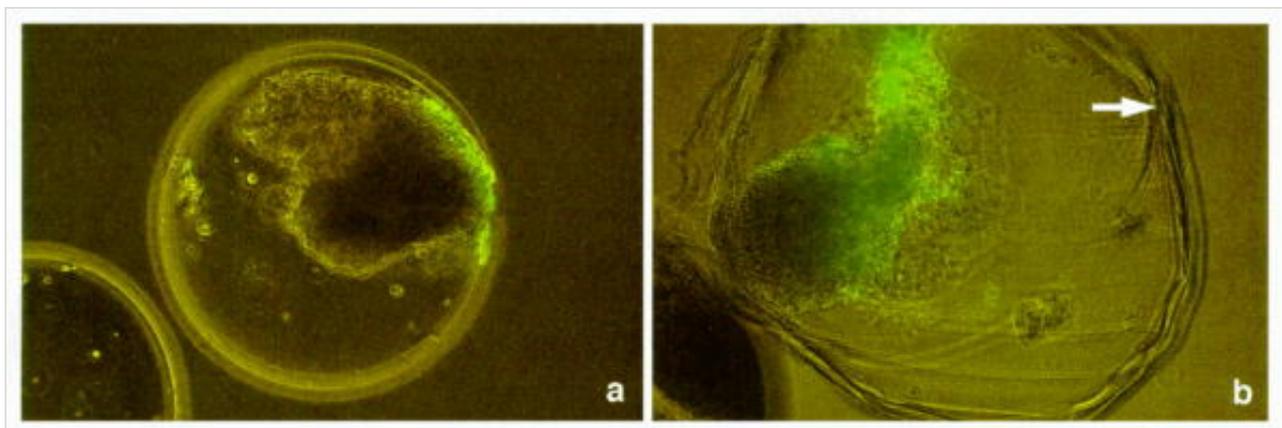


Figure 1. Manifestations of inadequate encapsulation. Capsules were prepared from a 3% Keltone LV solution. Pancreatic islets encapsulated in 500- $\mu\text{m}$  alginate-PLL microcapsules were suspended for 45 min in KRH containing FITC-labeled RCA-I. (A) Incomplete encapsulation: Islet cells protruding from the capsule are specifically labeled by RCA-I (original magnification  $\times 100$ ). (B) Defective encapsulation: Islet cells surrounded by a defective enveloping capsule are labeled by RCA-I. The arrow indicates the localized defect (original magnification  $\times 150$ ).

*Identification of inadequate beads.* In the absence of an enveloping microcapsule, the calcium-alginate beads were found to be permeable for ricinus communis agglutinin I (RCA-I), because the enclosed islets were readily labeled. To enlarge the molecular weight of RCA-I, small RCA-I aggregates were formed by coupling biotinylated RCA-I (Vector Laboratories, Burlingame, UK) to fluorescein isothiocyanate (FITC)-labeled streptavidin (Vector), which is a 68-kDa protein with four biotin binding sites (11). To prevent the formation of large aggregates, we ultrasonicated the biotinylated RCA-I and FITC-labeled streptavidin (RCA-I-S).

The RCA-I-S aggregates were formed by mixing biotinylated RCA-I and FITC-labeled streptavidin in a molar ratio of 2:1 in an ultrasonic water bath (Branson, 3200). During ultrasonication of a 0.42-nM biotinylated RCA-I solution in either 100 mM  $\text{CaCl}_2$  (in 10 mM HEPES buffer) or KRH, we added a 0.21-nM FITC-labeled streptavidin solution (also in either 100 mM  $\text{CaCl}_2$  solution or KRH). After 15 min of ultrasonication, the RCA-I-S had aggregated into small complexes (smaller than 1  $\mu\text{m}$ ). Aliquots of 300-500 islet-containing calcium-alginate beads or islet-containing capsules were incubated with 0.5 ml of RCA-I-S in either 100 mM  $\text{CaCl}_2$  or KRH, respectively, for 45 min at 4 °C, with gentle shaking every 5 min.

Results with RCA-I-S were identical to those obtained with RCA-I when the degrees of inadequate encapsulation with 500- $\mu\text{m}$  and 800- $\mu\text{m}$  microcapsules prepared from either 3% Manugel or 3% Keltone LV were assessed.

Just like capsules, beads with positive fluorescence were always microscopically examined. Again, we identified two different manifestations of inadequate bead formation. In one manifestation, the islet partly protrudes from the bead. We termed this “incomplete enclosure.” The other manifestation is that the bead is deformed or contains strains, which we have defined as “defective enclosure.”

*Determination of viscosity.* Viscosity measurements were performed by measuring the apparent viscosity ( $[\eta]_{\text{apparent}}$ ), as described by Rosen et al. (12).

*Statistics.* Statistical evaluation was performed using the paired and unpaired two-tailed Student's *t* test when appropriate. Values are expressed as mean  $\pm$  SEM. Only  $P < 0.05$  was considered to be significantly different.

## RESULTS

To investigate the influence of the diameter on the degree of inadequate encapsulation, capsules with 500, 600, 800, and 1800  $\mu\text{m}$  diameters were prepared from a 3% Keltone LV solution and tested with the lectin binding assay. The percentages of both incomplete and defective encapsulation were determined for each diameter, and the results are shown graphically in Figure 2.

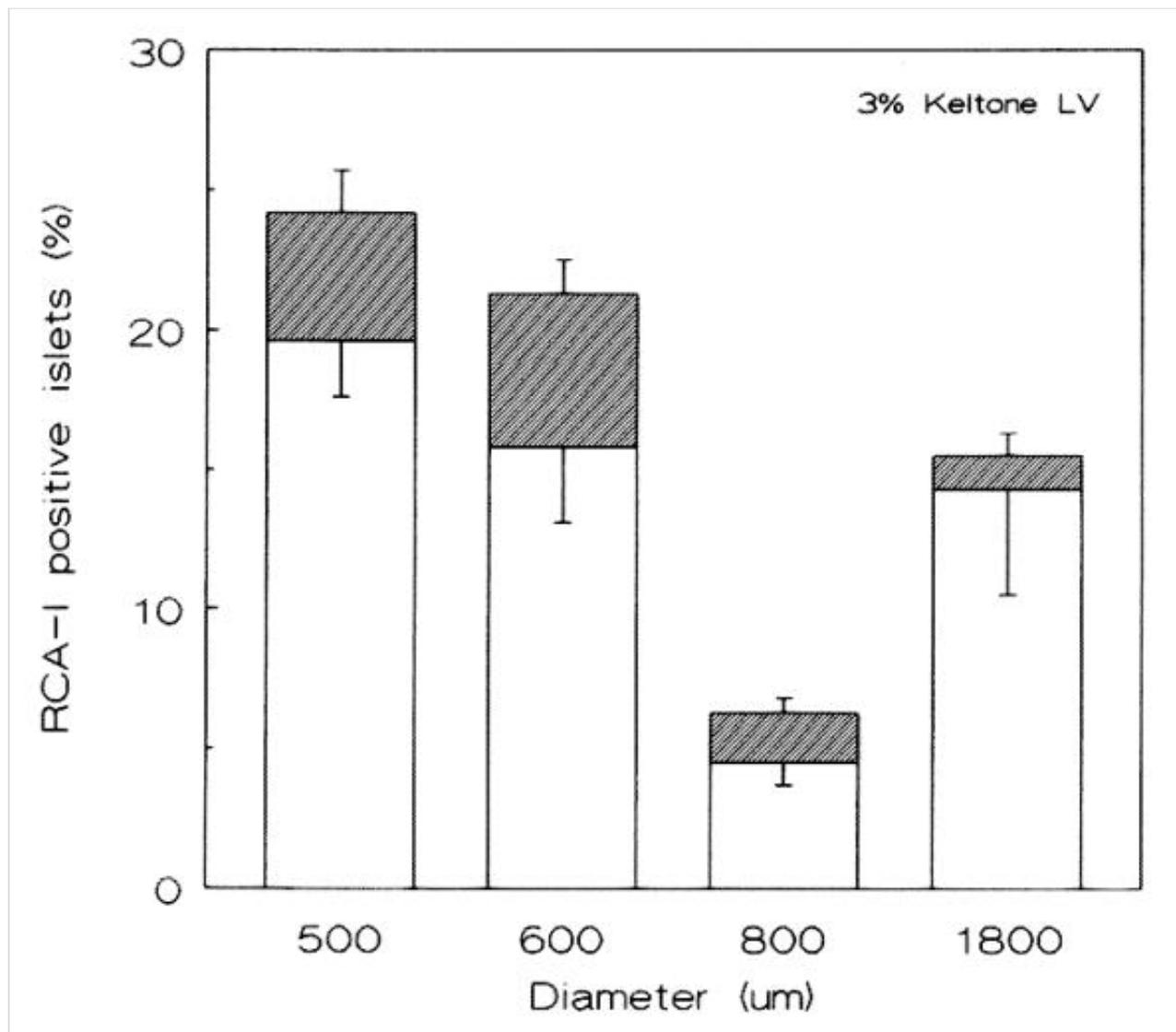


Figure 2. Influence of capsule diameter on adequacy of islet encapsulation. Capsules were prepared from a 3% Keltone LV solution. Pancreatic islets encapsulated in an alginate-PLL microcapsule were suspended for 45 min in KRH containing FITC-labeled RCA-I. Incomplete encapsulated islets ([white square]) and islets encapsulated in defective capsules ([square with upper right to lower left fill]) were labeled by RCA-I. Values represent mean  $\pm$  SEM of six or seven experiments.

The lowest percentage of incomplete encapsulation was obtained with the intermediate diameter of 800  $\mu\text{m}$  ( $4.5 \pm 0.8\%$ ). Higher percentages were observed with 500  $\mu\text{m}$  ( $19.6 \pm 2.0\%$ ,  $P < 0.002$ ), 600  $\mu\text{m}$  ( $15.8 \pm 2.7\%$ ,  $P < 0.004$ ), and 1800  $\mu\text{m}$  capsules ( $14.3 \pm 3.8\%$ ,  $P < 0.05$ ).

The percentage of islet-containing defective capsules, too, was significantly lower with 800  $\mu\text{m}$  ( $1.8 \pm 0.5\%$ ) than with 500  $\mu\text{m}$  ( $4.6 \pm 1.5\%$ ,  $P < 0.014$ ) or 600  $\mu\text{m}$  ( $5.5 \pm 1.2\%$ ,  $P < 0.018$ ) diameter capsules. With 1800- $\mu\text{m}$ -diameter capsules, the percentage of islet-containing defective microcapsules also tended to be lower ( $1.2 \pm 0.8\%$ ), but this difference was statistically significant only in comparison to 500- $\mu\text{m}$ -diameter capsules ( $P < 0.049$ ).

To investigate whether the composition of the alginate applied influences the degree of inadequate encapsulation, we repeated the above experiments not only with 3% Keltone LV, which holds a low G:M ratio, but also with 3% Manugel, which holds a high G:M ratio. In view of the results obtained above, we have restricted these experiments to 500- $\mu$ m- and 800- $\mu$ m-diameter capsules.

As shown in Figure 3, the percentage of incomplete encapsulation was lower with 3% Manugel ( $9.2 \pm 1.8\%$ ) than with 3% Keltone LV ( $19.6 \pm 2.0\%$ ,  $P < 0.02$ ) for 500- $\mu$ m-diameter capsules. With this diameter, the percentage of defective capsules was also lower with 3% Manugel ( $3.2 \pm 0.9\%$ ) than with 3% Keltone LV ( $4.6 \pm 1.5\%$ ,  $P < 0.005$ ). Similarly, when 800- $\mu$ m-diameter capsules were tested, the percentages of incomplete and defective capsules tended to be lower with 3% Manugel than with 3% Keltone LV, but these differences were not statistically significant.

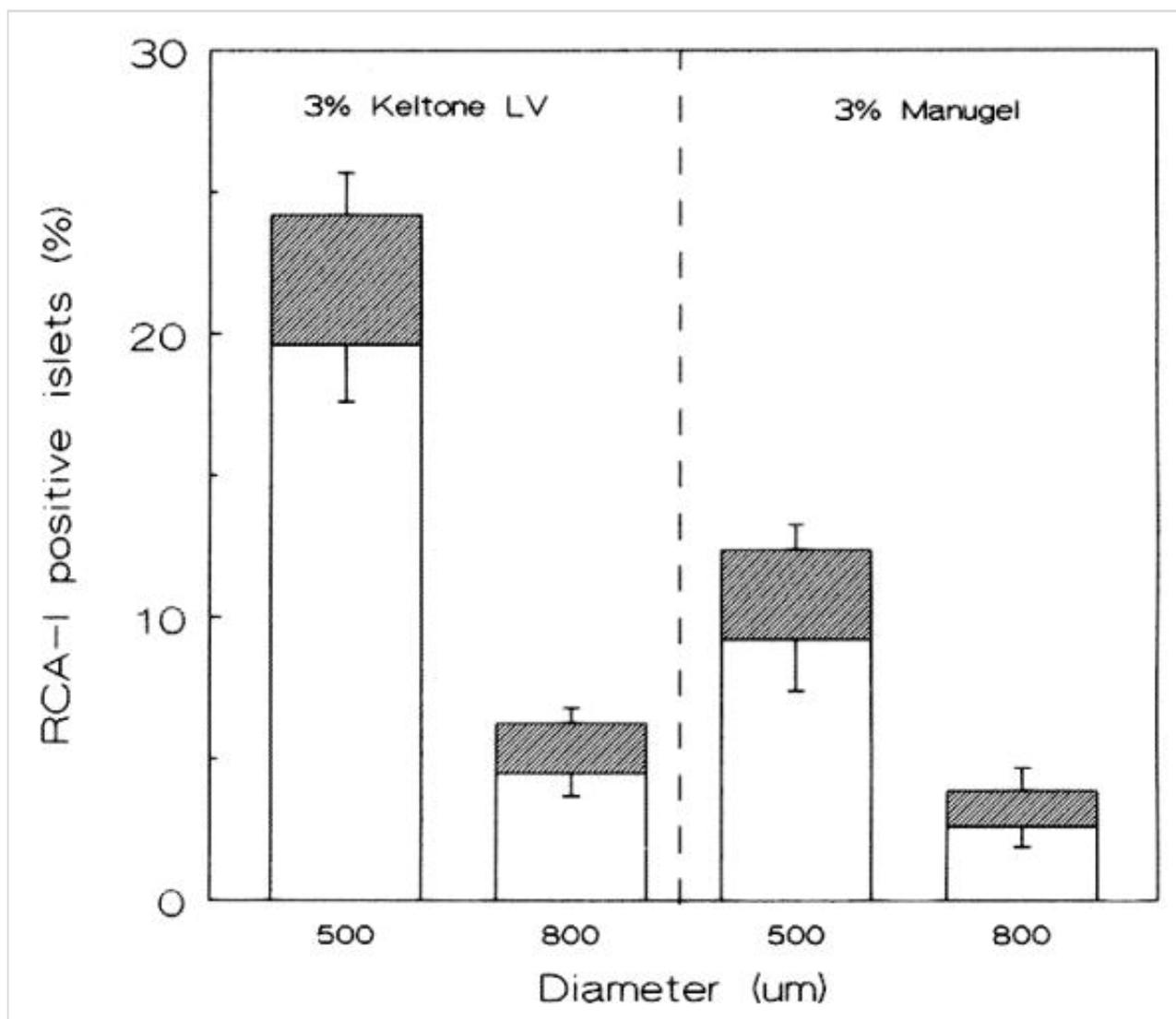


Figure 3. Influence of composition of alginate on adequacy of islet encapsulation. Capsules were prepared from a 3% Keltone LV solution or from a 3% Manugel solution. Pancreatic islets encapsulated in an alginate-PLL microcapsule were suspended for 45 min in KRH containing FITC-labeled RCA-I. Incomplete encapsulated islets ([white square]) and islets encapsulated in defective capsules ([square with upper right to lower left fill]) were labeled by RCA-I. Values represent mean  $\pm$  SEM of six or seven experiments.

As shown in a previous study (13), swelling and shrinkage occur during the process of bead and capsule formation, with a net result of a capsule diameter somewhat larger than the initial bead diameter. Thus, we have determined the bead diameters required for obtaining capsules with either a 500- $\mu\text{m}$  or an 800- $\mu\text{m}$  diameter, both with 3% Keltone LV and 3% Manugel.

As shown in Table 1, we found that substantially smaller beads are required with 3% Keltone LV than with 3% Manugel in order to obtain the same capsule sizes. The increase of diameter from bead to capsule with 3% Keltone LV is almost twice as high as with Manugel.

Alginate	Diameter of bead ( $\mu\text{m}$ )	Diameter of capsule ( $\mu\text{m}$ )	Diameter increase (%)
3% Keltone LV	375	500	33
	550	800	45
3% Manugel	425	500	17
	625	800	28

Table 1. Diameter increase during the formation of capsules from calcium-alginate beads

Next, we investigated whether these differences in swelling and shrinkage properties are actually associated with different degrees of inadequate encapsulation between 3% Keltone LV and 3% Manugel. To this end, islet-containing beads and islet-containing capsules were tested for inadequate bead formation (incomplete or defective enclosure) and inadequate (incomplete or defective) encapsulation, respectively, while either 3% Keltone LV or 3% Manugel was applied for the formation of capsules with a diameter of either 500  $\mu\text{m}$  (Fig. 4A) or 800  $\mu\text{m}$  (Fig. 4B).

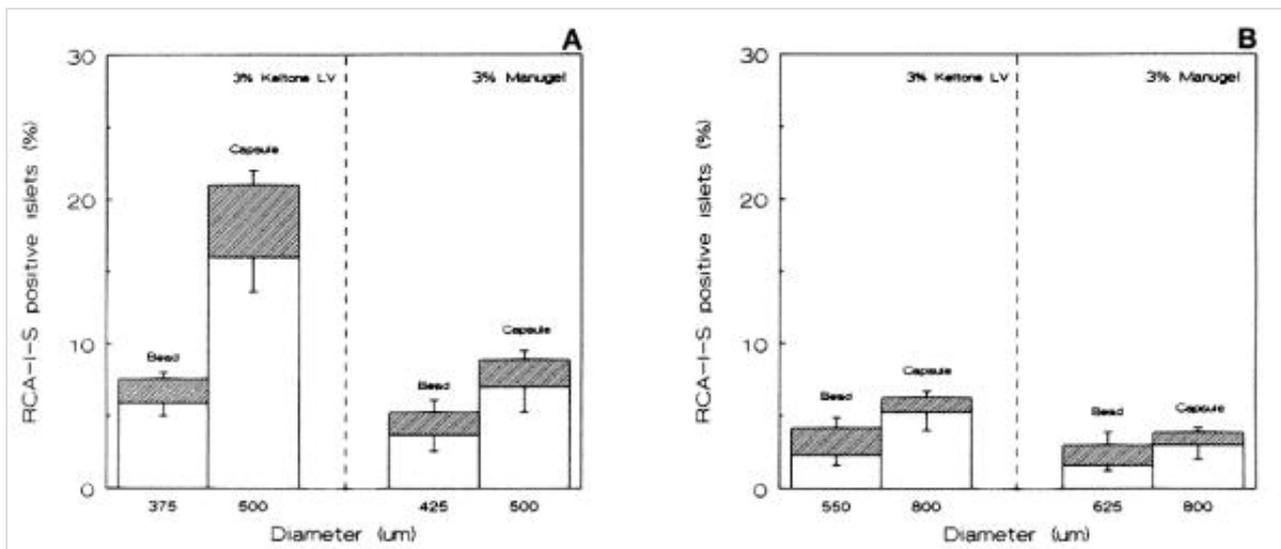


Figure 4. Influence of the encapsulation procedure on adequacy of islet encapsulation: (A) formation of 500- $\mu\text{m}$  capsules and (B) formation of 800- $\mu\text{m}$  capsules. Pancreatic islets were suspended in a 3% Keltone LV solution or in a 3% Manugel solution. After droplet formation and subsequent calcium bead formation in 100 mM  $\text{CaCl}_2$ , 50% of the calcium beads were alginate-PLL encapsulated. The alginate-PLL-alginate microcapsules were suspended for 45 min in KRH containing biotinylated RCA-I coupled to streptavidin (RCA-I-S) (right bars). The resulting 50% of the Ca-alginate beads were suspended for 45 min in 100 mM  $\text{CaCl}_2$  containing biotinylated RCA-I-S. Islets incompletely enclosed or encapsulated ([white square]) and islets enclosed or encapsulated in defective beads or capsules ([square with upper right to lower left fill]) were labeled by RCA-I-S. Values represent mean  $\pm$  SEM of seven experiments.

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During the formation of 500- $\mu\text{m}$ -diameter capsules with 3% Keltone LV, the percentage of incomplete enclosure ( $5.9\pm 0.9\%$ ) increased significantly to  $16\pm 2.4\%$  of incomplete encapsulation ( $P<0.002$ ), and the percentage of defective enclosure ( $1.7\pm 0.4\%$ ) increased significantly to  $5.0\pm 1.0\%$  of defective capsules ( $P<0.008$ ). Apparently, this increase is influenced by the swelling properties of the alginate applied, since it was much lower and statistically not significant with 3% Manugel (Fig. 4A). The increase is also influenced by the diameters of the beads and capsules formed, since it was not very outspoken when capsules with 800- $\mu\text{m}$  instead of 500- $\mu\text{m}$  diameters were produced, either with 3% Keltone LV or 3% Manugel (Fig. 4B).

A significant factor in the comparisons between 3% Keltone LV and 3% Manugel is possibly the difference between their viscosities of 4.3 cps and 8.2 cps, respectively. To decide upon the influence of viscosity on the degree of inadequate encapsulation, we compared islet-containing capsules produced with either 4.3% Keltone LV or 3% Manugel, since these alginate concentrations have identical viscosities.

As seen in Figure 5, the percentages of incomplete and defective capsules with 3% Manugel are similar to those found in previous experiments, both with 500- $\mu\text{m}$ - and 800- $\mu\text{m}$ -diameter capsules (see Fig. 3). With 4.3% Keltone LV, however, these percentages were significantly lower than with 3% Keltone LV (see Fig. 3), with both 500- $\mu\text{m}$ - and 800- $\mu\text{m}$ -diameter capsules. Although not statistically significant, the percentages were even somewhat lower than those obtained with 3% Manugel (Fig. 5).

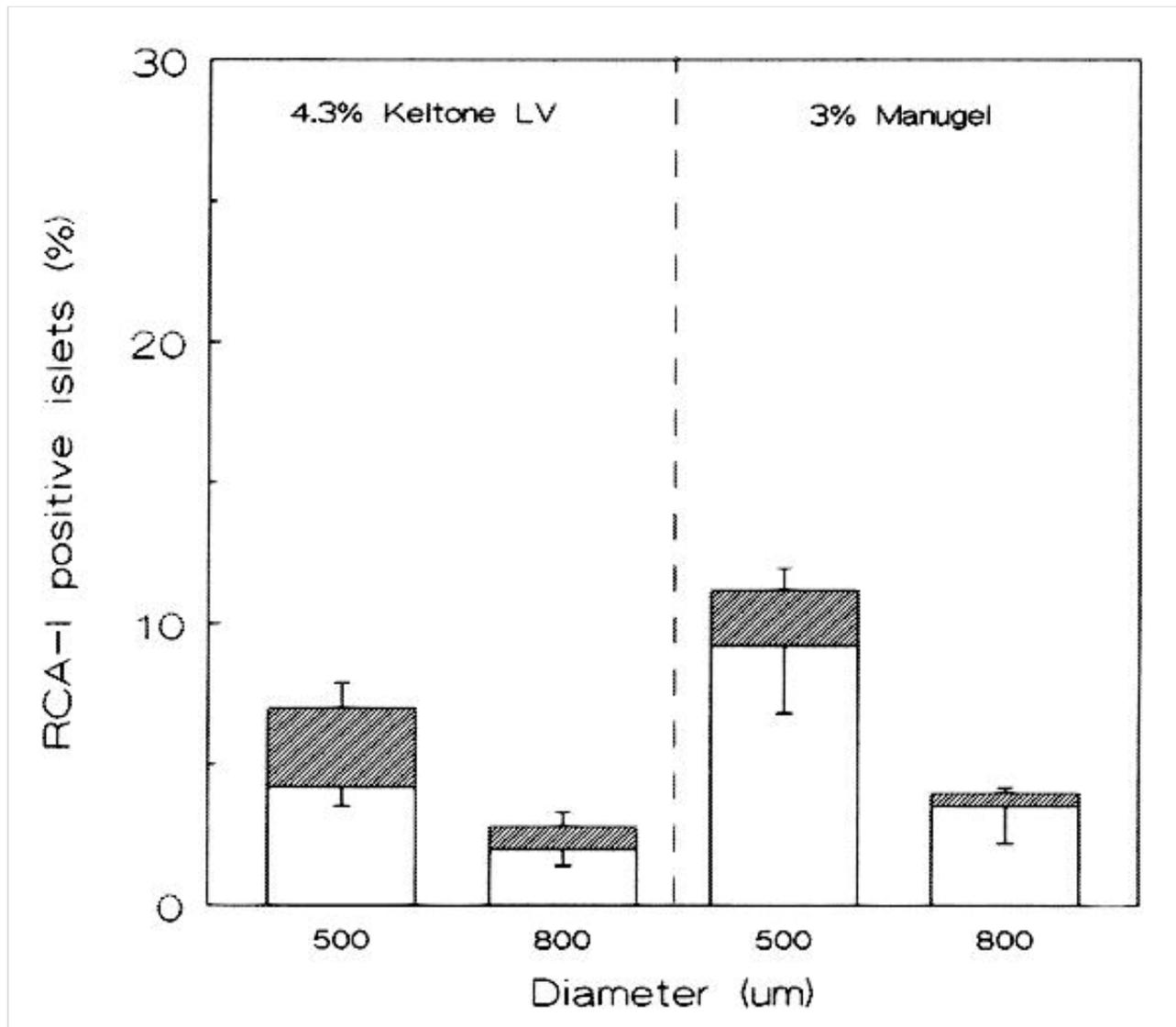


Figure 5. Effect of alginate viscosity on adequacy of islet encapsulation. Pancreatic islets were suspended in a 4.3% Keltone LV solution or in a 3% Manugel solution. Pancreatic islets encapsulated in 500- $\mu\text{m}$  and 800- $\mu\text{m}$  alginate-PLL microcapsules were suspended for 45 min in KRH containing FITC-labeled RCA-I. Incomplete encapsulated islets (white square) and islets encapsulated in defective capsules ([square with upper right to lower left fill]) were labeled by RCA-I. Values represent means  $\pm$  SEM of six experiments.

Finally, we questioned whether these improved results with high-viscosity Keltone LV should be explained by improved swelling properties. This, however, is not the case, because, with 4.3% Keltone LV, bead diameters of 375  $\mu\text{m}$  and 550  $\mu\text{m}$  are required to obtain capsule sizes of 500  $\mu\text{m}$  and 800  $\mu\text{m}$ , respectively. This increase is identical to that observed with 3% Keltone LV (see [Table 1](#)).

## DISCUSSION

This study clearly demonstrates that, with our lectin binding assay, inadequate islet encapsulation, be it incomplete or defective, is a readily demonstrable and quantitatively assessable phenomenon. Quantitatively, incomplete encapsulation is the more important manifestation, and incomplete rather than defective encapsulation appears to be influenced by the factors investigated, i.e., the diameter of the capsule, the composition of the alginate, and the viscosity of the alginate solution.

When capsule diameters of 500, 600, 800, and 1800  $\mu\text{m}$  were compared, the 800  $\mu\text{m}$  diameter was associated with the lowest rate of inadequate encapsulation. That not only smaller (6) but also larger capsule diameters were associated with an increased rate of inadequate encapsulation can probably be explained by the fact that 1800- $\mu\text{m}$  capsules contain three to five islets per capsule, as compared with one to two islets per 800- $\mu\text{m}$  capsule. The chance for protrusion of an islet from the capsule obviously increases with an increased islet load per capsule.

Our finding that the percentage of inadequate encapsulation is influenced by the composition of the alginate applied can be attributed to differences in swelling and shrinkage properties during the subsequent steps of the encapsulation procedure. The principle of this process was examined previously by using Sephadex beads (S-beads) instead of islets (13), and can be briefly summarized as follows (see Fig. 6). After gelatinization, the calcium-alginate beads are washed with Ca-free KRH buffer containing 135 mM NaCl, causing partial displacement of  $\text{Ca}^{2+}$  by  $\text{Na}^+$ , which results in the formation of an anionic interface by the carboxyl groups of alginate. The interaction of these carboxyl groups with the polycationic polymer PLL results in the formation of a polyionic semipermeable hydrogel membrane at the gel surface (14). In the meantime, however, the exchange of  $\text{Ca}^{2+}$  for  $\text{Na}^+$  also induces an increase of the alginate bead diameter. As a consequence, the S-beads tend to be displaced toward the periphery of the alginate bead. During the subsequent step of PLL binding, some shrinkage occurs as a consequence of cross-linking of alginate molecules. During this shrinking, the S-beads maintain their peripheral location and consequently tend to readily protrude from their capsules.

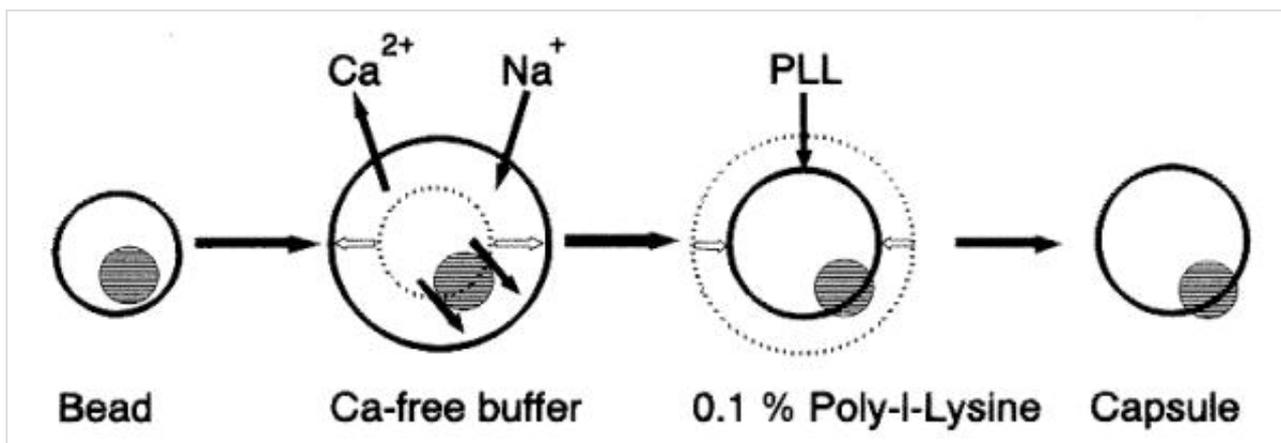


Figure 6. Schematic presentation of the process of swelling and shrinkage during the encapsulation procedure. Swelling and shrinkage contribute to the occurrence of incomplete encapsulation.

It is reasonable to suggest that the chance for protrusion and incomplete encapsulation increases with increasing ranges of swelling and shrinkage during the encapsulation procedure. This explains our finding that the rate of incomplete encapsulation was lower with Manugel than with Keltone LV, since Manugel has a higher G:M ratio than Keltone LV, and a higher G:M ratio is associated with less swelling in the absence of divalent cations like  $\text{Ca}^{2+}$  (15, 16).

The above holds for the comparison of solutions of Manugel and Keltone LV with the same alginate concentration of 3%. These concentrations, however, are associated with different viscosities. Our study seems to indicate that the drawback of Keltone LV associated swelling properties can be reduced, or even removed, by increasing the viscosity, since the adequacy of encapsulation was similar for Manugel and Keltone LV when they were used in concentrations with equal viscosities. We hypothesize that by using higher alginate viscosities, a higher intracapsular resistance for islets moving toward the periphery during the Ca-free washing step is induced. Unfortunately, however, the use of high-viscosity solutions is limited, because the only sterilization process that does not result in degradation of the alginate molecules, i.e., 0.2- $\mu$ m and 0.45- $\mu$ m filtration (17), requires the use of alginate solutions with low viscosities. This suggests that preferably alginates with a high G:M ratio should be used at a viscosity level near the filtration limit for optimal adequacy of encapsulation.

Again (1, 9), our findings emphasize that optimal mechanics of the encapsulation procedure and flawless physical features of the capsules are required for a minimal number of failing capsules and, consequently, a maximum chance for success of the encapsulated islet transplant.

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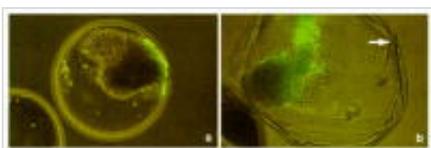


Figure 1

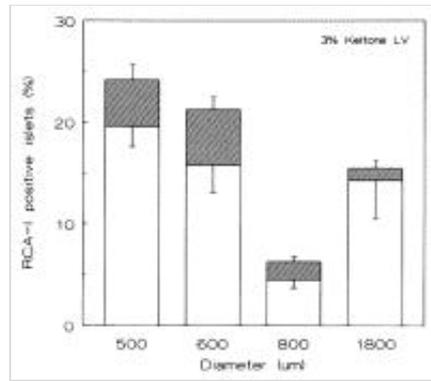


Figure 2

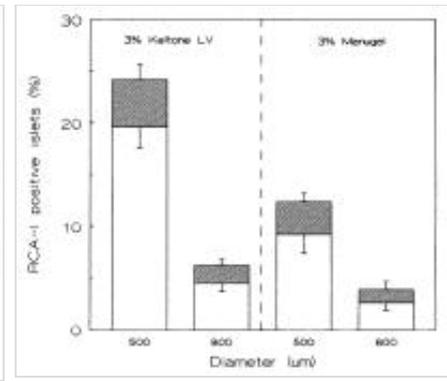


Figure 3

Alginate	Diameter of bead (μm)	Diameter of capsule (μm)	Diameter increase (%)
3% Keltone LV	375	500	33
	500	800	45
3% Menugel	425	500	17
	625	800	28

Table 1

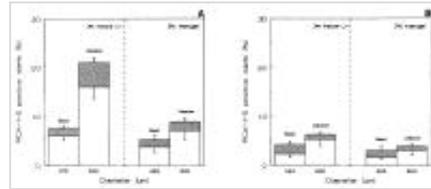


Figure 4

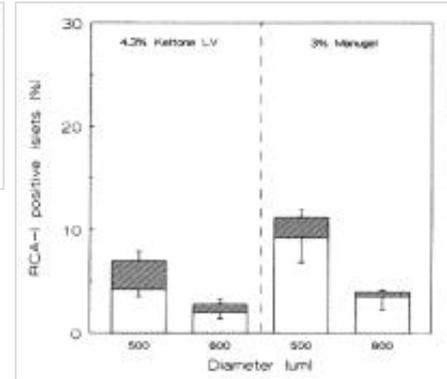


Figure 5

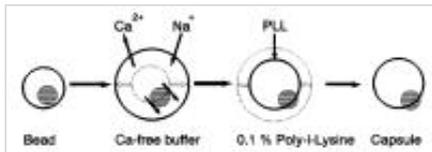


Figure 6

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