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Multilayer Nanoencapsulation. New Approach for Immune Protection of Human Pancreatic Islets

Silke Krol,^{*,†} Silvia del Guerra,[‡] Maria Grupillo,[‡] Alberto Diaspro,[§]
Alessandra Gliozzi,[†] and Piero Marchetti[‡]

*Department of Physics, University of Genoa, via Dodecaneso 33, 16146 Genoa, Italy,
Department of Endocrinology and Metabolism, University of Pisa, Via Paradisa 2,
56124 Pisa-Cisanello, Italy, and LAMBS-IFOM, MicroScoBIO Research Center,
University of Genoa, via Dodecaneso 33, 16146 Genoa, Italy*

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ABSTRACT

Immune protection of artificial tissue by means of pancreatic islet microencapsulation is a very ambitious new approach to avoid life-long immune suppression. But the success in the utilization of the alginate-beads with incorporated islets is unfortunately limited. Some of the problems cannot be solved by a two-component system, so polymer encapsulation of the microbeads was tested to improve the properties. In the present paper a pure nanoencapsulation multilayer approach was tested in order to reduce the size of the capsule and possibly apply in the future a multilayer capsule with individual properties in each layer or region of the capsule. Different polycations were attached in a self-assembly process. The advantage in using the surface charge of islets as binding site for the polyions is the guarantee of complete coverage after the second layer. Release of insulin was determined to characterize the function of the islets after encapsulation as well as the permeability of the capsule. Fluorescence microscopy was used to visualize the polyelectrolyte layers. Finally by means of an immune assay, the protection capability of the capsule was proved. In these first measurements the encapsulation with a multilayer nanocapsule was shown to be a possible alternative to the more space-consuming and random islet-trapping microencapsulation.

Immune protection of artificial tissue by means of microencapsulation is a very ambitious new approach to avoid life-long immune suppression especially in the case of juvenile recipients (type 1 diabetes). The aim is to transplant islets with a minimum or no immune suppression. Great progress has been achieved since the conception of this idea. We shall summarize here the most important obstacles and advantages in the use of the different coatings; an extensive analysis can be found in several good reviews.^{1–4}

Microencapsulation in the field of Langerhans islets usually means entrapment of cell clusters in high-viscose ultrapure alginate droplets stabilized with divalent positively charged ions such as, e.g., barium or calcium. As major problems for alginate, a marine polysaccharide, the researchers identified that the ratio between both alginate building blocks, L-guluronic (G) and D-mannuronic (M) acid, plays an important role in inflammation and fibrotic overgrowth after implantation. Fibrosis in the implantation site can lead to necrosis of the enveloped cells due to malnutrition or hypoxia. So the new generation of capsules was usually

prepared with ultrapure alginates with an intermediate (G) and a high (M) content to guarantee biocompatibility and to reduce fibrosis.⁵ Apart from preventing excessive fibrosis, also neovascularization of the transplant can increase the long-term viability and functionality. Only recently, it was shown for a glucose sensor that the functionality is increased significantly if revascularization occurs.⁶ Unfortunately, some alginates, especially those of low molecular weight⁷ or impurities, showed that surfaces finishing with the negative polymer prevent fibroblast adhesion⁸ which can be also an explanation of the fibrosis.⁹

Another problem is release of factors such as, e.g., cytokines, nitroxide (NO), or antibodies by the enveloped cells of allo- or xenografts. These factors lead for example to attraction of macrophage, antibody-mediated cytotoxicity, or again fibrosis. Microcapsules of alginate allow only for a limited tuning of the cutoff to prevent recognition by antibodies, but cytokines which have usually the same size as insulin cannot be excluded efficiently. This was the reason for applying multilayers on top of microbeads to change the permeability properties.^{10,11}

Nevertheless the first transplantations of microencapsulated human pancreatic islets into patients with type 1 diabetes were promising because they led to short-term independence

* Corresponding author. E-mail: krol@fisica.unige.it.

[†] Department of Physics, University of Genoa.

[‡] Department of Endocrinology and Metabolism, University of Pisa.

[§] LAMBS-IFOM, MicroScoBIO Research Center, University of Genoa.

(1–12 months)^{12–14} from exogenous insulin with a minimum of immune suppression. But there is still the problem with the shortage of human donor organs. So in the past few years, significant progress was made for xenotransplants from pigs.^{15,16} Especially after the most serious constriction, a possible infection with porcine endogenous retroviruses was proved to be without cause.^{17,18} Moreover recently some xenotransplantation of pig islets to humans was carried out.¹⁹

However, there are some drawbacks for microencapsulation with alginate gel droplets. The unfavorable ratio between encapsulated cell volume and overall capsule volume only allows transplantation subcutaneous or intraperitoneal. The random trapping of the islets sometimes leads to incomplete coverage, an undefined number of cells, and prolonged response times to external stimulation. The alginate system is not flexible enough to fulfill all the requirements demanded by islet cells inside, cutoff of the capsule, and biocompatibility and neovascularization of the transplant. In the past some work was focused on polyelectrolyte multilayers to vary the properties of the microcapsule or used multicomponent microbeads to encapsulate the islets or cells.^{20–24}

With the present study a new multilayered nanocapsule as a more flexible encapsulation for islets is introduced. Interactions with cells adhering on the multilayered surface are numerous and give evidence of how powerful this method is. Several groups modulate surfaces in a way that they induce or facilitate the binding of different cell types. The number of polyelectrolytes which can be used and combined to prepare multilayers is endless. Previous work, e.g., on the response of HUVEC (endothelial cells) on polyelectrolyte layers, was extremely interesting.²⁵ Here there was found that endothelial cells grow well on the polycation (polyallylamine) PAH and more functional than on the other tested surfaces. Another interesting biomedical application in this context is the multilayer deposition in order to repair damaged arteries with hyaluronan and chitosan.²⁶ In this work layer-wise deposition directly on the inside of an artery was performed in order to prevent platelet adhesion and a later thrombus. More biomedical applications can be found in the work of Salloum et al. in which they deal with the prevention of cell adhesion (smooth vascular muscle cells) on implant surfaces.²⁷ The authors tested several polyelectrolytes for the cell-repellent properties and found that this specific cell type preferentially grows on hydrophobic as well as negatively charged surfaces. In addition to use with blood vessel cells, multilayer materials were tested also for the deposition of fully functional photoreceptor cells as therapy for retinal or age-related macular degeneration.²⁸ So the use of multilayer systems in order to improve or change surface properties is well established. The underlying method to deposit multilayers onto surfaces or cells is the layer-by-layer (lbl) technique.^{29,30} It is based on the attachment of highly and oppositely charged polyions onto charged surfaces in a self-assembly process. Due to its ease of handling and cost effectiveness the method has attracted attention also in the field of biomedicine lately.

So the idea of applying multilayers via lbl directly to the Langerhans islets was fascinating. With this technique,

complete coverage of the islets can be guaranteed because the perinsular capsule serves as a template for polyion adhesion. Moreover, it is expected that a system based on electrostatic interactions of oppositely charged polymers can be tailored to support lower layer islet functionality and perhaps neovascularization or fibrosis reduction in the outermost layer. For these two functions, completely different polyelectrolyte pairs could be used. With specific polyelectrolytes, a defined cutoff of the capsule is possible or also inhibitor binding in order to prevent graft rejection, macrophage attacks, or antibody recognition. Some other features of the capsule which can be advantageous are the nanometer size in terms of faster response to stimulation and the possibility to bind factors to the polyelectrolyte so a slow release can be triggered. This was shown impressively in the work of Chluba et al.³¹ where a hormone was covalently bound to a polymer and slowly released. Another work deals with binding RGD covalently to polymers in order to improve cell adhesion and biomimic the extracellular matrix.³² Hence, it is expected that the lbl technique in the future could allow for a multifunctional nanometer thick capsule.

In the present paper to our knowledge for the first time polyelectrolyte multilayers were deposited on human pancreatic islets in order to receive immune protection. First an encapsulation protocol for the Langerhans islets was developed. After islet encapsulation, their functionality was characterized by release of insulin, sensitivity to stimulation intensity by static glucose stimulation, and long-term viability and functionality of the islets. Furthermore it was possible to show that the capsule prevents the recognition of the islets by antibodies. Several polyelectrolyte couples were tested for their suitability. From the results it can be concluded that the nanoencapsulation of pancreatic islets is a promising alternative to the commonly used microencapsulation with ultrapure alginate beads.

In the beginning it was necessary to investigate how cells were stressed by the encapsulation process itself with its repeated medium changes and if the cells survive the encapsulation process. Then it was also interesting to determine the maximum number of layers which can be applied without causing too much damage to the islets. Different polycations were used as first layer because from previous investigations it was known that cells have different sensitivities to diverse polyelectrolytes.³³

In Figure 1 the microscopy images of islets are shown after encapsulation with PAH (poly-(allylamine hydrochloride)) or PDADMAC (poly-(diallyldimethylammonium chloride)) as polycation and PSS (poly-(styrenesulfonate)) as polyanion in order to visualize the attached polyion, the intactness of the islet, and the number of dead cells after the coating process.

In Figure 1A, trypan blue-stained human islets are shown after encapsulation with three pairs of layers (PDADMAC/PSS)₃. The number of dead cells was estimated by screening microscopically through at least 10 islets by two independent observers. The number of dead cells detected was less than 5% of the complete islet volume. To control the attachment

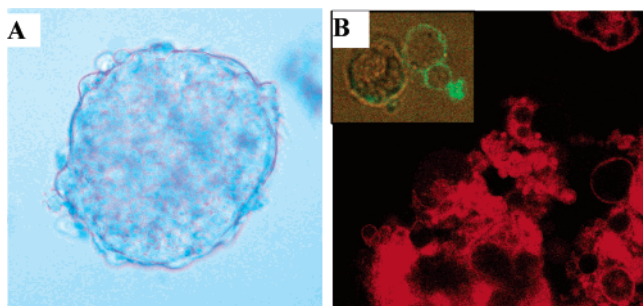


Figure 1. (A) Light microscopy image of trypan blue-stained islets after encapsulation with three pairs polyelectrolyte layers (PDADMAC/PSS)₃. The dead cells appear dark blue. (B) Fluorescence microscopy of the coated islets. Only imaging of the border region was possible due to the thickness of the islet. The applied PAH layer appears red due to covalently bound Alexa-555. The polymer penetrates also the intercellular space (bright red). Inset: Smaller fragment of a pancreatic islet. The merged images of transmission and fluorescence microscopy that the capsule surrounds the cells. Here the fluorescence is due to green-fluorescent FITC-PAH.

of the polycation to the islets, a fluorescently labeled polymer was used. In Figure 1B the border of an islet with the red fluorescent capsule was visualized. It can be seen that the polymer is attached to every part of the islets, but the amount is notably high in the intercellular space. The imaging of the complete islet in three dimensions is not possible because of the thickness of 200–300 μm . In the inset of Figure 1B a smaller fragment of islet is shown. The merged image of transmission and fluorescence microscopy revealed that the capsule follows completely the surface of the cells. The appearance of human islets in general was very compact with a smooth surface. Rarely islets with a fuzzy surface were observed, these islets have the tendency to dissociate during the polyelectrolyte encapsulation. This was observed with islets of another species (data not shown). Incubating islets for around 6 weeks in medium revealed a difference in vitality of islets with smooth and fuzzy surfaces. In the case where the surface was fuzzy with cells sticking out of the network, these cells were dead and the islet was necrotic. If the surface was smooth, cells of the islets were intact and no necrosis was observable (data not shown).

But the functionality test of the coated islets, based on the insulin release in dependence of the glucose level, revealed that the islets encapsulated with an even number of layers always release the same amount of insulin (Figure 2A,B) which is around 40% of the amount released by uncoated islets under stimulation with low glucose (3.3 M). In these sets of experiments, it can be seen that the polymer nature as well as the molecular weight plays an important role in the release behavior of the coated islet.

With the high molecular weight polycations PDADMAC (Figure 2A) and PAH (Figure 2B, striped column), only a limited insulin release and relation to the stimulation intensity was detected. For both molecules the released amount of insulin for low glucose was comparable to that of high glucose levels. The situation is only slightly better if the lower molecular weight of PAH was used. This effect was significant for an even number of applied layers. In the case where an odd number of layers is attached to the islet surface

(Figure 2C), a stronger difference between the quantity of insulin released after low glucose and that after high glucose concentration was measured. Moreover the release from coated islets with three layers is only slightly lower than that of uncoated islets. These coated islets showed a good functionality for at least 1 week. So in the following we focused our interest on capsules constructed with PAH with a molecular weight of 15 kDa and refer to it as PAH.

The three-layer coated Langerhans islets did not show any morphologically distinctive features (Figure 3).

Their appearance (Figure 3B) visualized by means of electron microscopy is comparable to that of untreated cell clusters (Figure 3A).

The same capsule was also investigated in terms of protection of the islets against antibody recognition (Figure 4). Uncoated islets that were recognized by the antibody are visible as a strong fluorescence signal (Figure 4A). After the islets were coated with three polyelectrolyte layers, no antibody recognition of the islets was detected (Figure 4B).

In summary, we were able to show that the layer-wise-deposited nanometer-thick shell with an odd number of layers influences only slightly the passage of insulin and provides a protection against islet-specific antibody recognition. Only with an odd number of layers (positive net charge), a relationship between stimulation intensity and response intensity could be measured (Figure 2B). With an even number of layers (negative net charge) the amount of insulin released is independent of glucose level (Figure 2A). This effect is still under investigation because we assume an unspecific stimulation due to the negative charge. Moreover, it is noteworthy that no significant difference in the insulin release was observable with two or with six layers applied to the islet surface. This can be understood if one takes into account that up to six layers, the structure of the capsule is part of the so-called precursor zone which is less ordered and more permeable also to larger molecules as defined in the model developed by Decher.³⁴

Interesting is also the difference in insulin release if a high (PDADMAC, 240 kDa; PAH, 70 kDa) or low molecular weight (PAH, 15 kDa) polycation is used. This gives a hint that the assumption that a modulation of the capsule properties in terms of permeability by varying the polymers is reasonable. From polyelectrolyte theory³⁵ it is known that the conformation of weak polymers (PAH) depends on the ionic strength of the solution while strong polyions (PDADMAC) are independent from ion concentration. Due to ions present in the medium, a random coil structure of the polycations is reasonable. But the structure induced in the high molecular weight seems to be less coiled, so the nanopores³⁶ are smaller and the permeability for insulin (5.8 kDa) is reduced. This also true for the strong polyelectrolyte PDADMAC which presumably exists in a more extended form, and due to this a capsule with it as polycation only showed a limited insulin release (Figure 2A). That a variation of the cutoff is possible by application of polymer multilayers was also proved by Sakai et al.³⁷ Furthermore it cannot be excluded that the binding of polyelectrolytes directly on the islet surface also influences the insulin release mechanism

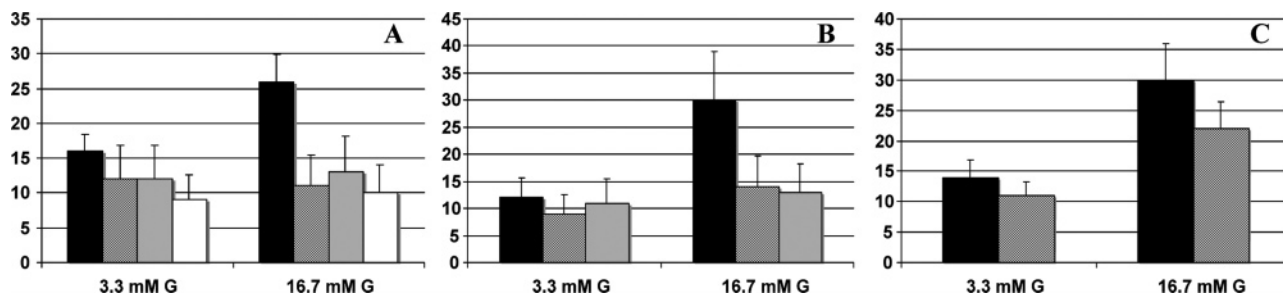


Figure 2. Static insulin release for human pancreatic islets to different glucose (G) levels. The islets were coated with polyelectrolytes with an odd or even number of layers as well as with different polymers at different molecular weights. y -axis is insulin ($\mu\text{U/mL}$); x -axis is the stimulating substance. The bars indicate the SD. (A) Uncoated (filled column) and islets coated with PDADMAC/PSS (thickly striped column), (PDADMAC/PSS)₂ (thinly striped column), and (PDADMAC/PSS)₃ (empty column) polyelectrolyte layers. (B) Uncoated (filled column) and islets coated with PAH(15 kDa)/PSS (thickly striped column) and PAH(70 kDa)/PSS (thinly striped column). (C) Uncoated (filled column) and PAH(15 kDa)/PSS/PAH(15 kDa)-coated islets (striped column).

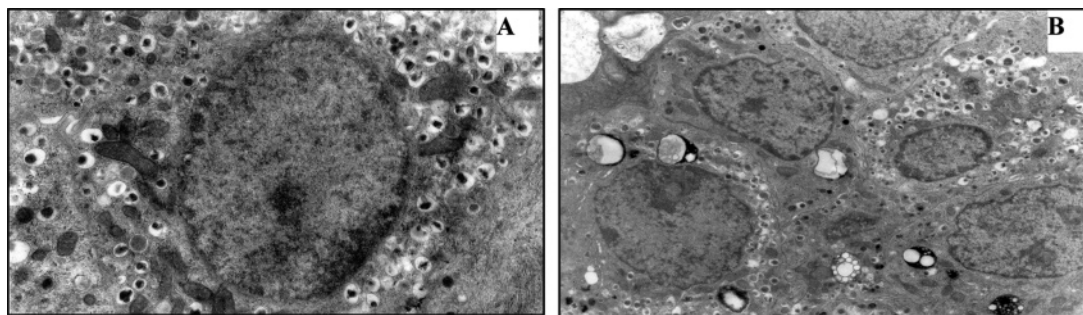


Figure 3. Electronmicrographs of (A) uncoated and (B) PAH/PSS/PAH-coated islets. Morphology of the coated cells is comparable to the uncoated ones.

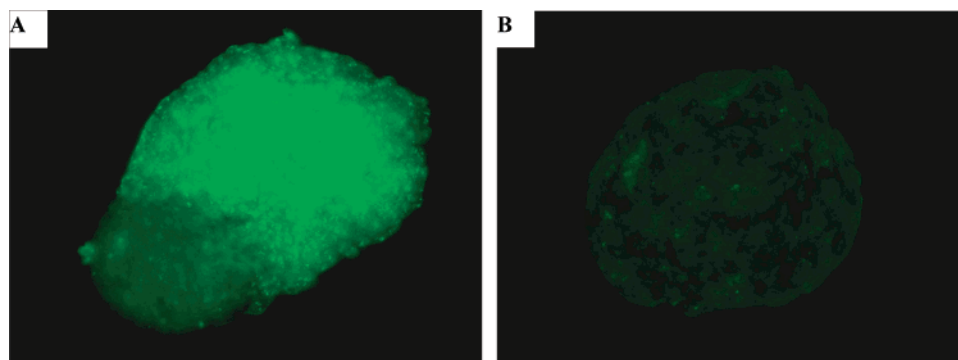


Figure 4. Fluorescence microscopy of (A) uncoated islets and (B) PAH/PSS/PAH coated human pancreatic islets. The fluorescence is due to FITC-labeled anti-GAD(+) antibodies and autofluorescence of the islets. The coating prevents the recognition of the islets by the antibody.

from the β -cells by capping regions necessary for the exocytose and partly interact with the glucose by attractive forces. But the effect on the islets was not so high, as can be seen from Figure 2C.

Macroscopically no significant changes in the morphology of the islets was observed, and by the insulin release, functionality was proved. But more interesting for a later use as immune barrier is the protection against antibodies or cytokines. As a first step we checked if the three-layer capsule was able to prevent antibody recognition, and this was successfully proved. Two possible mechanisms are reasonable explanations this: (1) an exclusion of the antibody (MW 65 kDa^{38,39}) due to smaller cutoff of the multilayer capsule; (2) masking of the epitope on the cell surface for

antibody recognition. The determination of the cutoff of hollow capsules prepared under the same conditions (medium, ionic strength, polyelectrolyte composition) like the islets revealed a cutoff of >150 kDa,⁴⁰ so a masking of the epitope is more probable.

As far as we know this is the first time that a nanometer-thick multilayer capsule was used to cover and protect human pancreatic islets. From the multilayer polyelectrolyte capsule, we expect that in future we will be able to construct a capsule with discrete zones. The first zone should be tuned to optimize the environment of the coated islets in terms to guarantee a long-term viability and functionality. The middle zone should determine the permeability of the capsule as well as offer binding sites for cytotoxic cytokines in order

to prevent graft failure. The outermost zone in direct contact to the environmental tissue should moderate the connection to the blood circuit in order of revascularization and reduced fibrosis.

Another pressing question that must be answered is can a nanometer thick shell withstand shear forces during an injection process during transplantation and the forces they are exposed to at the transplantation site. Future experiments will be focusing on this issue. Until now it can be stated that yeast cells coated with a four-layer capsule of the same composition used in the present work reveal a Young's modulus of a cross-linked gel on the nanometric level.⁴¹ From measurements on hollow multilayer capsules, it can be concluded that the overall behavior of these shells is more that of material in a glassy state.^{42–44} Hence an electrostatically cross-linked multilayer capsule offers a rigid system at the microscopic level, which is advantageous for protection against friction during injection, and nanometrically a shell which is quite soft and prevents damage of the coated cells.

Summary and Outlook. The results led to the conclusion that encapsulation with only a nanometer thin but multilayered coating can be a new approach to create multifunctional capsule for immune protection of artificial tissue. Future experiments should show that a capsule with three regions which fulfill different functions is an intelligent system to overcome several problems in protection of the graft and also prevention of transplant rejection for xenotransplants.

Methods. Islet Isolation. Pancreata were received from 14 donors ranging in age between 23 and 85 years, 9 men and 5 women. All manipulations on the pancreata were processed in a P1000 clean room, under vertical laminar flow hoods. Methods for islet preparation have been previously described.^{39,45,46} Immediately after excision, every pancreas was transported to the clean room in the transport solution on ice. The gland was trimmed by careful dissection of surrounding fat tissue, lymphnodes, vessels, and membranes and divided in two portions: the head and the body with tail. The last one was used for islet isolation. The pancreatic duct was cannulated with a 16–18 gauge angiocatheter, and about 200 mL of Hank's solution (HBSS), doped with 2% human albumin and 1.5 mg/mL collagenase P (Roche), at room temperature was injected into the pancreatic duct until complete distension of the gland, using a 50 mL syringe. Then, the pancreas was incubated with the rest of collagenase solution in a water bath at 37 °C for 12–15 min. Successively, the gland is gently shaken at room temperature until free islets can be observed in a sample. At this point, the digestate was filtered through a 300 μm and a 90 μm mesh stainless steel filters. The solution that has passed through the filters and the tissue entrapped on the 300 μm mesh filter was again treated with collagenase solution, for further digestion. The tissue remaining on the 90 μm mesh filter was washed with HBSS and 2% human albumin at room temperature in order to stop the digest. The procedure of filtration, washing, and settling in HBSS solution was repeated every 8–10 min up to 40–50 min. For the purification, the islet suspension was aliquoted in 50 mL Falcon tubes and centrifuged at 1000 rpm for 2 min at 4 °C.

Supernatant was discarded, and the pellet was suspended in 15 mL of 80:20 Lymphoprep:HBSS (v:v; with 2% human albumin). Then 10 mL of HBSS was layered over the Lymphoprep-HBSS volume. The tubes are centrifuged at 1800 rpm for 5 min at 4 °C. Islets, concentrated at the interface between the two layers, were collected and recentrifuged at 1800 rpm for 2 min at 4 °C. Supernatant was discarded, and the pelleted islets were aliquoted in 75 cm² suspension flasks with M199 culture medium (5.5 mmol/L glucose), supplemented with penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), gentamicin (50 $\mu\text{g/mL}$), and amphotericin B (0.25 $\mu\text{g/mL}$).

Islet Encapsulation. The isolated islets were incubated at least for 1 day in RPMI 1640 medium at 24 °C and 5% CO₂ to recover them. The poly-(allylamine, hydrochloride) (PAH; MW 15 kDa and 70 kDa), poly-(diallyldimethylammonium chloride) (PDADMAC; 240 kDa), and poly-(styrenesulfonate, sodium salt) (PSS; MW 70 kDa) were obtained from Aldrich (Milan, Italy). All synthetic polyelectrolyte solutions were prepared with a concentration of 2 mg/mL by solving it in RPMI 1640 medium 1 day in advance.

To visualize the PE-coating by fluorescence microscopy FITC (fluoresceine isothiocyanate, (λ_{exc} = 488 nm; Fluka, Germany) or Alexa Fluor-555 (Molecular Probes, Eugene, OR) was covalently bound to PAH or PDADMAC. The method is described in detail elsewhere.⁴⁷ In brief, the polycations were solved in a NaHCO₃ buffer (2%, pH = 9) up to a concentration of 4 mg/mL. The dye was solved in DMSO (1 mg/200 μL). Then the dye was mixed with the polycation in a 1:12 (w:w) ratio and incubated in the dark for 1 h. Removal of unbound dye was performed by dialysis against Milli-Q-grade water for at least 1 week by means of a 3.5 kDa cutoff dialysis membrane (Spectrum Laboratory, Rancho Dominguez, CA).

For the encapsulation the islets were pelleted by low-speed centrifugation and the medium was exchanged with polycation solution. If not mentioned otherwise, PAH means always that the capsule was constructed by 15 kDa PAH as polycation. After resuspension of the islets, they were incubated for 5 min in the polyelectrolyte solution. The unbound polyelectrolytes were removed by pelleting the islets by slow speed centrifugation for 5 min and washing the cells twice with RPMI medium without polyelectrolyte. Then usually the polyanion layer was applied by following the procedure described above. This process can be repeated until the desired number of layers is reached. For the deposition of one double layer, around 1 h of preparation time is needed. Experiments were performed with up to six layers on the islets. At the end of the encapsulation process the coated islets were recovered in 30 mL of RPMI 1640 medium at 24 °C and 5% CO₂ for at least one night.

Viability Staining. To determine the amount of dead cells after the encapsulation procedure, live/dead staining with trypan blue (0.4%, Sigma, Milan, Italy) was performed. For this, an aliquot of 5–10 islets were taken with 1 mL of medium, and 20 μL of undiluted trypan blue solution was added. The dead cells were stained dark blue because the cell membranes lost their integrity and the dye can enter.

The number of dead cells in a islets was estimated by two independent observers.

Fluorescence Microscopy of the Polyelectrolyte Capsule. Fluorescence microscopy was performed using a Leica TCS SP2 AOBS equipped with a 405 nm 10 mW laser diode. Imaging of the coated islets or cell clusters was obtained by using the 488 or 543 nm line of a 20 mW argon ion laser. Images were collected using a 100× oil NA = 1.4 objective HCX PL APO (Leica Microsystems S.p.A., Milan, Italy).

Static Insulin Measurement. Islets were studied within 3–4 days after isolation. Insulin secretion studies were performed by the batch incubation method, as previously described.^{39,45,46} Following a 45 min preincubation period at 3.3 mmol/L glucose, groups of approximately Y islets of comparable size were kept at 37 °C for 45 min in Krebs-Ringer bicarbonate solution (KRB; 0.5% albumin, pH 7.4) containing 3.3 mmol/L glucose. At the end of this period, medium is completely removed and replaced with KRB containing either 3.3 mmol/L glucose, or 16.7 mmol/L glucose. After an additional 45 min incubation, medium samples are collected and stored at –20 °C until insulin concentrations were measured by IRMA (Pantec Forniture Biomediche, Turin, Italy).

Indirect Immunofluorescence on Pancreatic Islets. The antibody anti-GAD+ (autoantibodies to glutamic acid decarboxylase; 6.75 U/mL) for the immunofluorescence was obtained from the serum of patients with insulin-dependent diabetes mellitus type 1. Glutamic acid decarboxylase (GAD₆₅) is an enzyme that is produced primarily by pancreatic islet cells.⁴⁸ Then the islets were incubated with 10% of this serum for 24 h at 37 °C. The islets were harvested by centrifugation for 3 min at 800 rpm. The pellet was suspended in 70% ethanol/double-distilled water solution and applied on microscope slides (SuperFrost Plus, Menzel-Glasser, Braunschweig, Germany).

Slides were dried at room temperature for 5 min and then washed twice with phosphate-buffered saline (PBS) (pH 7.2). After being washed, the bound GAD-antibody was visualized by incubation with appropriate FITC-labeled secondary anti-human antibody and diluted with a ratio of 1:30 in PBS buffer for 30 min at room temperature. The slides were washed with PBS twice, then an aqueous mounting media (Immunotech, Marseille, France) was added to improve the visibility. The immunofluorescence was analyzed by means of a fluorescence microscope (excitation wavelength 488 nm).

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