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Upscaling the production of microencapsulated pancreatic islets

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Presently used single-needle air-driven droplet generators are incapable of producing sufficient numbers of islet-containing droplets in a sufficiently short time-period to allow for successfully grafting alginate–poly-L-lysine encapsulated islets in large animals or humans. We have designed an air-driven multineedle droplet generator, which increases the production rate by simultaneously producing multiple droplets. Although we have tested a four-needle device, the construction is such that the number of needles, and thereby the production rate, can be readily extended. The production rate can be further extended by increasing the number of islets per millilitre alginate in the reservoir. When tested with 500 and 800 μm capsules, an increase in the number of islets per millilitre alginate was found to be associated with an increase in the number of inadequately encapsulated islets in a diameter-dependent fashion. When small instead of large capsules are produced from a given volume of alginate, larger numbers of capsules are obtained, but also a larger portion of inadequate capsules. With 10 000 islets per millimetre alginate, these combined effects can be calculated to result in a two-fold increase in the production rate of adequate capsules when 500 μm instead of 800 μm capsules are produced. Hence, substantial upscaling of the production can be achieved by combining an increase in the number of needles with a decrease in the capsule diameter.

Keywords: Droplet generator, microencapsulation, alginate, inadequate capsules, islets

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ABBREVIATIONS

EGTA, ethylenebis(oxyethylene-nitrilo) teraacetic acid
HBSS, Hanks balanced salt solution
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
KRH, Krebs–Ringer Hepes
NBCS, newborn calf serum
PLL, poly-L-lysine
RCA-I, *Ricinus communis* agglutinin I
RPMI medium, Rothwell Park Memorial Institute medium
UW, University of Wisconsin solution

Alginate–poly-L-lysine (PLL) microencapsulation allows for successful transplantation of pancreatic islets into diabetic animals in the absence of immunosuppressive therapy[1–4] and is, therefore, a promising technique for application in man[5]. The technique involves the entrapment of islets in alginate droplets which, subsequently, are transformed into spherical beads and coated with a polycation[6–8].

Like others[4,8–11], we produce the islet-containing droplets with a droplet generator composed of an alginate reservoir (i.e. a syringe) connected with regulated air pressure, and a droplet sizer, which is composed of an air chamber and an alginate nozzle (i.e. a needle) surrounded by an air jacket[12]. A major disadvantage of this device is that it is incapable of producing sufficient numbers of islet-containing droplets needed for successfully grafting encapsulated islets in large animals or humans[6,12] in a sufficiently short time-period. For example, if one million islets are required for human islet transplantation, one would need 101 h[6] to provide all islets with a bead, under the assumption that each bead contains one islet. The production time, however, is probably closer to 200 h, since not all but approximately 50% of the capsules contain an islet[13] when the usual amount of 2000 islets per millilitre is transformed into droplets[4,14].

The production of droplets can be enhanced by the simultaneous production of more than one droplet. The production can also be enhanced by increasing the number of islets per millilitre alginate in the alginate syringe, but this is not without risk. The location of an islet within an alginate bead is determined by chance[13], which implies that an islet may be located in the centre but also in the periphery of the bead, and thereby may even be found to protrude from the bead[13,15]. If we increase the number of islets per millilitre alginate we also increase the chance for

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beads to contain more than one islet which is associated with more protruding and, thus, inadequately encapsulated islets[15].

The present study describes the design and construction of a multineedle droplet generator. It also includes our investigations of the effects of increasing the number of islets per millilitre alginate on the adequacy of encapsulation.

MATERIAL AND METHODS

Design of the muiltineedle droplet generator

Testing of the droplet production by the multineedle droplet generator involves manual counting of the production of beads. Therefore, we provided the multineedle droplet generator with only four needles.

The multineedle droplet generator is composed of an alginate reservoir (i.e. a syringe) and a droplet sizer, which are both connected to compressed air by silicon tubing. The droplet sizer, as shown in *Figures 1* and *2*, is composed of two cylindrical chambers, topped by the so-called alginate dispenser (component 3, *Figure 1*), i.e. a cone-shaped connection part for the syringe. The upper cylindrical chamber (component 4, *Figure 1*) holds the four alginate nozzles (i.e. blunted hypodermic needles) and the air inlet (component E, *Figure 2*). The alginate dispenser not only serves to direct the alginate from the syringe to the needles, but also keeps the needles fixed in the sizer. The lower chamber (component 5, *Figure 1*) holds four air jackets (component F, *Figure 2*) with an inner diameter of 1.7 mm. The lower chamber also holds the centre plate (component D, *Figure 2*), provided with a total of 16 holes. Twelve holes, each with a diameter of 2.0 mm, serve as the air inlet to the lower cylindrical chamber, and four holes each with a diameter of 0.65 mm serve the tight fit of the 25G needles and are positioned exactly above the middle of the four air jackets. By this means, the needles are fixed in the centre of the air jackets. The upper and lower chambers are connected by four screws. Leakage of air at the connection between the dispenser and upper chamber and between the upper and lower chambers is prevented by tightly fitting rubber rings (component 9, *Figure 1*).

We chose to apply a 10 ml syringe in our study. The air pressure in the syringe was measured manometrically (component 7, *Figure 1*), and the air flow rate through the air chamber was measured by an air flow meter (component 8, *Figure 1*).

The multineedle droplet generator is made of stainless steel and is autoclavable.

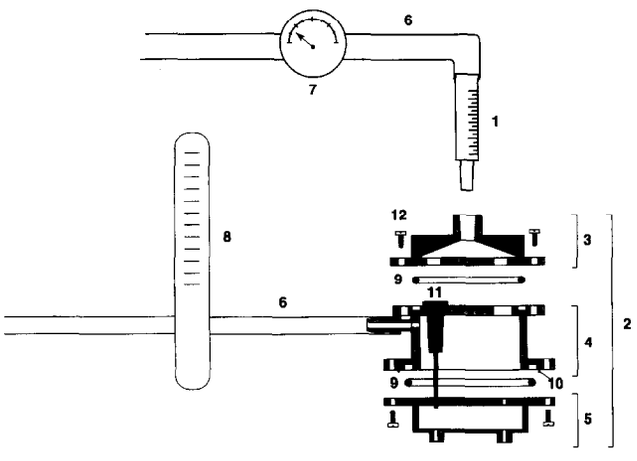

Figure 1 Schematic presentation of the multineedle droplet generator: 1, alginate reservoir (syringe); 2, droplet sizer; 3, alginate dispenser, with connection for syringe; 4, upper cylindrical chamber; 5, lower cylindrical chamber, including centre plate; 6, silicon tubes; 7, manometer; 8, air flow meter; 9, rubber rings; 10, fitting pin (to position the screw holes exactly over each other); 11, one of the four blunted 25G hypodermic needles; 12, screws.

Islet isolation

Islets were isolated from the pancreas of 2–4-year-old female slaughterhouse pigs. The pancreases were excised immediately following exsanguination of the pig, which in total took approximately 5 min. The pancreases were transported to the laboratory in Hanks' balanced salt solution (HBSS) on melting ice. Only the splenic portion of the pancreas was used for islet isolation. After cannulation of the pancreatic duct, 2 ml HBSS containing 1 mg ml^{-1} collagenase (Sigma type XI, lot number 104H68681) was infused per gram pancreas. After this infusion, which induced distension, the pancreas was divided into 10 approximately equal portions and brought into a digestion chamber as described by Ricordi[16]. This digestion chamber was provided with a screen (mesh 400 μm) and five beads of glass. The chamber was connected to a circulation system including a reservoir, a heating circuit and a temperature recorder. The chamber was filled with collagenase solution and, subsequently, recirculation was started at a flow rate of 50 ml min^{-1}. The temperature of the perfusate was increased at 1°C min^{-1}. The digestion was considered to start when the temperature of the perfusate was 36°C. During the digestion the temperature in the chamber varied between 35 and 37°C. After the start of the digestion, the chamber was gently shaken by hand for 20 s min^{-1}. After 30 min of digestion, samples of 5 ml were taken from the chamber every other minute. These samples were stained with dithizone and screened microscopically for the presence of intact islets without obvious adherent exocrine tissue. When more than 40 of these intact islets were counted, the elution of the digested tissue was started. The elution was performed with HBSS, supplemented with 25 mM HEPES and 10% newborn calf serum (NBCS) at 33°C. Monitoring of the number of intact islets was continued during the elution phase. When the number of intact islets decreased or when the number of fragmented islets increased the elution was continued at a higher flow rate of 250 ml min^{-1}. After the elution, the digested tissue was collected and kept for 30 min in University of Wisconsin solution (UW) at 4°C. Subsequently, islets were separated from 6–12 ml digested tissue on a discontinuous gradient of 11, 15, 17 and 25% dextran in Krebs–Ringer HEPES (KRH). In our experiments we only used islets obtained from the 15–17% dextran interface as this interface was the only interface without obvious contamination with exocrine, ducts or other non-islet tissue.

The total number of islets after the gradient separation was determined by assessing the number of islets in a 4% aliquot of the islet suspension.

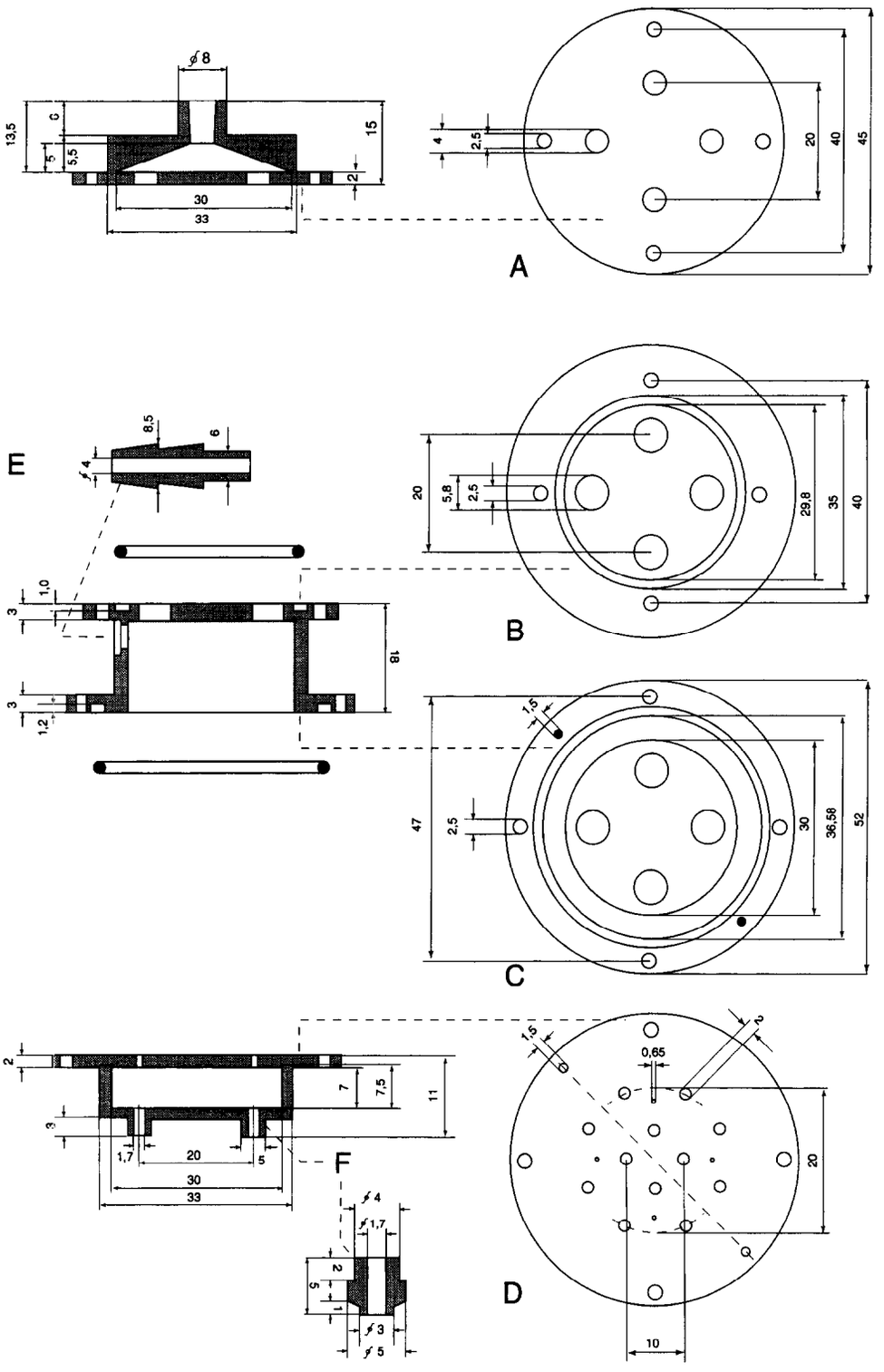

Figure 2 Detailed drawing of the multineedle droplet generator: A, underside of the alginate reservoir; B, topside of the upper cylindrical chamber; C, underside of the upper cylindrical chamber; D, centre plate; E, air inlet pipe; F, air packet. The numbers represent the sizes in millimetres.

Islet microencapsulation

Prior to encapsulation the islets were washed three times with HBSS containing 10% NBCS. In order to obtain alginate–islet suspensions of 1000, 2000, 4000, 6000 and 10000 islets per millilitre alginate, the appropriate number of islets were mixed with an appropriate volume of 3% alginate solution (Keltone LV, Kelco International, London, UK).

Polylysine–alginate encapsulation was performed as described elsewhere[7]. 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 3 min in 50 ml Ca^{2+}-free KRH buffer containing 135 mM NaCl. A PLL membrane was formed by suspending the alginate beads in 10 ml 0.1% PLL solution for 10 min (PLL–HCl, M_w 22000, Sigma). Non-bound PLL was removed by three successive washings with 10 ml Ca^{2+}-free KRH for

3 min. The outer alginate layer was subsequently applied by 5 min incubation in 10 ml ten-times diluted alginate solution. In order to liquify their inner core, the microcapsules were suspended in 50 ml of 1 mM EGTA in Ca^{2+}-free KRH for 10 min, and finally washed three times with KRH containing 2.5 mM $CaCl_2$. Microencapsulated islets and control islets were cultured in RPMI medium (Gibco, no. 22511) for 1 h at 37°C in humidified air containing 5% CO_2.

Assessment of diameter of beads and capsules and production rate of droplets

The bead or capsule diameters were measured with 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 μm. In each experiment, 30–60 beads were measured. The variation in diameter was always less than 5%.

The production of alginate droplets was assessed by collecting the droplets for 30 s in 100 mM $CaCl_2$ and subsequent counting of the beads with the dissection microscope. The production was expressed as the number of beads produced per hour.

Identification of inadequate capsules

The adequacy of encapsulation was assessed by identifying the capsules that provide inadequate immunoisolation of the islet as described previously[13]. Briefly, the microencapsulated islets were incubated with 0.5 ml RCA-I solution (0.42 nM RCA-I) in KRH for 45 min at 4°C. Subsequently, the microcapsules were washed four times with KRH to remove unbound RCA-I and were stored at 4°C. Positive fluorescence was assessed by using a fluorescence microscope (Olympus TMT-2) equipped with inverted phase contrast. This inversion was required to focus on the islet which, as a consequence of its eccentric location within the capsule, usually caused some rotation of the capsule until gravity maintained the islet at the lowest point. Microscopic examination was always performed, to confirm that the fluorescence was caused by labelling of islet cells and not by non-specific labelling.

The adequacy of encapsulation was quantified by expressing the number of inadequate capsules as the percentage of the total number of islet containing capsules.

Statistical analysis

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

RESULTS

The effect of the air flow rate through the droplet sizer on the diameter and production rate of beads

These experiments were performed at a constant pressure on the alginate of 20 kPa. This forces the alginate through the needles and droplets are formed at the tips of the needles[12]. The droplets detach from the needles when the drag forces of the air flow around the needle tip and the weight of the droplet exceeds the surface tension that keeps the droplet attached to the needle tip. An increase in the air flow through the air jacket increases the drag forces on the droplet and thereby decreases the diameter of the beads. Quite in accordance with this principle, we observed that an increase in the air flow from 8 to 24 l min^{-1} through the air jacket decreases the diameter of the beads in a linear way (*Figure 3*, open symbols), and concomitantly increases the number of beads produced (*Figure 3*, closed symbols). An air flow of 24 l min^{-1} through the air jacket was associated with the production of high quantities of beads with a diameter as small as 470 μm. Smaller beads could not be produced while maintaining less than 5% variation in diameter, since air flow rates higher than 24 l min^{-1} were associated with the formation of satellite beads (so-called fragmentation), i.e. not normal sized and undesired very small droplets[12,17].

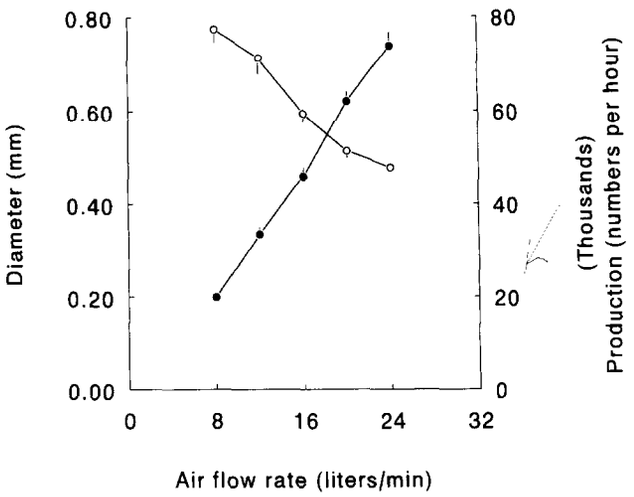

Figure 3 Influence of the air flow rate through the droplet sizer on the diameter (open symbols) and production rate (filled symbols) of beads. Values represent mean ± s.e.m. of six experiments.

The effect of the pressure on the alginate on the production rate of beads

These experiments were performed with beads of diameter 500 and 800 μm. With both bead diameters, an increase in pressure on the alginate induced an almost linear increase in the production of the beads (*Figure 4*). However, far more 500 μm than 800 μ beads were formed, at each alginate pressure tested. In addition, the increase in pressure was associated with a more pronounced increase in production of 500 μm beads than 800 μm beads. This is illustrated by the steeper slope of the line representing the production of 500 μm beads (*Figure 4*, filled symbols) than that of the line representing the production of 800 μm beads (*Figure 4*, open symbols).

The effect of the number of islets per millilitre alginate on the adequacy of islet encapsulation

A portion of the capsules always contains inadequately encapsulated islets both with 500 and 800 μm capsules (*Figure 5*). With the lowest number of islets per millilitre alginate tested, i.e. 1000 islets per millilitre, this portion amounts to 33 ± 3.4 and 6.7 ± 2.2% for 500 and 800 μm capsules, respectively. The rise in the number of inadequately encapsulated islets with higher numbers of islets per millilitre alginate is

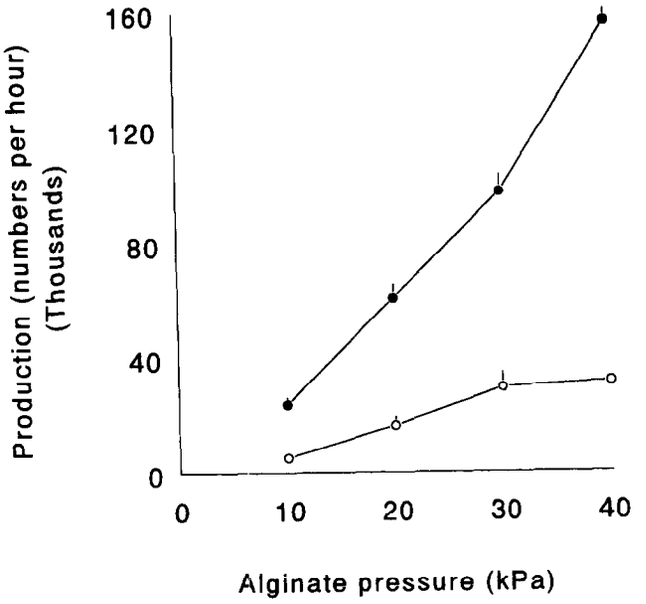

Figure 4 Influence of the alginate pressure on the production rate of beads with a diameter of 500 μm (filled symbols) and 800 μm (open symbols). Values represent mean ± s.e.m. of six experiments.

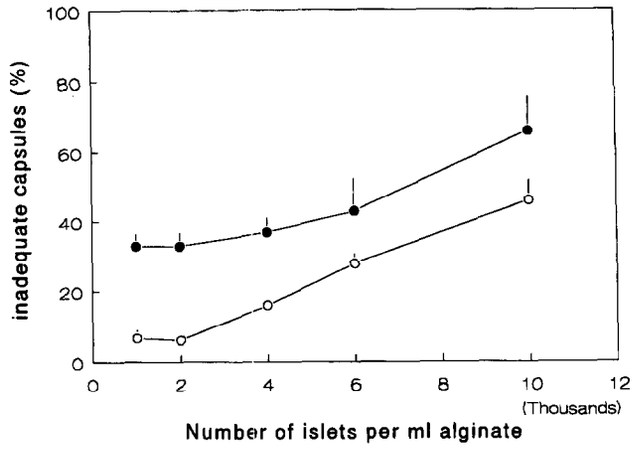

Figure 5 Influence of the number of porcine islets per millilitre alginate on the adequacy of islet encapsulation in capsules of 500 μm (filled symbols) and 800 μm (open symbols). Values represent mean ± s.e.m. of five experiments.

dependent on the capsule diameter applied. With 500 μm capsules there are no significant differences between the portions of inadequately encapsulated islets in the range from 1000 to 6000 islets per millilitre alginate, but with 10 000 islets per millilitre alginate the portion rises to 66 ± 9.7%, which is significantly larger than with 6000 islets per millilitre alginate ($P < 0.05$). When 800 μm capsules are prepared, the portion of inadequately encapsulated islets is much lower than with 500 μm capsules, but it starts to rise significantly ($P < 0.05$) when 4000 or larger numbers of islets per millilitre alginate are fed to the alginate reservoir.

DISCUSSION

The design of the multineedle droplet generator is based on our previously developed single-needle droplet generator, and its simple mode of operation was maintained in spite of the differences in construction[12]. An important aspect of the new construction is the centre plate, which replaces the setscrews for the needle in the single-needle droplet generator[12]. The centre plate sets the needles tight by fixation of the shafts, while the setscrews only hold the plastic upper part of the needle, i.e. the connector for the syringe[12]. The centre plate is quite effective in limiting the vibration of the needles at high air flow rates, as illustrated by our observation that the multineedle droplet generator allowed for production of beads somewhat smaller than 500 μm, which bead diameter could not be produced without generating undesired, satellite beads, with a single-needle droplet generator provided with a 25G needle[12]. Another important aspect of the multineedle droplet generator is the alginate dispenser, which allows for the supply of alginate to the needles not by four syringes but by one syringe only. This dispenser not only maintains the simplicity of the mode of operation, but it also allows for a redistribution of the islet–alginate suspension over the other needles in case of a stopped-up needle, which prevents eventual waste of islets. Finally, we have applied stainless steel instead of copper for the construction of the device to prevent oxidation after repeated autoclaving.

The current microencapsulation procedure is not fully efficacious since approximately 50% of the capsules with the usual diameter of 800 μm remain empty[13]. Theoretically, this percentage can quite easily be reduced by increasing the number of islets per millilitre alginate in the syringe. This number should not exceed a certain maximum, since increasing the number of islets per millilitre alginate increases the chance for beads to contain more than one islet, which is associated with an increase in the number of inadequately encapsulated islets[13]. We found the maximum number of islets per millilitre alginate to be higher with 500 μm than with 800 μm capsules, which is explained by the fact that from the same volume of alginate approximately three times more 500 μm than 800 μm capsules can be prepared[13].

The three-fold increase in the total number of capsules does not imply that the number of adequate islet containing capsules also shows a three-fold increase, as shown by the following exemplary calculation. When 10 000 islets per millilitre alginate are fed to the syringe, the percentage of adequate capsules is 34% with 500 μm capsules and 54% with 800 μm capsules (*Figure 5*). The total number of capsules available is three times higher with 500 μm than with 800 μm capsules. Thus, when 10 000 islets per millilitre alginate are applied, the same volume of alginate required for the production of 54 adequate 800 μm capsules can be predicted to suffice for the production of three times 34, i.e. 102, adequate 500 μm capsules. Since the time period required to process a certain volume of alginate does not depend upon the diameter of the capsules produced, this indicates that the production rate of adequate islet-containing capsules can be increased approximately two-fold by reducing the capsule diameter from 800 to 500 μm. This factor of two can be increased to eight when the capsules of 500 μm are produced with the four-needle droplet generator. The construction of the droplet generator is such that it can be readily adapted to

contain more than four needles to further increase the production rate. When combined, these measures reduce the production time for application in man from several days[6] to several hours. Finally, our study represents a worst-case scenario, since we used an alginate solution with a high viscosity, and a needle with the smallest diameter to accommodate large islets. Thus, the production time may be further reduced by applying alginate solutions with lower viscosities and needles with larger diameters[12].

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