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Developing a Rapid Algorithm to Enable Rapid Characterization of Alginate Microcapsules

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The islets of Langerhans are endocrine tissue clusters that secrete hormones that regulate the body's glucose, carbohydrate, and fat metabolism, the most important of which is insulin, a hormone secreted by β-cells within the islets. In certain instances, a person's own immune system attacks and destroys them, leading to the development of type 1 diabetes (T1D), a life-long condition that needs daily insulin administration to maintain health and prolong survival. Islet transplantation is a surgical procedure that has demonstrated the ability to normalize blood sugar levels for up to a few years, but the need for chronic immunosuppression relegates it to a last resort that is often only used sparingly and in seriously ill patients. Islet microencapsulation is a biomedical innovation designed to protect islets from the immune system by coating them with a biocompatible polymer, and this new technology has demonstrated various degrees of success in small- and large-animal studies. This success is significantly impacted by microcapsule morphology and encapsulation efficiency. Since hundreds of thousands of microcapsules are generated during the process, characterization of encapsulated islets without the help of some degree of automation would be difficult, time-consuming, and error prone due to inherent observer bias. We have developed an image analysis algorithm that can analyze hundreds of microencapsulated islets and characterize their size, shape, circularity, and distortion with minimal observer bias. This algorithm can be easily adapted to similar nano- or microencapsulation technologies to implement stricter quality control and improve biomaterial device design and success.

Key words: Alginate; Microcapsules; Type 1 diabetes (T1D); Image analysis; Automation

INTRODUCTION

Over 3 million people in North America have type 1 diabetes (T1D), with 18,000 new cases annually (4,28). With over $245 billion spent in managing problems caused by diabetes nationwide and average medical expenditures for those diagnosed with diabetes more than twice as costly than those undiagnosed, diabetes is a crippling disease (1). T1D is an autoimmune disorder that results in the destruction of insulin-secreting endocrine cells (β-cells) inside the "islets of Langerhans," which are found scattered all over the pancreas. β-Cells produce insulin in response to rising blood glucose levels, thus maintaining glucose homeostasis and facilitating carbohydrate metabolism. The autoimmune destruction of these insulin-producing β-cells results in a loss of glucose homeostasis, leading to perpetually elevated blood glucose levels in the absence of exogenous insulin therapy. However, this treatment regimen can frequently cause low blood glucose levels, leading to dizziness, palpitations, and tremors; in serious cases, even seizures, coma, or death may ensue. Without this treatment, however, untreated T1D can lead to the development of diabetic ketoacidosis, a potentially life-threatening complication in the short term, and eventually cause irreparable eye damage, organ failure, and death (30).

Islet Transplantation in T1D Patients

Islet transplantation involves harvesting the pancreas from a deceased organ donor, isolating and purifying the islets from the pancreas, and infusing them into the portal vein of a transplant recipient after the performance of appropriate functional assays to evaluate graft function (2). While islet transplantation has been demonstrated to be a temporary cure for T1D, it has been problematic due to transplant rejection by the recipient's immune system. Therefore, transplant patients are required to be under chronic immunosuppression. Although it has been shown that transplant recipients remain insulin independent for

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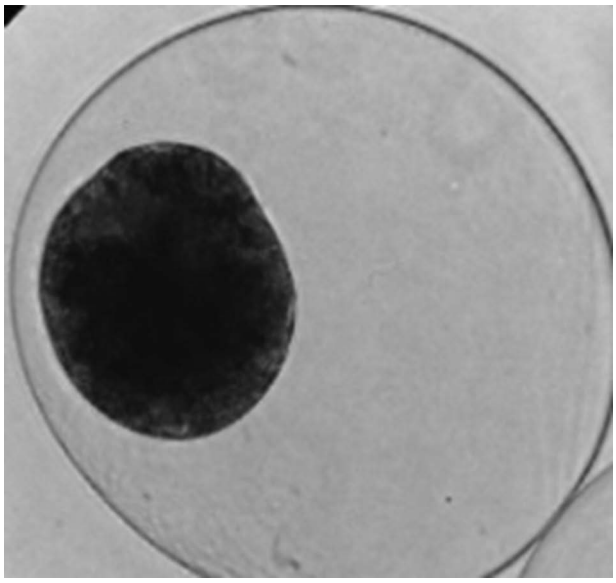

Figure 1. Young porcine islet encapsulated within an alginate microcapsule. Porcine islets are encapsulated in 3% ultrapure low-viscosity mannuronate (UPLVM) alginate, stained positive using dithizone (DTZ), and displayed an unperturbed structure.

more than a year, 80%–90% of patients eventually revert to exogenous insulin therapy, implying that islet transplantation, in its current form, is not sustainable in the long term (16,21). With immunosuppression, the immune response against islets is not completely eliminated; implanted islets last inside the recipient's body ranging from a few months to a few years. The search for a suitable organ donor is a painstaking process owing to a scarcity of healthy donors that are a near-perfect biological match; often, multiple donors are needed for successful reversal of hyperglycemia—the hallmark of a successful islet transplantation (9,12,20,21,24). Thus, the problems of islet transplantation are twofold: transplant rejection by the recipient's immune system and the relative lack of transplant-worthy, compatible islet donors.

The scarcity of suitable human islets often is the rate-determining step of the islet transplantation process. Because porcine insulin only differs from human insulin by a single amino acid, islets isolated from pigs hold prospects to be a possible alternative to human islets. As porcine insulin has been mass produced and used to treat diabetes in human patients for decades, porcine islets could be a safe and scalable alternative source of islets for transplantation into human recipients (11,13,14,26,27).

Islet Microencapsulation: An Alternative to Immunosuppression

To address the immune response that is triggered against transplanted islets, islet microencapsulation, where the islets are coated with a biocompatible polymer using electrostatic microcapsule generators, has demonstrated various degrees of success in small- and large-animal studies (3,6). Encapsulation of islets within hydrogels (Fig. 1) prevents cell–cell contact between the islets and the host immune system, thus providing a selectively permeable barrier from the host immune system while allowing for diffusion of nutrients, oxygen, and insulin. Encapsulation of islets within these microcapsules eliminates, or at the very least reduces, the need for transplant recipients to be under chronic immunosuppression (23). To improve the prevention of immune system molecular permeability to the capsules, the addition of polylysine to the alginate hydrogel structure has been suggested (7). However, there have been more recent studies that demonstrated increased binding of macrophage to polylysine-coated capsules (8,15). More recently, chitosan has been shown to improve permselectivity of alginate capsules (18), with minimal immune response in an encapsulated islet xenotransplant model (29). The success of alginate capsule-mediated protection against immune system binding and molecule permeation to reach the islets depends greatly on microcapsule morphology, the degree of islet coverage, and the location of the islets within the encapsulating hydrogel. It has been suggested that capsule size affects biocompatibility as well as the ability for microencapsulated islets to respond to glucose and secrete insulin (5). Small microcapsules are prone to osmotic stress and swelling compared to their larger counterparts, affecting the microcapsule permeability and the ability for nutrients and insulin to pass through (19). The ability of microcapsules to withstand physical stress during and after transplantation dramatically improves transplant outcomes. Small microcapsules have been reported to be able to withstand physical stress much better than big microcapsules, significantly improving graft

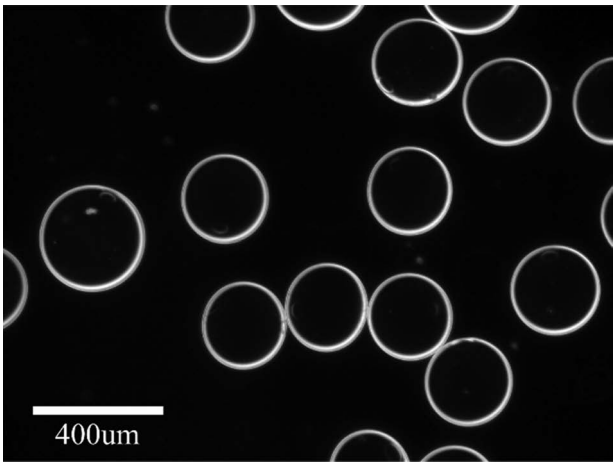

Figure 2. Microscopy image of alginate microcapsules taken with phase-contrast settings.

survival rates (10,25). Microcapsule size also impacts the efficiency with which oxygen and nutrients can diffuse in and insulin can diffuse out. Since patients would require hundreds of thousands, and in some cases even millions, of microencapsulated islets to reverse diabetes, it is imperative that techniques employed in cell encapsulation are able to produce large numbers of microcapsules of the desired size with high efficiency and consistency. Following microencapsulation, the islets will then need to be subjected to rigorous quality control analysis before they can be transplanted into diabetic recipients.

Since hundreds of thousands, and in most cases millions, of microencapsulated islets will be generated during the process, characterization of encapsulated islets without the help of some degree of automation would be painstakingly difficult, time-consuming, and error prone due to inherent observer bias. In our study, we developed and evaluated an algorithm using conventional, user-friendly imaging modalities that enables analysis of alginate microcapsules and characterization of their morphology and consistency. With this innovation, microencapsulated islets can be rapidly characterized with great consistency for pretransplant assessment within a fraction of the time it would otherwise require. This innovation will find wide applicability in automating quality control assays to evaluate microcapsules used in the cell and tissue encapsulation research and a variety of other fields where large quantities of micro- or nanospheres of consistent size and shape are generated.

MATERIALS AND METHODS

Concentrations of 1.5% and 3% (w/v) ultrapure low-viscosity D-mannuronate (UPLVM) alginate (NovaMatrix®, Sandvika, Norway) were made with endotoxin-free sterile water and filtered using a 0.2-μm filter to exclude microbial contaminants. The alginate mixture was transferred into a 2-ml glass syringe, fitted with a steel 25-gauge needle produced by Staedtler Mars GmbH & Co. (Nuremberg, Germany), and mounted on an air-driven electrostatic microcapsule generator produced by Nisco Engineering Inc. (Oslo, Norway). The alginate solution was allowed to drip from a height of 25 mm into a gelling solution composed of sterile 120 mM calcium chloride, which was gently and continuously agitated using a magnetic stirrer. The air pressure and voltage settings were maintained at 3 pounds per square inch (psi) and 9 kV, respectively, to generate consistently circular microcapsules. The alginate microcapsules were polymerized into circular bead-like structures. Encapsulated islet was made by mixing alginate with Yorkshire pig (S&S Farms, Ramona, CA, USA) islets that were isolated in our laboratory, under University of California, Irvine-approved Institutional Animal Use and Care Committee (IACUC) protocol No. 2008–2823.

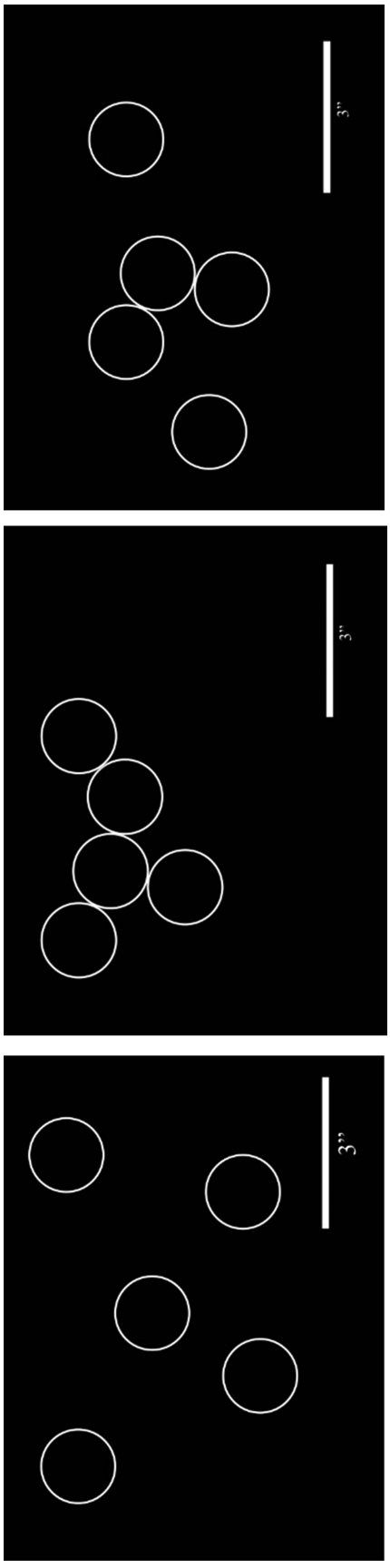

Figure 3. Sample images of generated circular outlines. (Left) Free circular outlines with outer diameter of 1.5 inches. (Middle) Circular outlines in complete contact with outer diameter of 1.5 inches. (Right) Circular outlines in incomplete contact with outer diameter of 1.5 inches. Scale bar: 3 inches.

Conventional characterization methodology involves the use of the ImageJ [National Institutes of Health (NIH), Bethesda, MD, USA] line tool to manually measure the diameter of microcapsules. The algorithm developed and evaluated in this study uses ImageJ custom coding language and built-in image analysis tools to count and measure the size distribution, shape, and morphology of microcapsule using images obtained with a simple microscope at bright-field and phase-contrast settings.

To determine the consistency and accuracy of the algorithm, circular outlines were created to mimic microscopy images of microcapsules (Fig. 2). Using a photo-editing software, three sets of 50 images were generated containing circular outlines of varying diameters (0.5, 1.0, 1.5, 2, 2.5, and 3.0 in.) (Fig. 3). To simulate microcapsules in solution, one set of images contain circular outlines that are completely separated from each other, while the other two sets of images contain circular outlines that are touching other microcapsules at varying degrees (Fig. 3). Each individual image contains 5 circular outlines, and a total of 50 microcapsules were generated for each sample, for a total of 900 circular outlines.

For quality control evaluation, microcapsule size, size distribution, and circularity were evaluated using the algorithm. Because the images obtained are two-dimensional, it does not allow us to calculate sphericity, which requires three-dimensional measurement. Conventional characterization of microcapsule size and circularity was performed using three independent observers (each making three nonconsecutive attempts at characterizing the microcapsule images), and the results obtained from these were tabulated on Microsoft Excel (MS Office v. 2010; Microsoft, Redmond, WA, USA) and were then compared against those calculated by ImageJ (NIH).

Statistical Analysis

All data are presented as mean±SEM. Statistical analysis was done using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) using a one-way analysis of variance (ANOVA), followed by post hoc Tukey's HSD test to determine significance of differences between experimental groups, with the level of significance set at $p<0.05$.

RESULTS

Evaluation of Microcapsule Size

Blank microcapsules (Fig. 4A) created with an electrostatic microcapsule generator (Fig. 4B) and polymerized in a 120 mM calcium chloride solution were analyzed using the conventional characterization processes and compared with an algorithm created for automated microcapsule analysis. Using the conventional method, three independent observers required 11.6±1.1 min (Fig. 5A) to measure the diameter of 50 microcapsules, producing results with significant variation: 393±1.06, 390±1.83, and 392±1.35 μm (Fig. 5B). When using the algorithm, the same results were obtained within 37±3 s and produced results with zero variation (391±1.19 μm). Characterization by the algorithm is faster compared to manual characterization (an 18-fold reduction in the required time).

We created circular outlines to mimic microscopy images of microcapsules and assessed the accuracy of the algorithm. We generated a total of 150 images containing circular outlines of varying diameters (0.5, 1.0, 1.5,

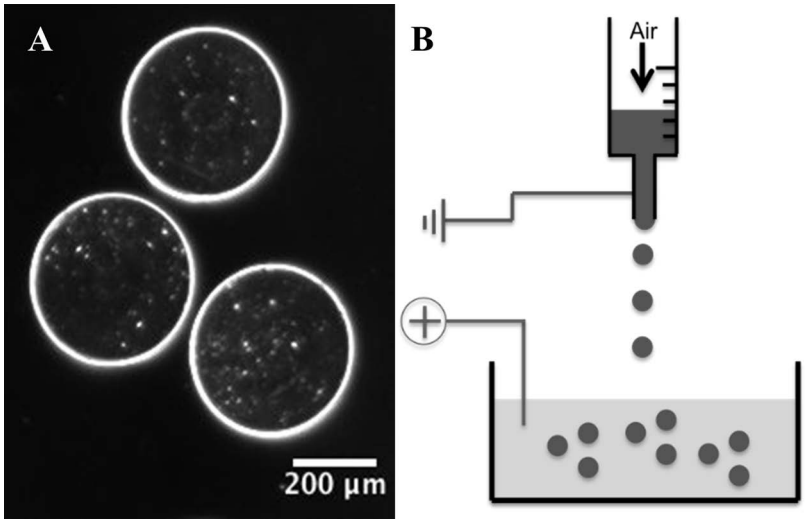

Figure 4. Empty microcapsules. Empty microcapsules (A) are created by an airflow, high-voltage electrostatic bead generator with 3% ultrapure low-viscosity mannuronate (UPLVM) alginate in a 120 mM calcium chloride solution (B). Image of capsules taken with phase-contrast microscopy to maximize algorithm efficiency.

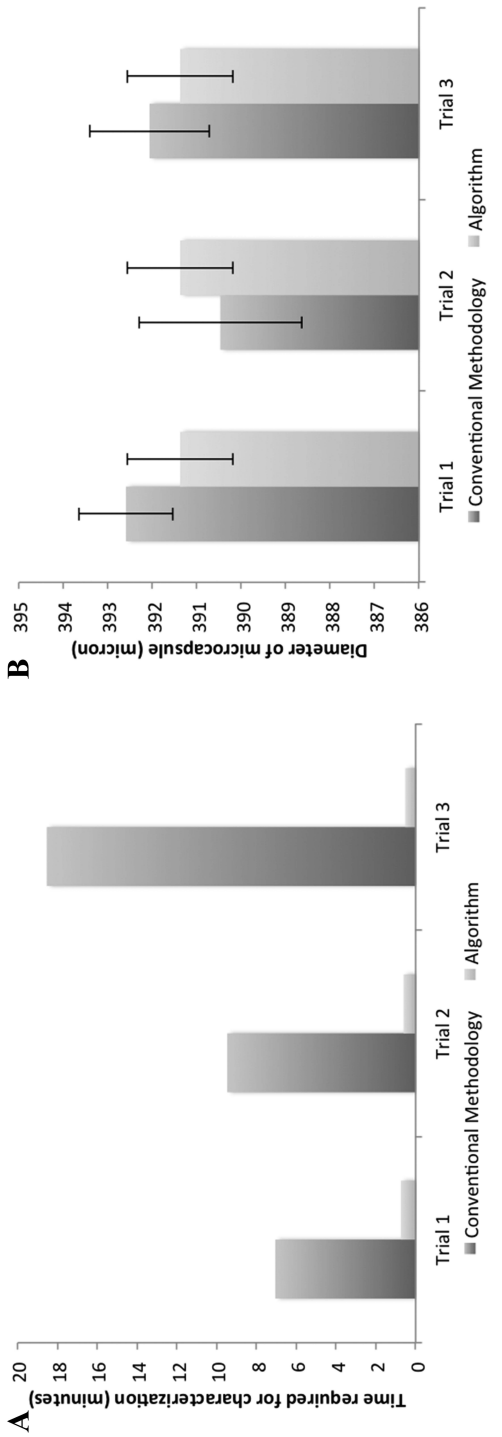

Figure 5. Empty microcapsules created with a 3% ultrapure low-viscosity mannuronate (UPLVM) alginate in a 120 mM calcium chloride solution are characterized manually with conventional methodology compared to characterization with an algorithm. The time required for manual characterization is 1,874% of the time required for the algorithm (A). Measurements of microcapsule diameter are obtained, and variations in results are recorded with manual characterization in different trials where there is no variation observed with automated characterization (B).

2, 2.5, and 3.0 in.) and analyzed them using the same algorithm. In our samples of free circular outlines (that do not touch each other), there was no significant difference between the measured size of the circular outline and the predetermined size ($p = 0.2$) of the circular outlines in all samples (Fig. 6). In our samples where there was complete contact between the circular outlines (Fig. 7A), we found that in samples of smaller diameters (500 μm and 1 mm), a difference of $0.6 \pm 0.1\%$ was observed between the measured and predetermined diameters ($p = 0.08$). At larger diameters, no significant differences were noted between the measured and predetermined diameters ($p = 0.1$). In samples where the circular outlines were only partially in contact (Fig. 7B), we found that in smaller samples (500 μm), a difference of $0.6 \pm 0.1\%$ was observed between the measured and predetermined diameters ($p = 0.1$), while in the remaining samples (>500 μm), no difference was noted ($p = 0.5$).

Evaluation of Microcapsule Size Distribution

Using the conventional method, three independent observers required an additional 5 ± 2 min (Fig. 5A) to evaluate the size distribution of 50 microcapsules (Fig. 5B). When using the algorithm, the same results were obtained along with microcapsule size measurements obtained as discussed earlier. No additional time was required to obtain this additional information. Evaluation of microcapsule size distribution by the algorithm was thus several-fold faster compared to manual characterization ($p < 0.01$).

Evaluation of Microcapsule Circularity

Using the conventional method, three independent observers required an additional 8 ± 1 min to evaluate the circularity of 50 microcapsules (Fig. 5B). When using the algorithm, the same results were obtained along with microcapsule size measurements obtained as discussed earlier. No additional time was required to obtain this additional information. Evaluation of microcapsule size distribution by the algorithm was thus several-fold faster compared to manual characterization ($p < 0.01$).

DISCUSSION

When conventional characterization techniques were compared to the algorithm developed in our laboratory, we found that the algorithm was orders of magnitude faster. The results generated using the algorithm were also consistent and did not demonstrate user-dependent variations, a drawback that was noted with conventional characterization, which depended on the skill and experience of the technician. This establishes the consistency and efficiency of the algorithm.

In addition to these advantages, the algorithm has also demonstrated to be highly user friendly and can be adapted to different microscope settings with minimal

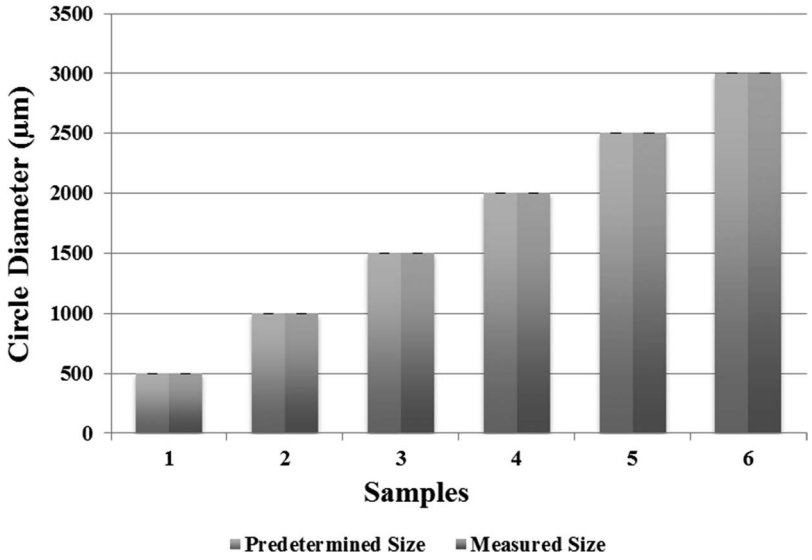

Figure 6. The circular outlines (free) characterized with an algorithm compared with the predetermined size of the circular outline. The difference between the measured size and predetermined size is 0% in all samples ($n = 50$ for all samples).

changes made to the original code. The use of this software removes observer bias that is often a problem with conventional characterization techniques, which often rely on measurements by technicians.

One of the main advantages of using an automated algorithm was to be able to scale up the number of microcapsules that can be imaged for quality control purposes. In our experience, a trained technician would require over an hour to measure, analyze, and generate a report on a minimum of 100 microcapsules. However, the sample size is so small that it is not representative of the microcapsule samples that are being evaluated, as hundreds of thousands of microcapsules are being evaluated using a miniscule sample of 100 microcapsules. Using this algorithm, we were able to evaluate up to 10,000 alginate microcapsules, complete the analysis, and generate a report within 5 min of imaging. In addition to size and circularity analysis, a size distribution histogram was also available with appropriate means of central tendency (mean, median, and mode) and dispersion (standard deviation) being included in the final report.

Conventional microencapsulation for islet transplantation results in the production of many blank microcapsules (those with no islets in them), which if transplanted would interfere with the ability of microencapsulated islets to obtain nutrients and secrete insulin (22). Alginate biocompatibility after transplantation is also largely determined by the morphology of microcapsules, specifically its geometry and smoothness (17). No current technology exists where the outcome of an encapsulation procedure (the morphological characteristics of the capsules), the efficiency of encapsulation (the percentage of islets that are encapsulated within alginate), and the quality of the final encapsulation product (the extent of "damaged" or undesirably misshapen microcapsules) can be rapidly evaluated. Therefore, efficient identification of blank microcapsule and characterization of their morphology are paramount in pretransplant analysis to ensure a successful transplantation. Our algorithm has been developed specifically to address all these issues while eliminating human error and expediting this tedious process.

Limitations

In our evaluation of the accuracy of the algorithm, we found that the algorithm provided an error-free analysis of free circular outlines. However, when samples of circular outlines with varying degrees of contact were evaluated, an error rate of 6 in 1,000 was observed. This error rate can be attributed to the overlapping of pixel data when the circular outlines are in direct contact, where the program failed to attribute the overlapping diameter distance to the appropriate circular outline, creating a small discrepancy between the measured diameter and the predetermined diameter. While efforts are being expended in minimizing and eliminating this error, an error rate of 0.6% is well within the acceptable limits and did not significantly impact our analysis of large numbers of microcapsules.

CONCLUSION

A comprehensive characterization of microencapsulated islets is crucial for quality control in research studies as well as in clinical islet transplantation in human recipients. Because typical islet transplant for humans requires an average of 500,000–1,000,000 islets per

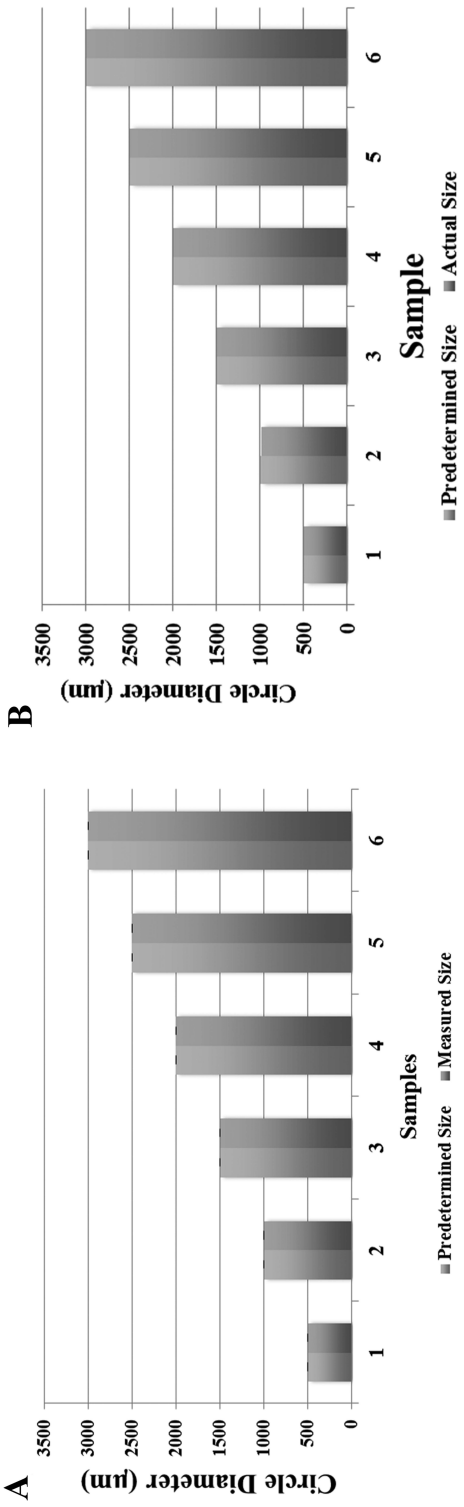

Figure 7. The circular outlines in complete contact (A) and incomplete contact (B) characterized with an algorithm compared with the predetermined size of the circular outline. (A) For circular outlines in complete contact, the difference between the measured size and the predetermined size for sample 1 and sample 2 is 0.6%, while for the remaining samples is 0%. (B) For circular outlines in incomplete contact, the difference between the measured size and the predetermined size for sample 1 is 0.6%, while for the remaining samples is 0.6% (n=50 for all samples).

infusion, conventional characterization techniques are not a feasible way to characterize encapsulated islets for statistically significant sample sizes. The need for a process of automation will not only eliminate human error and reduce analysis time but also accurately quantify data that were not possible before. Strict characterization of microencapsulated islets is necessary to develop guidelines and standards in microencapsulated islet transplantation. We have developed an algorithm that can analyze hundreds of microencapsulated islets and characterize their size, shape, circularity, and distortion. This innovation will find wide applicability in the fields of islet and stem cell encapsulation and other microencapsulation processes by eliminating human error and reducing analysis time.

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