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Rapid Communication

3D printed vascularized device for subcutaneous transplantation of human islets

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Keywords: 3D printing, Diabetes, Encapsulations, Islets, Transplantations

Abbreviations: FDM, Fused Deposition Method; **H&E**, hematoxylin and eosin; **IEQ**, Islet equivalent; **PLM**, platelet lysate matrix; **PLA**, polylactic acid; **SEM**, scanning electron microscopy; **VEGF**, vascular endothelial growth factor.

Abstract

Transplantation of pancreatic islets or stem cell derived insulin secreting cells is an attractive treatment strategy for diabetes. However, islet transplantation is associated with several challenges including function-loss associated with dispersion and limited vascularization as well as the need for continuous immunosuppression. To overcome these limitations, here we present a novel 3D printed and functionalized encapsulation system for subcutaneous engraftment of islets or islet like cells. The devices were 3D printed with polylactic acid and the surfaces treated and patterned to increase the hydrophilicity, cell attachment and proliferation. Surface treated encapsulation systems were implanted with growth factor enriched platelet gel, which helped to create a vascularized environment before loading human islets. The device protected the encapsulated islets from acute hypoxia and kept them functional. The adaptability of the encapsulation system was demonstrated by refilling some of the experimental groups transcutaneously with additional islets.

Introduction

Transplantation of pancreatic islets or stem cell derived insulin secreting cells is an attractive treatment strategy for diabetes[1,2]. However, islet transplantation is associated with several challenges including function-loss associated with dispersion and limited vascularization as well as the continuous need for immunosuppression[3]. In spite of early success with intrahepatic transplantation, widespread use of islet transplantation has been hampered by poor long-term survival of the graft[3,4]. After intra-hepatic or intravascular islet transplantation, graft function is lost rapidly due to dispersion of transplanted tissue, damage to graft caused by blood-mediated inflammatory reaction, and the hypoxic stress due to the limited ingrowth of new blood vessels[5]. High oxygen demand and the need for physiological architecture necessitate a highly vascularized and three-dimensional system for the long-term survival and function of transplanted islets. In addition, a minimally invasive and accessible site is fundamental for implantation, replenishment and graft retrieval. Retrievability is important when using engineered stem cells, whose long term fate and potential for tumor formation are not well known.[6] The subcutaneous space could serve as an ideal implantation site, if we can overcome challenges of low oxygen tension and poor vascularity and offer mechanical protection to transplanted cells[7,8]. To meet these needs, we designed an adaptable, scalable and refillable encapsulation system for subcutaneous transplantation of cells. In this work, we demonstrate the feasibility to 3D print an innovative encapsulation system and evaluate its vascularization after subcutaneous implantation. The system was loaded transcutaneously with human pancreatic islets and their long-term survival and function were studied in an immunocompromised mouse model.

Materials & Methods

Discoidal encapsulation devices (8 mm in diameter and 2.5 mm in thickness, Fig. 1a) suitable for holding up to 5,000 islets were printed adopting a Fused Deposition Method (FDM) based 3D printer (Replicator™ 2X, MakerBot Industries) and medical grade polylactic acid (PLA, Foster Corporation). The two inner surfaces were composed of an array of micro-reservoirs (300 μm x 300 μm) to house the transplanted islets individually, maintaining them in close proximity while avoiding clustering. These micro-reservoirs are connected to surrounding tissues by an array of square

microchannels (100 µm x 100 µm cross section, 50 µm length) to allow for the growth of transmembrane blood vessels in view of graft vascularization. The devices featured a loading port (1 mm diameter) for transcutaneous cell loading. Device surfaces were treated with Argon and Oxygen plasma (March plasma etcher, Nordson). The power (30W) and gas flow (150mTorr) were kept constant, while changing the exposure time (30, 90, 120 and 150 seconds). The nanopattering of the device surfaces was evaluated before and after treatment by scanning electron microscopy (SEM) (Nova NanoSEM, FEI) for channel quality and size (Fig. 1b-c). Hydrophilicity was evaluated by measuring the water contact angle and surface roughness was evaluated by atomic-force microscopy set in tapping mode (BioScope Catalyst, Bruker Instruments, Texas).[9]

The encapsulation systems were evaluated in nude mice (Nu/Nu, female, 8-10 week old), after receiving approval by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute. Surface treated and sterilized devices, loaded with platelet-lysate matrix (PLM) enriched with VEGF at two different concentrations (0.5 and 5 µg/ml), were implanted subcutaneously in the mice dorsum (n=12 per group). At 1, 2, and 4 weeks post implantation, 4 mice per group were euthanized and the graft explanted for the histological assessment of vascularization and innervation. The implant and surrounding tissues were harvested, and processed for histopathology evaluation of tissue response to the implanted device. CD31 antibody (Abcam, ab28364) and a pan-axonal antibody (Cambridge Bioscience, SMI-312R-100) were used to assess vascularization and innervation, respectively. A second experiment was performed in nude mice (n=5 per group) to evaluate insulin release from human islets transplanted into a prevascularized device. Human pancreatic islets (2,000 IEQ per mice) were injected with a 22 G needle transcutaneously into the encapsulation system 4 weeks after device implantation. Islets implanted under the kidney capsule served as positive control.[10] Human insulin (ultra-sensitive human insulin ELISA kit, Alpco) and blood glucose (OneTouch® Glucometer, Johnson and Johnson) levels were assessed weekly and body weight was monitored throughout the experiment. Intra peritoneal glucose tolerance test (IPGTT) was performed weekly to assess insulin secretion, in response to stimuli, from the transplanted islets. A subsequent test was performed on the same animal cohort to demonstrate the refillability of the implant. Twelve

weeks after the first injection, additional human islets (2,000 IEQ per mice) were injected trancutaneously in all groups where the insulin production was lower than 0.125 μIU/ml.

All data are represented as average and standard error of the mean (SEM) and statistical analysis performed using Student's paired t-test. A value of $p<0.05$ was considered statistically significant. GraphPad Software, Inc. was used for the analysis.

Results & Discussion

Transplantation of islets on porous biomaterials has emerged as a promising strategy for long-term islet function facilitating rapid tissue ingrowth, vascularization and innervation providing oxygen, nutrition, and waste removal[11]. Recognizing such needs for an islet and cell encapsulation system, we are working on new strategies to deliver cells subcutaneously[9,12]. The architecture of the proposed device is designed to maintain pancreatic islets close to blood vessels in a growth factor enriched environment, but separated from each other to mimic the physiological architecture in the pancreas and avoid cell crowding.

To increase the biointegration of the encapsulation system, we decided to use PLA, which is a widely adopted polymer in biomedical devices, biocompatible, and presents good elasticity and mechanical strength suitable for subcutaneous implantation[13–15]. Due to the chiral nature of lactic acid, PLA is hydrophobic with low cell adhesion properties. Surface treatment with plasma improves the low surface free energy of different materials and offers a solvent-free technique capable of changing the wettability, surface roughness and surface chemistry of polymers, enhancing cell proliferation and viability[16–18]. Plasma activation also increases PLA's surface free energy forming a broad variety of functional groups on the surface, including polar groups, which drastically change wettability and have a positive effect on material–cell interactions. Previous work from our group demonstrated that plasma treatment substantially increased the hydrophilicity of the surface and reduced the contact angle, which remained stable over 30 days in phosphate buffered saline (PBS)[9]. Here we looked at the effect of plasma exposure time on surface patterning and roughness and compared Oxygen and Argon treatments. As shown in Fig. 1d, both treatments increased surface roughness, reaching a maximum value, after which the roughness diminished with continued exposure, possibly due to eventual

etching of the crystalline regions. It was also noticed that the Oxygen treatment cause deeper patterns (4.63 nm with Argon and 26.45 nm with Oxygen, $p<0.01$, Fig. 1e), which has been demonstrated to be beneficial for cell attachment and proliferation[19].

We then investigated the in vivo vascularization and innervation of Oxygen treated devices after subcutaneous implantation in nude mice. We used nude mice as they show an inflammatory response to a foreign body, but allow for transplantation with human islets without the need for immunosuppression. It has been broadly demonstrated that vascularization of the graft is the key for successful engraftment of islet transplants[7,20]. Prevascularization could mitigate the issue of acute hypoxia which was shown to result in islet apoptosis and graft failure[3]. To stimulate neovascularization and to support the islet viability and function for a period of time after transplantation we used devices loaded with biological gels with different VEGF concentrations[21–23]. An acute inflammatory response to the foreign body was present in the first week after device implantation, mainly in the VEGF groups, and subsided with time, leaving a rim of vascularized connective tissue around the device at week 4 (Fig. 2). Indeed, the neutrophils, which are considered of impact for the development of the inflammatory response which leads to the formation of the fibrotic capsule[24], were almost absent from the second week (Fig. 2 a-i). The red arrows in Fig. 2 indicate the protrusion of the surrounding subcutaneous tissue into the devices. Tissue samples were taken from the side of the device closer to the skin, representing the subcutaneous environment. We noticed a positive trend between VEGF concentration and the number of vessels stained by CD31 (Fig. 2j-m). However, after 4 weeks, we noticed calcification in the VEGF 5 µg/ml sample (Fig. 2i) as evident by the dark spots in high density close to the tissue-implant interface. It was observed that in 4 weeks the subcutaneous tissue and the inside of the device were vascularized (Fig. n-o). Based on these results, 0.5 µg/ml VEGF was selected for further studies. Finally, we also found nerve bundles in the proximity of the device (Figure 2p-q), indicating their potential to reach the transplanted islets. Further studies are necessary to prove and quantify islet innervation. After proving the device vascularization, we performed a second experiment injecting human islet into a prevascularized device. We observed detectable levels of human insulin from week 4, but at lower levels compared to the positive control ($p<0.001$). As described in various islet encapsulation models, there is a lag time

for adequate function until the transplanted islets are vascularized[7,25]. The prevascularization of the device appeared to have helped encapsulated islets to overcome the initial post implant injury, but it takes a few weeks for the transplanted cells to develop mature vasculature and be functional.

A second load of islets (2,000 IEQ) into vascularized devices increased the insulin levels (~10 μIU/ml) to values that were comparable to the kidney capsule transplantation ($p>0.05$). Additionally, the devices were well tolerated and animals showed comparable basal glucose level and weight, demonstrating that the presence of additional islets is not associated with hypoglycemia.

Conclusion

In this study, we showed that the subcutaneous implantation of a 3D printed and functionalized cell encapsulation system generates adequate and prompt vascularization of the graft. Vascularization was enhanced by the ability to dispense pro-angiogenic factors, such as VEGF, which is also known to increase islet viability and function[22]. In addition, the device could protect the graft, while the islets are being vascularized, from initial transplant site stressors and support their long-term survival. The transcutaneous refillability of the device offers opportunities for cell supplementation, without surgical retrieval and re-implantation, to accommodate changing physiological needs. This will be of significant advantage in the case of the growing children with diabetes. Moreover, the reservoir structure permits the potential retrievability of the graft, which is important for stem cell derived engineered cells, undergoing malignant or other unwanted transformations. Though the current studies were done in immunodeficient animal models, the device can be incorporated with local delivery of immunomodulators, which will expand its evaluation in immunocompetent diabetic animals[26,27]. Further studies in diabetic animal models are required to prove the efficacy of this versatile encapsulation system for diabetes cell therapy.

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Conflict of interest

Authors declare that they have competing interests as the encapsulation system described in this paper is currently under invention disclosure.

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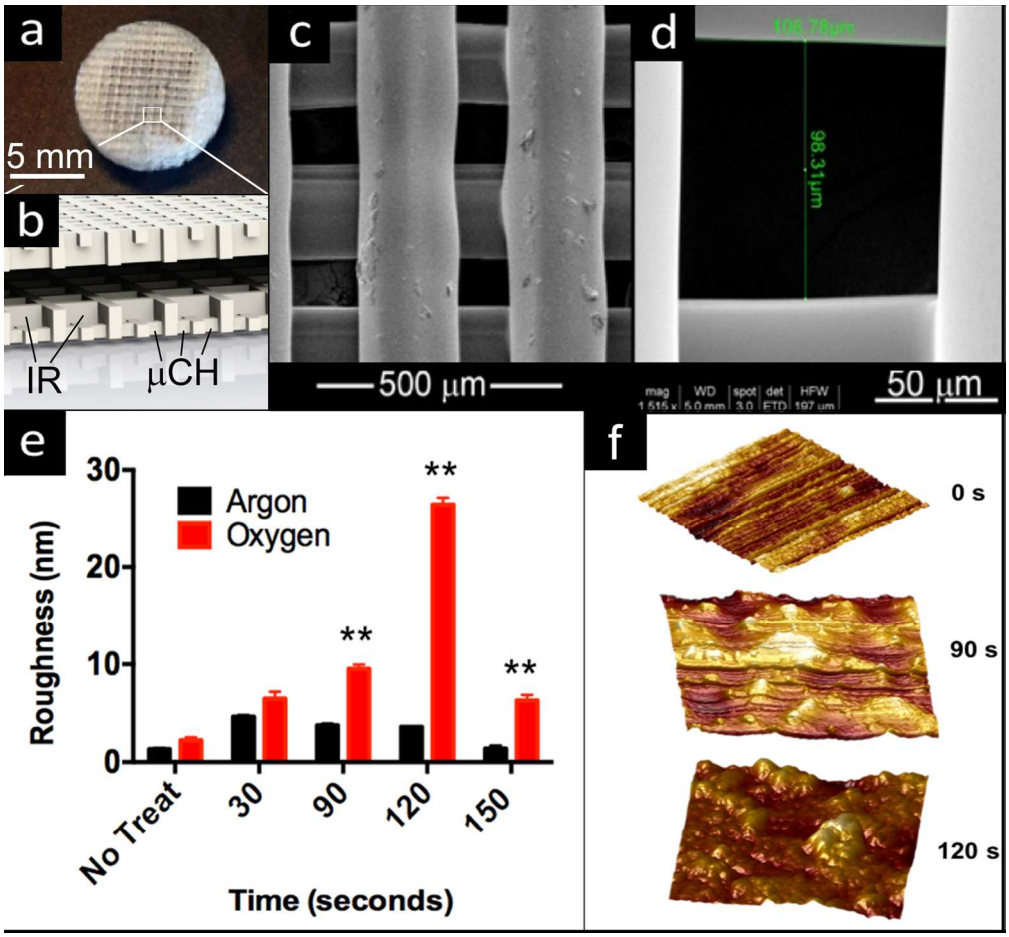
Figure 1. Mouse prototype of the 3D-printed PLA encapsulation system (a) with magnification under SEM (b, c). PLA superficial roughness measurements after Argon and Oxygen plasma treatments, changing time of application (0, 30, 90, 120, and 150 seconds) (d). AFM pictures of PLA surface treated with Oxygen plasma after 0, 90 and 120 seconds (e). Average and Standard Error of the Mean are represented. ** $p<0.01$.

Figure 2. H&E of the tissue surrounding the device at 4x. Tissue response at the device-subcutaneous tissue interface is shown (red arrow, a-i). Different concentrations of VEGF were tested and device retrieved after 1, 2, and 4 weeks from the implantation. CD31 staining of tissue (j-l). Scale bar is 50 μm. CD31 count for each group (m). Optical microscope visualization of the tissue collected from the device reservoir, showing the presence of mature vessels (n-o). Neurofilament staining (SMI312-R, in green) indicates the proximity of subcutaneous nerve bundles to the encapsulation system (p-q).

Figure 3. Insulin release from kidney capsule (black) and encapsulation system (red) after glucose stimulation (a). On week 12 all the animal with insulin level below 0.125 uU/ml where treated by refilling the implant with additional 2000 IEQ (red arrow). Basal blood glucose levels (b) and body weight (c). Average and Standard Error of the Mean are represented. * $p<0.05$; *** $p<0.001$.



Mouse prototype of the 3D-printed PLA encapsulation device (a) and rendering of the structure (b) that includes insulin reservoirs (IR) and microchannels (µCH). Magnification of the microchannels under SEM (c and d). PLA superficial roughness measurements after Argon and Oxygen plasma treatments, changing time of application (0, 30, 90, 120, and 150 seconds) (e). AFM pictures of PLA surface treated with Oxygen plasma after 0, 90 and 120 seconds (f). Average and SEM are represented. ** $p<0.01$.

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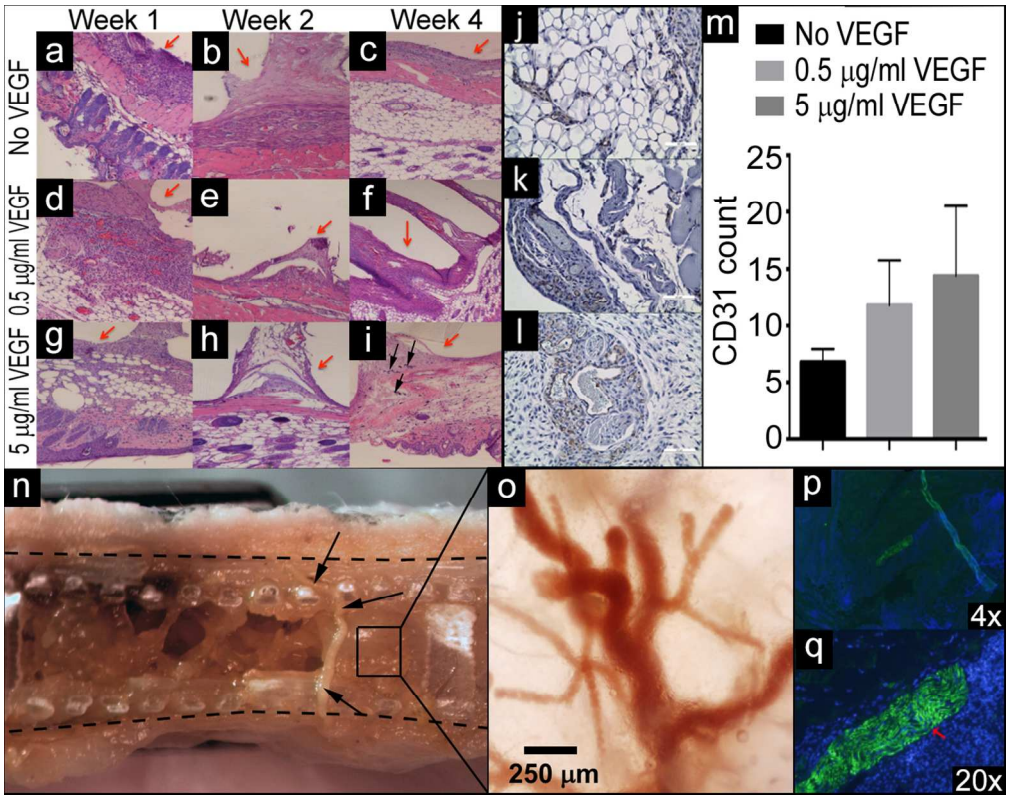

H&E of the tissue surrounding the device at 4x. Tissue response at the device-subcutaneous tissue interface is shown (red arrow, a-i). Different concentrations of VEGF were tested and device retrieved after 1, 2, and 4 weeks from the implantation. Diffuse spots of calcification (black arrows) were present at week 4 with the highest VEGF concentration (i). CD31 staining of tissue (j-l). Scale bar is 50 µm. Average and SEM of the CD31 count for high power field (m). Representative optical microscope visualization of the tissue collected from the device reservoir (VEGF 0.5 µg/ml group), showing the presence of mature vessels (n-o) Dotted lines represent device-subcutaneous tissue interface and the black arrows follow the path of a vessel through the polymeric scaffold. Neurofilament staining (SMI312-R, in green) indicates the proximity of subcutaneous nerve bundles to the encapsulation device (p-q).

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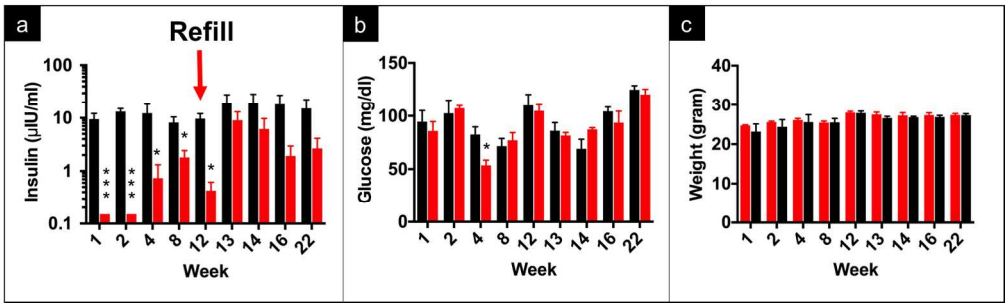

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318x95mm (150 x 150 DPI)

3D printed vascularized device for subcutaneous transplantation of human islets

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Keywords: 3D printing, Diabetes, Encapsulation, Islets, Transplantation

Abbreviations: FDM, Fused Deposition Method; **H&E**, hematoxylin and eosin; **IEQ**, Islet equivalent; **PLM**, platelet lysate matrix; **PLA**, polylactic acid; **SEM**, scanning electron microscopy; **VEGF**, vascular endothelial growth factor.

Abstract

Transplantation of pancreatic islets or stem cell derived insulin secreting cells is an attractive treatment strategy for diabetes. However, islet transplantation is associated with several challenges including function-loss associated with dispersion and limited vascularization as well as the need for continuous immunosuppression. To overcome these limitations, here we present a novel 3D printed and functionalized encapsulation device for subcutaneous engraftment of islets or islet like cells. The devices were 3D printed with polylactic acid and the surfaces treated and patterned to increase the hydrophilicity, cell attachment and proliferation. Surface treated encapsulation systems were implanted with growth factor enriched platelet gel, which helped to create a vascularized environment before loading human islets. The device protected the encapsulated islets from acute hypoxia and kept them functional. The adaptability of the encapsulation system was demonstrated by refilling some of the experimental groups transcutaneously with additional islets.

Introduction

Transplantation of pancreatic islets or stem cell derived insulin secreting cells is an attractive treatment strategy for diabetes[1,2]. However, islet transplantation is associated with several challenges including function-loss associated with dispersion and limited vascularization as well as the continuous need for immunosuppression[3]. In spite of early success with intrahepatic transplantation, widespread use of islet transplantation has been hampered by poor long-term survival of the graft[3,4]. After intra-hepatic or intravascular islet transplantation, graft function is lost rapidly due to dispersion of transplanted tissue, damage to graft caused by blood-mediated inflammatory reaction, and the hypoxic stress due to the limited ingrowth of new blood vessels[5]. High oxygen demand and the need for physiological architecture necessitate a highly vascularized and three-dimensional system for the long-term survival and function of transplanted islets. In addition, a minimally invasive and accessible site is fundamental for implantation, replenishment and graft retrieval. Retrievability is important when using engineered stem cells, whose long term fate and potential for tumor formation are not well known.[6] The subcutaneous space could serve as an ideal implantation site, if we can overcome challenges of low oxygen tension and poor vascularity and offer mechanical protection to transplanted cells[7,8]. To meet these needs, we designed an adaptable, scalable and refillable encapsulation system for subcutaneous transplantation of cells. In this work, we demonstrate the feasibility to 3D print an innovative encapsulation system and evaluate its vascularization after subcutaneous implantation. The system was loaded transcutaneously with human pancreatic islets and their long-term survival and function were studied in an immunocompromised mouse model.

Materials & Methods

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microchannels (100 µm x 100 µm cross section, 50 µm length) to allow for the growth of transmembrane blood vessels in view of graft vascularization. A rendering of this microstructure was designed with a 3D CAD software (SolidWorks®) and is represented in Figure 1b. The devices featured a loading port (1 mm diameter) for transcutaneous cell loading. Device surfaces were treated with Argon and Oxygen plasma (March plasma etcher, Nordson). The power (30W) and gas flow (150mTorr) were kept constant, while changing the exposure time (30, 90, 120 and 150 seconds). The structure of the device was evaluated at different magnification by scanning electron microscopy (SEM) (Nova NanoSEM, FEI) for channel quality and size (Fig. 1c-d). Hydrophilicity was evaluated by measuring the water contact angle, as described previously[9] and surface nanopattering and roughness were evaluated by atomic-force microscopy set in tapping mode (Fig. 1e-f, BioScope Catalyst, Bruker Instruments, Texas).

The encapsulation devices were evaluated in nude mice (Nu/Nu, female, 8-10 week old), after receiving approval by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute. The devices were sterilized with 70% ethanol and UV before performing all the surface modification treatment with oxygen or argon plasma in a sterile environment (clean room of the Houston Methodist Research Institute). Thereafter, devices were filled with a platelet-lysate solution (ZenBio), under aseptic conditions and incubated overnight at 37 °C in a cell incubator to form the gel, with the addition of bovine thrombin (BioPharm Laboratories, LLC). The ready to be implanted devices were then packaged and transferred to the animal facility and the package opened in a sterile environment just before the implantation. Surface treated and sterilized devices, loaded with platelet-lysate matrix (PLM) enriched with VEGF at two different concentrations (0.5 and 5 µg/ml), were implanted subcutaneously in the mice dorsum (n=12 per group). At 1, 2, and 4 weeks post implantation, 4 mice per group were euthanized and the graft explanted for histological assessment of vascularization and innervation. The implant and surrounding tissues were harvested, and processed for histopathology evaluation of tissue response to the implanted device with hematoxylin and eosin (H&E). This staining was used to analyse tissue morphology and to evaluate the presence of immune cells, vessels infiltration, and fibrotic response at the tissue-device interface. CD31 antibody (Abcam, ab28364) was used for immunohistochemistry (IHC) analysis and a pan-

axonal antibody (Cambridge Bioscience, SMI-312R-100) with immunofluorescence (IF) to assess device vascularization and innervation respectively. CD31 expressing cells were counted separately in 10 high-power fields. (HPFs).

A second experiment was performed in nude mice (n=5 per group) to evaluate insulin release from human islets transplanted into a prevascularized device. Human pancreatic islets (2,000 IEQ per mice) were injected subcutaneously with a 22 G needle into the encapsulation system 4 weeks after device implantation or were inserted under the kidney capsule (positive control).[10] Human insulin (ultra-sensitive human insulin ELISA kit, Alpco) and blood glucose (OneTouch® Glucometer, Johnson and Johnson) levels were assessed weekly and body weight was monitored throughout the experiment. Intra peritoneal glucose tolerance test (IPGTT) was performed weekly to stimulate insulin secretion from the transplanted islets. Blood was collected using retro-orbital method, under topical anaesthesia and aseptic techniques. A subsequent test was performed on the same animal cohort to demonstrate the subcutaneous refillability of the implant. Twelve weeks after the first injection, additional human islets (2,000 IEQ per mice) were injected trancutaneously in all groups where the insulin production was lower than 0.125 μIU/ml.

Statistical analysis was performed using Student's two tailed paired t-test. Results are presented as mean and standard error of the mean (SEM). A value of $p<0.05$ was considered statistically significant. GraphPad Software, Inc. was used for the analysis.

Results & Discussion

Transplantation of cells on porous biomaterials has emerged as a promising strategy for long-term function facilitating rapid tissue ingrowth, vascularization and innervation providing oxygen, nutrition, and waste removal[11]. Recognizing such needs for an islet and cell encapsulation system, we are working on new strategies to deliver cells subcutaneously[9,12]. The architecture of the proposed device is designed to maintain pancreatic islets close to blood vessels, but separated from each other to mimic the physiological architecture in the pancreas and avoid cell crowding.

To increase the biointegration of the encapsulation system, we decided to use PLA, which is a widely adopted polymer in biomedical devices, biocompatible, and presents good elasticity and

mechanical strength suitable for subcutaneous implantation[13–15]. Due to the chiral nature of lactic acid, PLA is hydrophobic with low cell adhesion properties. Surface treatment with plasma improves the low surface free energy of different materials and offers a solvent-free technique capable of changing the wettability, surface roughness and surface chemistry of polymers, enhancing cell proliferation and viability[16–18]. Plasma activation also increases PLA's surface free energy forming a broad variety of functional groups on the surface, including polar groups, which drastically change wettability and have a positive effect on material–cell interactions. Previous work from our group demonstrated that plasma treatment substantially increased the hydrophilicity of the surface and reduced the contact angle, which remained stable over 30 days in phosphate buffered saline (PBS)[9]. Here we looked at the effect of plasma exposure time on surface patterning and roughness and compared Oxygen and Argon treatments. Since UV light sterilization methods showed to increase polymer wettability without affecting morphology and mechanical properties, we decide to evaluate the effect of the surface modification only at the end of plasma treatment[19]. As shown in Fig. 1e, both treatments increased surface roughness, reaching a maximum value, after which the roughness diminished with continued exposure, possibly due to eventual etching of the crystalline regions. It was also noticed that the Oxygen treatment cause deeper patterns (4.63 nm with Argon and 26.45 nm with Oxygen, $p<0.01$, Fig. 1f), which has been demonstrated to be beneficial for cell attachment and proliferation. For this reason, a 120 seconds Oxygen treatment was selected for the following experiments.

We then investigated the in vivo vascularization and innervation of Oxygen treated devices after subcutaneous implantation in nude mice. Nude mice were selected for these studies with the novel encapsulation device as they show an inflammatory response to a foreign body, but allow for transplantation with human islets without the need for immunosuppression. It has been broadly demonstrated that vascularization of the graft is the key for successful engraftment of islet transplants[7,20]. Prevascularization could mitigate the issue of acute hypoxia which was shown to result in islet apoptosis and graft failure[3]. To stimulate neovascularization and to support the islet viability and function for a period of time after transplantation we tested devices loaded with biological gels with different VEGF concentrations[21–23]. An acute inflammatory response to the foreign body was present in the first week after device implantation, mainly in the VEGF groups, and subsided with time,

leaving a rim of vascularized connective tissue around the device at week 4 (Fig. 2). Indeed, the neutrophils, which are considered to have an impact on the development of the inflammatory response leading to the formation of the fibrotic capsule[24], were almost absent from the second week (Fig. 2 a-i). The red arrows in Fig. 2 indicate the protrusion of the surrounding subcutaneous tissue into the devices. Tissue samples were taken from the side of the device closer to the skin, representing the subcutaneous environment. We noticed a positive trend between VEGF concentration and the number of vessels stained by CD31 (Fig. 2j-m), but there was no statistically significant difference between the groups after 1 week, probably for the limited number of animals per group. After 4 weeks, we noticed calcification in the VEGF 5 µg/ml sample as evident by the dark spots in high density close to the tissue-implant interface (Fig. 2i, black arrow). It has been already shown that VEGF is involved in the calcification process of cartilage[25] and at similar concentration it was tested to induce bone response to implanted craniofacial implants[26]. It was observed that in 4 weeks the subcutaneous tissue and the inside of the device were also vascularized with lower concentration of VEGF (Fig. 2n-o). Based on these results, 0.5 µg/ml VEGF was selected for further studies.

Finally, we also found nerve bundles in the proximity of the device (Fig. 2p-q). These may have reached the device and the transplanted islets. However, further long term studies are necessary to prove and quantify islet innervation, considering the slower rate of growth of nerves compared to vessels.

After proving the device vascularization, we performed a second experiment injecting human islet into a prevascularized device previously loaded with the VEGF rich matrix. By the time of the islet injection the gel was already degraded, releasing growth factors (Fig. 2n). We observed detectable levels of human insulin from week 4, but at lower levels compared to the positive control ($p<0.001$). As described in various islet encapsulation models, there is a lag time for adequate function until the transplanted islets are vascularized[7,27]. The prevascularization of the device appeared to have helped encapsulated islets to overcome the initial post implant injury, but it takes longer for the transplanted cells to develop mature vasculature and be functional. The lower insulin level detected after IPGTT in the device group as compared to the kidney capsule control could be ascribed to a non-complete vascularization of islets in the device. Although prevascularized, it is expected that islets will need to

fully connect to the vasculature post transplantation. The duration of this process is compatible with the 2 weeks timeframe observed in Fig.3 for an increase in insulin secretion in the device group. As the platelet lysate matrix is expected to be resorbed within few days from device implantation, we do not expect that islets were affected in their function by residual PLM. A second load of islets (2,000 IEQ) into vascularized devices increased the insulin levels (~10 µIU/ml) to values that were comparable to the kidney capsule transplantation (Fig. 3a, $p>0.05$). The total number of islets loaded was 4,000 IEQ per device, less than the theoretical maximal loadable volume according to the three-dimensional structure of device and the presence of a vascularized environment. Nevertheless, it was not possible to estimate exact volume of vessels and tissue present in the device at the time of the second islet loading, and this could have an effect on the distribution and the packing density of the islets in the device. Further studies are required to understand the optimal timing and the amount of islets needed to obtain a valid insulin secretion after multiple loading of islets. Achieving this could help adapting the insulin output to patient physiology and clinical need. Additionally, the devices were well tolerated and animals showed comparable basal glucose level (Fig. 3b) and weight (Fig. 3c), demonstrating that the presence of additional islets is not associated with hypoglycemia.

Conclusion

In this study, we showed that the subcutaneous implantation of a 3D printed and functionalized cell encapsulation device generates adequate and prompt vascularization of the graft. Vascularization was enhanced by the ability to dispense pro-angiogenic factors, such as VEGF, which is also known to increase islet viability and function[22]. In addition, the device could protect the graft, while the islets are being vascularized, from initial transplant site stressors and support their long-term survival. The transcutaneous refillability of the device offers opportunities for cell supplementation, without surgical retrieval and re-implantation, to accommodate for changing physiological needs. This will be of significant advantage in the case of the growing children with diabetes. Moreover, the reservoir structure permits the potential retrievability of the graft, which is important for stem cell derived engineered cells, undergoing malignant or other unwanted transformations. A current limitation of this device is the lack of immunoprotection of the transplanted cells. The priority of this work was to

prove the vascularization and function of the device without compounding with the risk of immune rejection. For this reason, we used an immunodeficient animal model. A different device design can incorporate a strategy for local delivery of immunomodulators, which will expand its use in immunocompetent diabetic animals[28,29]. Further studies in diabetic animal models are required to evaluate the full potential of this versatile encapsulation device for diabetes cell therapy.

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Conflict of interest

Authors declare that they have competing interests as the encapsulation system described in this paper is currently under invention disclosure.

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Figure 1. Mouse prototype of the 3D-printed PLA encapsulation device (a) and rendering of the structure (b) that includes insulin reservoirs (IR) and microchannels (μCH). Magnification of the microchannels under SEM (c and d). PLA superficial roughness measurements after Argon and Oxygen plasma treatments, changing time of application (0, 30, 90, 120, and 150 seconds) (e). AFM pictures of PLA surface treated with Oxygen plasma after 0, 90 and 120 seconds (f). Average and SEM are represented. ** $p<0.01$.

Figure 2. H&E of the tissue surrounding the device at 4x. Tissue response at the device-subcutaneous tissue interface is shown (red arrow, a-i). Different concentrations of VEGF were tested and device retrieved after 1, 2, and 4 weeks from the implantation. Diffuse spots of calcification (black arrows) were present at week 4 with the highest VEGF concentration (i). CD31 staining of tissue (j-l). Scale bar is 50 μm. Average and SEM of the CD31 count for high power field (m). Representative optical microscope visualization of the tissue collected from the device reservoir (VEGF 0.5 μg/ml group), showing the presence of mature vessels (n-o) Dotted lines represent device-subcutaneous tissue interface and the black arrows follow the path of a vessel through the polymeric scaffold. Neurofilament staining (SMI312-R, in green) indicates the proximity of subcutaneous nerve bundles to the encapsulation device (p-q).

Figure 3. Insulin release from kidney capsule (black) and encapsulation device (red) after glucose stimulation (a). On week 12 all the animal with insulin level below 0.125 uU/ml where treated by refilling the implant with additional 2000 IEQ (red arrow). Basal blood glucose levels (b) and body weight (c). Average and SEM are represented. * $p<0.05$; *** $p<0.001$.