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Islet encapsulation with polyphenol coatings decreases pro-inflammatory chemokine synthesis and T cell trafficking

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Abstract

Type 1 Diabetes (T1D) is a chronic pro-inflammatory autoimmune disease consisting of islet-infiltrating leukocytes involved in pancreatic β -cell lysis. One promising treatment for T1D is islet transplantation; however, clinical application is constrained due to limited islet availability, adverse effects of immunosuppressants, and declining graft survival. Islet encapsulation may provide an immunoprotective barrier to preserve islet function and prevent immune-mediated rejection after transplantation. We previously demonstrated that a novel cytoprotective nanothin multilayer coating for islet encapsulation consisting of tannic acid (TA), an immunomodulatory antioxidant, and poly(N-vinylpyrrolidone) (PVPON), was efficacious in dampening *in vitro* immune responses involved in transplant rejection and preserving *in vitro* islet function. However, the ability of (PVPON/TA) to maintain islet function *in vivo* and reverse diabetes has not been tested. Recent evidence has demonstrated that modulation of redox status can affect pro-inflammatory immune responses. Therefore, we hypothesized that transplanted (PVPON/TA)-

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encapsulated islets can restore euglycemia to diabetic mice and provide an immunoprotective barrier. Our results demonstrate that (PVPON/TA) nanothin coatings can significantly decrease *in vitro* chemokine synthesis and diabetogenic T cell migration. Importantly, (PVPON/TA)-encapsulated islets restored euglycemia after transplantation into diabetic mice. Our results demonstrate that (PVPON/TA)-encapsulated islets may suppress immune responses and enhance islet allograft acceptance in patients with T1D.

Keywords

Reactive oxygen species; islet transplantation; chemokines; macrophage; Type 1 diabetes; and antioxidant

INTRODUCTION

Type 1 Diabetes (T1D) is an autoimmune disease characterized by the targeted lysis of insulin-producing pancreatic β -cells, in which patients are unable to maintain euglycemia without daily insulin injections or an insulin pump. Even the most meticulous methods of providing exogenous insulin can allow wide fluctuations in blood glucose that significantly alter metabolism and contribute to life threatening diabetic complications including cardiovascular disease, nephropathy, and retinopathy [1]. A viable alternative to exogenous insulin injection is pancreatic islet transplantation, a process that requires isolating islets from human cadaveric or porcine donors [2, 3]. The main advantage of islet transplantation is that the pancreatic β -cell is finely tuned to properly regulate blood glucose levels, and episodes of hyperglycemia (elevated blood glucose) and hypoglycemia (low blood glucose) are less frequent compared to exogenous insulin injection. Unfortunately, numerous challenges still exist with islet transplantation including islet viability, efficient engraftment, islet function, and preventing immune recognition of transplanted islet allo- or xenografts [4]. T1D patients require immunosuppressants to protect donor islets from rejection, but these immunotherapies can be toxic, decrease islet function, and increase the susceptibility to life threatening microbial infections [5].

One promising method to circumvent immune recognition after islet transplantation is to encapsulate islets in an immunoprotective coating that will prevent immune-mediated pancreatic islet destruction, but still afford islets the ability to maintain euglycemia. We previously demonstrated that islet encapsulation with a layer-by-layer conformal coating of tannic acid (TA), a natural polyphenol with antioxidant activity, hydrogen-bonded with the non-ionic poly(N-vinylpyrrolidone) (PVPON) polymer, was non-toxic and maintained *in vitro* islet function [6, 7]. TA is an antioxidant that scavenges free radicals, inhibits free radical-induced oxidation, and can elicit immunomodulation [6]. Importantly, TA is also involved in the assembly of multilayer films, capsules, and coatings of biomedical relevance [8–16]. PVPON is biocompatible and has been used in drug delivery [17]. Similar to poly(ethylene glycol), PVPON has been shown to prevent protein absorption on the surfaces due to its hydrophilic nature [18]. In addition to dissipating reactive oxygen species (ROS) synthesis involved in pathological islet cell destruction, (PVPON/TA) can also influence the activation of redox-dependent signaling pathways that contribute to pro-inflammatory

cytokine synthesis [19, 20]. More importantly, we showed that (PVPON/TA) multilayer coatings attenuated the synthesis of innate immune-derived pro-inflammatory cytokines and adaptive immune T cell effector responses involved in autoimmunity and islet rejection *in vitro* [6, 7]. As a natural antioxidant, TA assembled with PVPON may afford additional protection to insulin-secreting β -cells during oxidative stress since β -cells display an inherent decrease in antioxidant protection [21–24]. Similarly, dissipating local concentrations of ROS may also prevent the maturation of effector T cell responses involved in islet graft rejection including T cell activation markers and IFN- γ , a pro-inflammatory Th1 cytokine [25–29]. Therefore, studies to further define the role of (PVPON/TA) multilayer biomaterial on immune modulation and protection for pancreatic islet transplantation are highly warranted.

We recently demonstrated the importance of NADPH oxidase (NOX)-derived superoxide to promote a pro-inflammatory M1 macrophage phenotype involved in autoimmune destruction of pancreatic β -cells in T1D [30]. Our results and others [30, 31], provide evidence that oxidative stress during spontaneous autoimmune diabetes can influence the differentiation of classically-activated pro-inflammatory M1 macrophages and promote the synthesis of pancreatic β -cell damaging cytokines such as TNF- α , IL-1 β , IL-12p70, Type I interferons, and cell surface co-stimulatory molecules such as CD40, CD80, and CD86. Macrophages can facilitate the recruitment of other immune cells to sites of inflammation by the secretion of chemokines [32]. These secreted proteins play an integral role in the pathogenesis of T1D and islet transplant rejection by promoting cellular chemotaxis and immune cell migration to sites of newly transplanted islets and enhancing pancreatic β -cell necrosis. Many chemokines are associated with T1D pathogenesis, but one widely known β -cell destructive chemokine is CXCL10, which has been identified as a dominant chemokine involved in murine and human T1D [33]. The chemokine CCL5, also known as RANTES, plays a key role in T cell proliferation and recruitment of T cells in patients with T1D [34].

Despite the immunotherapeutic potential of hydrogen-bonded (PVPON/TA) multilayers for encapsulated islet transplants, little is known of the effects on chemokine production and pro-inflammatory macrophage differentiation in the presence of (PVPON/TA) multilayers. In the current study, we further demonstrate the ability of (PVPON/TA) multilayer capsules to decrease pro-inflammatory M1 macrophage responses and chemokine synthesis involved in leukocyte recruitment to potentially mitigate islet graft rejection. In addition, (PVPON/TA) multilayer encapsulation does not compromise *in vivo* islet function as demonstrated by the restoration of euglycemia following transplantation into immunodeficient diabetic mice. Our results demonstrate that the antioxidant and immunomodulatory properties of (PVPON/TA) nanothin multilayer coatings can provide an immunoprotective shield on encapsulated islets to reduce diabetogenic T cell responses, and potentially protect encapsulated islets from transplant rejection.

MATERIALS AND METHODS

Materials

Poly(N-vinylpyrrolidone) (PVPON), (average M_w 1 300 000 g mol⁻¹), tannic acid (TA), (M_w 1700 g mol⁻¹), poly(methacrylic acid) (PMAA) (average M_w 21000 g mol⁻¹), and

mono- and dibasic sodium phosphate were purchased from Fisher Scientific. Ultrapure (Siemens) water with a resistivity of 18.2 MΩ cm was used for preparation of buffered solutions. Silica micro particles of 4.0±0.1 μm in diameter were purchased from Cospheric. The BDC-2.5 mimotope (EKAHRPIWARMDAKK) was synthesized by Sigma Genosys. CCL2, CCL3, CCL4, and CCL5 DuoSet ELISA kits and CCL17 and CXCL10 antibody pairs were purchased from R&D Biosystems. Fluorochrome-conjugated anti-CD40, -CD80, -CD86, and -F4/80 antibodies were purchased from eBioscience, while biotin anti-mouse CD4, in addition to anti-F4/80-fluorochrome-conjugated and live/dead fluorochrome-conjugated antibodies were purchased from Invitrogen.

Mice

NOD/ShiLtJ, NOD.Cg-Tg(TcrαBDC2.5,TcrβBDC2.5)/DoiJ (BDC-2.5), NOD.C6.Cg-Tg(TcrαBDC6.9,TcrβBDC6.9)/DoiJ (C6.BDC-6.9), NOD.*scid*, and NOD.*Rag* mice were bred and housed under specific pathogen-free conditions at the Research Support Building of the University of Alabama at Birmingham. BDC-2.5 and C6.BDC-6.9 mice were originally obtained from Dr. Kathryn Haskins at National Jewish Hospital (Denver, CO). NOD.*scid* and NOD.*Rag* mice purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a light/dark (12hr/12hr) cycle at 23°C and received continuous access to standard lab chow and acidified water. Male and female mice between 7–9 weeks of age were used in all experiments in accordance with the University of Alabama-Birmingham and observing IACUC-approved mouse protocols and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Preparation of hydrogen-bonded (PVPON/TA) multilayer capsules

(PVPON/TA) multilayer hollow capsules were synthesized as previously described [6, 35] by incubating 1.5 mL of 10%-aqueous suspension of silica microspheres to PVPON (1 mg mL⁻¹) solution (0.01 M sodium phosphate, pH=3.5) for 10 min. The silica particle suspension was subsequently pelleted and rinsed two times with 0.01 M sodium phosphate (pH=3.5) to remove unbound excess of polymer. Then, TA was allowed to adsorb onto particle surfaces from 0.5 mg mL⁻¹ solution for 10 min. Following each deposited layer, particles were centrifuged (2 min, 2000 rcf) and rinsed two times with the rinsing solution (0.01 M sodium phosphate, pH=3.5). Alternating coating of particles with the polymers was continued until the desired number of layers was achieved. Multilayer capsules were obtained by dissolving (PVPON/TA) multilayer-coated silica cores in aqueous hydrofluoric acid (8% w/v) followed by their dialysis in de-ionized water for three days in the dark (Float-A-Lyzer MWCO=20 kDa, Spectrum Labs). Capsule shell configuration was (PVPON/TA)_n where the subscript denotes a number of (PVPON/TA) bilayers deposited within the multilayer with TA as the outmost layer and was labeled as “4–5” for five-bilayer (PVPON/TA)₅ capsules of 4 μm in diameter. An additional set of the (PVPON/TA)_{5,5} capsules was prepared using 4 μm sacrificial silica microspheres with PVPON as an outside layer, and labeled as “4–5.5”. A set of control capsules (PVPON)₅ containing only PVPON was prepared using poly(N-vinylpyrrolidone)-co-(aminopropyl)methacrylamide (PVPON-NH₂) with weight-average molecular weight of 81000 g mol⁻¹ and Đ = 1.54, and 4 μm sacrificial silica spheres as described previously [36]. Briefly, hydrogen-bonded multilayers

PVPON-NH₂-*n*, where *n* denotes a molar percentage of amine group-containing polymer units (*n*=7) were deposited on silica microspheres at pH=3.5, starting from PVPON-NH₂. Each deposition cycle was followed by three rinses with a pH=3.5 buffer solution (0.01 M) to remove excess polymer, followed by centrifugation at 2000 rpm for 2 min to remove supernatant. After five bilayers of (PVPON-NH₂/PMAA) were deposited, chemical cross-linking of PVPON-NH₂ layers was performed using glutaraldehyde solution (5 wt%) at pH=5 for 12 hours. After that, the coated particles were exposed to pH=8.5 for 4 hours to release PMAA, followed by rinsing at pH=4. The core dissolution was performed as described above and resultant capsules were purified by dialysis in deionized water for 3 days. All capsules were vortexed and sonicated (15 s each) three times before use. Concentration of the capsule suspensions (shells/microliter) was measured by capsule counting using a hemocytometer. Scanning electron microscopy (SEM) of the hollow capsules was performed using an FEI Quanta FEG SEM microscope at 10 kV. Samples were prepared by depositing a drop of a particle suspension on a silicon wafer and allowing it to dry overnight at room temperature. Before imaging, dried specimens were sputter-coated with a ~5 nm thick silver layer using a Denton sputter-coater. The chemical composition, size, and capsule top layer of the (PVPON/TA) multilayer capsules are defined in Table 1.

Islet isolation, encapsulation, and MTT assay

Islets from NOD.*scid* or NOD.*Rag* mice were isolated and encapsulated as previously described [7]. Non-encapsulated and encapsulated islet viability was assessed with an MTT assay (Sigma Aldrich) as described [6]. The conformal coating of islets with hydrogen-bonded (PVPON/TA)_{*n*} multilayer film was performed at 25° C inside the laminar hood. Before deposition of (PVPON/TA)_{*n*} multilayer coating, where *n* denotes the number of deposited bilayers, islets were pelleted in 1.5 mL Eppendorf centrifuge tubes and washed two times with rinsing solutions of islet culture media. PVPON was allowed to adsorb first onto islet surfaces from 1 mg mL⁻¹ solution (RPMI 1640 containing 10% FBS, 20mM HEPES, penicillin/streptomycin (100U/100mg/mL), 2mM L-glutamine, 50μM 2-mercapto-ethanol, 0.5% BSA w/v, pH = 7.4) for 7 min followed by the deposition of TA layer from 0.3 mg mL⁻¹ solution (Freshly dissolved in the media before coating procedure, pH = 7.4) for 3 min. After each deposited layer, islets were collected by centrifugation for 2 min at 2000 rpm and rinsed twice with the islet media. Alternating coating of islets with the polymers was continued until the desired number of layers was achieved. All solutions were filter-sterilized with polystyrene non-pyrogenic membrane systems (0.22 μm pore size) (Corning) before use. Islets were encapsulated with 5 bilayers of (PVPON/TA) (the 4–5 configuration) with tannic acid on the outer layer prior to transplantation into diabetic recipient NOD.*Rag* mice.

Differentiation and stimulation of bone marrow-derived macrophages (BM-Mφ)

Bone marrow hematopoietic stem cells were isolated from femurs and tibias of NOD mice and differentiated into macrophages using L-929 conditioned macrophage media as previously described [37]. Stem cells were plated in 24-well plates, petri dishes, and chamber slides at a concentration of 1.0 × 10⁶ cells/mL. After 7 days, differentiated macrophages were cultured in macrophage media depleted of L-929 conditioned media for 24 hours prior to stimulation. Cells were then treated with 25 μg/mL of the TLR3 ligand,

poly(I:C) (low-molecular-weight double stranded RNA synthetic analog, InvivoGen), and co-treated with 1.0×10^7 counts/mL of (PVPON/TA) capsules at various time intervals [38].

Chemotaxis assay

To assess CD4 T cell migration, BM-M ϕ were seeded and differentiated onto the lower chambers of a 24-well Corning transwell plate containing 8 μ m pores. Splenic CD4 T cells from diabetogenic NOD.C6.BDC-6.9 mice were purified by negative selection according to the manufacturer's protocol using the EasySep CD4 T cell enrichment kit (STEMCELL Technologies). CD4 T cell purity was routinely assessed by flow cytometry and found to be greater than 90% (data not shown). Following stimulation with p(I:C) in the presence or absence of (PVPON/TA) capsules for 24 hours, 2×10^5 C6.BDC-6.9 CD4 T cells were added to the upper chamber. Following incubation at 37°C for 24 hours, non-adherent T cells were collected from the lower chambers and counted via trypan blue exclusion.

Immuno-spin trapping and immunofluorescence

Macromolecule-centered free radicals were detected upon stimulating NOD bone marrow-derived macrophages with 25 μ g/mL p(I:C) in the presence of 1mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Dojindo) in tissue culture-treated chamber slides. Cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, blocked with 5% BSA in PBS, and incubated with 20 μ g/mL of chicken IgY anti-DMPO as described [39, 40]. DMPO adducts were detected with Alexa Fluor 488-conjugated goat anti-chicken IgY secondary antibody (1:500; Invitrogen). Macrophages were identified with anti-F4/80 Alexa Fluor 647-conjugated antibody (1:500; Life Technologies). Images were obtained with an Olympus IX81 Inverted Microscope at a 40X objective and analyzed with cellSens Dimension imaging software version 1.12. To quantitate fluorescence intensity, 3–6 images were obtained for each data point. Each image was collected at the same exposure time, adjusted to the same intensity level for standardization, and the fluorescence intensity was measured using ImageJ Software (NIH).

ELISA and quantitative RT-PCR (qRT-PCR)

Chemokine expression was measured in the supernatants of stimulated macrophages for 72 hours as described [41]. Pro-inflammatory CCL5 and CXCL10 chemokines were detected with DuoSet ELISA kit and antibody pairs (R&D Systems) according to the manufacturer's instructions. ELISA plates were read on a Synergy 2 microplate reader (BioTek) using Gen5 software. RNA was isolated from poly(I:C)-stimulated and (PVPON/TA) capsule-treated BM-M ϕ after 48 hours incubation using TRIzol (Invitrogen) and cDNA prepared by SuperScript III (Invitrogen) according to the manufacturer's protocol. The generated cDNA was amplified on a Roche LightCycler 480 instrument by qRT-PCR using the following TaqMan gene expression assays (Applied Biosystems): *Emr1* (Mm00802529), *Ccl2* (Mm00441242), *Ccl3* (Mm00441259), *Ccl4* (Mm00443111), *Ccl5* (Mm01302428), *Ccl17* (Mm00516136), *Cxcl10* (Mm00445235). The relative mRNA levels were calculated with 2^{-Ct} method and *Emr1* was used as a housekeeping control gene for normalization [38]. The unstimulated samples were used as calibrator controls and set as 1.

Flow cytometric analysis of macrophage markers

Following p(I:C) stimulation, 1×10^6 BM-M ϕ s were stained with fluorochrome-conjugated antibodies specific for F4/80 (BM8), CD40 (1C10), CD80 (16-10A1), and CD86 (PO3.1) (BD Biosciences, eBiosciences) for 30 minutes at 4°C as described previously [38]. Cells were collected on the Attune NxT Flow Cytometer (ThermoFisher) with at least 100,000 events collected for each sample and analyzed with FlowJo (10.0.8r1) software (Tree Star, Inc.).

Differentiation of NOD dendritic cells and co-culture with BDC-2.5 CD4 T cells and encapsulated NOD.Rag islets

Bone marrow hematopoietic stem cells were isolated from femurs and tibias of NOD mice and differentiated into dendritic cells (DCs) using IL-4 and GM-CSF, as described previously [42]. Before culture with CD4 T cells, DCs were stimulated with 1 μ g/mL LPS for 6 hours at 37°C with 5% CO₂. Splenic CD4 T cells from NOD.BDC-2.5 mice were purified by negative selection according to the manufacturer's protocol using the EasySep CD4 T cell enrichment kit (STEMCELL Technologies). CD4 T cell purity was routinely assessed by flow cytometry and found to be greater than 90% (data not shown). CD4 T cells (5×10^5) were cultured with 3.75×10^5 DCs in the presence or absence of non-encapsulated and encapsulated (4–5 and 4–5.5 PVPON/TA multilayers) NOD.*Rag* islets, in addition to the BDC-2.5 mimotope. Culture supernatants were harvested at 48 and 72 hours post-stimulation. Culture supernatants were harvested at 48 hours post-stimulation.

Streptozotocin (STZ) induction of diabetes, islet transplantation, and histology

To induce diabetes in NOD.*Rag* mice, 175 mg/kg STZ (Sigma-Aldrich) in PBS (pH 7.2) was injected intraperitoneally. Mice were considered diabetic after two consecutive positive glucosuria tests with Diastix (Bayer) and confirmed with blood glucose readings ≥ 300 mg/dL (19.8 mM) with a Breeze 2 blood glucose meter (Bayer). Euglycemia was restored by transplanting 500 (PVPON/TA)-encapsulated NOD.*scid* islets into the epididymal fat pad of diabetic NOD.*Rag* mice ($n=5$ for each group of transplanted or non-transplanted recipient mice) under isoflurane anesthesia as described [43]. Epididymal fat pads containing transplanted islets were excised, fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin or Optimal Cutting Temperature (OCT, Tissue-Tek), sectioned at 6–8 μ m, and stained with hematoxylin and eosin as described [44]. Cut paraffin sections were rehydrated and then blocked with 5% normal donkey serum in 1% BSA/1X PBS. Sections were incubated with primary antibodies overnight at 4°C: mouse anti-glucagon (1:4000, #G2654, Sigma), guinea pig anti-insulin (1:1000, #A056401-2, Dako). Cy2-, Cy3, or Cy5-conjugated donkey anti-guinea pig, anti-mouse, or anti-goat IgG secondary antibodies (1:500, Jackson ImmunoResearch Laboratories) were used for detection. Slides were imaged using an Olympus IX81 inverted microscope (Olympus) and the images were processed by CellSens Dimensions software version 1.12 (Olympus).

Intraperitoneal glucose tolerance test (IPGTT)

An IPGTT assay was performed as we described [26]. Male NOD.*scid* mice were fasted for 6 hours, weighed, and injected intraperitoneally with 2 g/kg body weight of 20% D-glucose

(Sigma-Aldrich). Blood was obtained from the tail vein before and at 5, 15, 30, 60, and 120 min after glucose injection and blood glucose was measured with a Breeze 2 blood glucose meter (Bayer).

Statistical analysis

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Determination of the difference between mean values and standard deviation for each experimental group was assessed using the 2-tailed Student's *t* test, with $p < 0.05$ considered significant. All experiments were performed at least three separate times with data obtained in a minimum of triplicate wells in each experiment.

RESULTS

(PVPON/TA) multilayer capsules elicit a reduction in macromolecular free radical adducts in p(I:C)-stimulated bone marrow-derived macrophages (BM-M ϕ)

To investigate the antioxidant effect of (PVPON/TA) multilayer capsules on p(I:C)-stimulated bone marrow-derived macrophages (BM-M ϕ), hollow multilayer capsules with varied composition were prepared using a hydrogen-bonded assembly of PVPON and TA on sacrificial silica particles of 4 μ m in diameter as we previously described [6, 7] (Figure 1A). After dissolution of the silica cores followed by their dialysis in water, hollow multilayer capsules were obtained. Scanning electron microscopy analysis confirmed a complete removal of the inorganic templates and the (PVPON/TA) hollow shells collapsed upon drying on surfaces of a Si wafer (Figure 1B). Figure 1C demonstrates a rough surface topography of the (PVPON/TA) shell in agreement with our previous data [35]. The average bilayer thickness of the (PVPON/TA) capsules produced at pH 5 (0.01 M) is 7.5 nm as previously measured by atomic force microscopy [35]. The 4 μ m capsules with the capsule top layer being either TA as in (PVPON/TA)₅, or PVPON as in (PVPON/TA)₅PVPON were prepared and labeled as '4-5' and '4-5.5', respectively (Table 1). As a non-functional control, we used 5-layer (PVPON)₅ multilayer capsules that did not contain any TA as a capsule wall constituent and were labeled as 'PVPON'.

To confirm that the inner layer location of TA in the 4-5.5 multilayer capsules can effectively dissipate NOX-derived superoxide synthesis, immuno-spin trapping was performed with the free radical spin trap, dimethyl pyrroline oxide (DMPO), to verify the generation of free radicals in macromolecules in BM-M ϕ upon stimulation with Toll-like receptor (TLR) agonists [28, 44]. Corroborating our previous studies, there was a 1.5-fold increase in DMPO-adducts after stimulation with p(I:C), a TLR3 agonist [7]. Co-treatment with (PVPON/TA) elicited a significant 6.5-fold decrease in DMPO-adducts via immunofluorescence with p(I:C)-treated macrophages in contrast to p(I:C) stimulation alone (Figure 2A). In Figure 2B, quantitation of DMPO-adduct fluorescence intensity revealed a significant reduction when p(I:C)-stimulated macrophages were treated in the presence of (PVPON/TA) capsules. The decrease in fluorescence intensity demonstrates that (PVPON/TA) capsules are effective in dissipating free radical synthesis and may be efficacious in modulating pro-inflammatory innate immune responses of macrophages.

Pro-inflammatory chemokine expression was dampened in the presence of (PVPON/TA) multilayer capsules

As pro-inflammatory chemokine synthesis is redox-regulated [41] and (PVPON/TA) nanothin multilayer coatings can function as an antioxidant to dissipate free radicals (Figure 1 and [7]), the ability of (PVPON/TA)_n to regulate pro-inflammatory chemokines implicated in islet transplant rejection within p(I:C)-stimulated BM-M ϕ was examined by qRT-PCR and ELISA. Expression of CCL5 and CXCL10 mRNA accumulation and protein expression by qRT-PCR and ELISA, respectively, was significantly lowered when p(I:C)-stimulated samples were treated with (PVPON/TA)_n capsules (Figure 3). Interestingly, differences were observed when macrophages were co-treated with (PVPON/TA)₅ capsules containing TA on the top (4–5) or shielded by the PVPON outer layer (4–5.5). At 48 hours post-stimulation, *Ccl5* mRNA levels were significantly reduced 1.4-fold with capsule 4–5, but no significant difference was observed with capsule 4–5.5 (Figure 3A). Similarly, in Figure 3B, *Cxcl10* mRNA accumulation was attenuated 2.6- and 1.7-fold with 4–5 and 4–5.5, respectively. To corroborate the decrease in chemokine mRNA, CCL5 and CXCL10 protein levels were suppressed consistently over a period of 96 hours (data not shown) by 4–5 and 4–5.5 capsules. At 72 hours post-stimulation, CCL5 was reduced with capsules 4–5 and 4–5.5 by 20.6- and 2-fold, respectively (Figure 3C). CXCL10 protein levels followed similar results with a 1.6- and 1.4-fold reduction with capsules 4–5 and 4–5.5, respectively (Figure 3D). To further demonstrate that CXCL10 chemokine levels were redox-regulated, p(I:C)-stimulated macrophages were also stimulated with PVPON capsules alone and unlike capsules 4–5 and 4–5.5, the absence of tannic acid was not able to diminish *Cxcl10* mRNA accumulation or CXCL10 expression (Supplemental Figure 2). CCL2, CCL3, CCL4, and CCL17 mRNA and chemokine levels did not differ when p(I:C)-stimulated macrophages were treated with 4–5 and 4–5.5 capsules (data not shown).

(PVPON/TA) multilayers can prevent T cell migration and chemotaxis

To corroborate the observed attenuated levels of pro-inflammatory chemokines elicited by (PVPON/TA) capsule treatment on p(I:C)-stimulated macrophages, a chemotaxis assay was performed with purified autoreactive CD4 T cells from the NOD.C6.BDC-6.9 mouse [45, 46] on a trans-well plate. The ability of diabetogenic C6.BDC-6.9 CD4 T cells to migrate from the top trans-well to the bottom chamber was assessed after stimulating NOD BM-M ϕ with p(I:C) in the presence or absence of 4–5 and 4–5.5 capsules by cell counting. As shown in Figure 3E, treatment of p(I:C)-stimulated macrophages with capsules 4–5 or 4–5.5 significantly decreased the number of migrated CD4⁺ T cells to the bottom chamber of the trans-well plate by 1.5-fold.

M1 activation markers were reduced in the presence of (PVPON/TA) multilayers

To further characterize the functional effects of (PVPON/TA) nanothin coatings on pro-inflammatory macrophage responses, surface expression of macrophage activation markers CD40, CD80, and CD86 were examined by flow cytometry at 24 hours post-stimulation with p(I:C) according to the gating strategy in Supplementary Figure 1. BM-M ϕ were immunophenotyped based on their size (FSC), granularity (SSC), single cells, live cells, and then gated on F4/80, a mouse macrophage-specific cell surface marker. Expression of CD40,

CD80, and CD86, extracellular pro-inflammatory M1 macrophage cell surface markers that indicate these innate immune cells are activated, were diminished upon (PVPON/TA) co-treatment. Capsules 4-5 and 4-5.5 induced a 2.5- and 4.7-fold reduction in the geometric mean fluorescence intensity (gMFI) and a 1.2- and 2-fold decrease, respectively, in the percentage of F4/80 and CD40 positive cells in contrast to p(I:C) stimulation alone at 24 hours post-stimulation (Figure 4A). Interestingly, only capsule 4-5.5 elicited a statistically significant 2-fold decrease in the percentage of F4/80⁺ and CD40⁺ cells upon quantitation in Figure 4B. Similarly, treatment of p(I:C)-stimulated macrophages with capsules 4-5 and 4-5.5 exhibited a 1.5- and 1.4-fold reduction in the gMFI and 3.2- and 2.9-fold decrease, respectively, in the percentage of F4/80 and CD80 expression, a macrophage co-stimulatory molecule necessary for T cell activation (Figure 5A). Quantitation of the percentage of F4/80⁺ and CD80⁺ macrophages in comparison to p(I:C) treatment alone demonstrates that both capsules 4-5 and 4-5.5 significantly decreased expression by 2.5-fold (Figure 5B). With respect to CD86 expression, this co-stimulatory molecule was upregulated after p(I:C) stimulation on F4/80⁺ macrophages (Figure 6A), but co-treatment with capsules 4-5 and 4-5.5 elicited a trend toward a decrease that was not statistically significant (Figure 6B). In addition to decreasing pro-inflammatory chemokine synthesis, treatment with (PVPON/TA) capsules diminished M1 macrophage activation markers, thereby demonstrating the potential to immunomodulate *in vivo* innate immune responses.

(PVPON/TA)-encapsulated islets are immuno-protected in co-culture assays with diabetogenic splenocytes

We previously demonstrated that (PVPON/TA)_n multilayers with $n \geq 3.5$ can conformally coat the surfaces of murine, non-human primate, and human islets using confocal laser microscopy [7]. Transmission electron analysis of coated islets displayed a conformal coating with an average thickness of 7 nm per (PVPON/TA) bilayer which correlated well with atomic force microscopy analysis of the thickness of (PVPON/TA) hollow shells produced at physiological conditions of pH=7.2 (0.1 M) with a (PVPON/TA) capsule bilayer thickness of 8 nm [35]. Our data demonstrated the ability of (PVPON/TA) multilayers to diminish both innate and adaptive immune responses involved in pancreatic β -cell destruction. We have previously shown that (PVPON/TA) encapsulation of murine, non-human primate, and human islets did not alter β -cell function and the secretion of insulin in response to hyperglycemic conditions [7], but whether (PVPON/TA) multilayers applied to islet surfaces could function as an immunoprotective “shield” in the presence of autoreactive immune cells is not known. To address this question, NOD.*Rag* islets were encapsulated with 4-5 or 4-5.5 multilayers and incubated with diabetogenic BDC-2.5 splenocytes. As we previously described [6, 7], islet encapsulation with (PVPON/TA) multilayers did not affect the viability of NOD.*Rag* islets as shown by an MTT assay (data not shown) nor compromise islet function [7]. In contrast to non-encapsulated islets, 4-5- and 4-5.5-encapsulated NOD.*Rag* islets were more immuno-protected as *Ifng* mRNA accumulation was undetected (Fig. 7A), and protein synthesis was blunted 1.4- and 1.3-fold, with 4-5 and 4-5.5 multilayers, respectively (Fig. 7B). *Cxcl10* mRNA was similarly reduced 1.8- and 6.5-fold, respectively upon islet encapsulation with 4-5 and 4-5.5 biomaterials (Fig. 7C), and CXCL10 synthesis was attenuated 3.3-fold upon encapsulation with biomaterials containing TA as the outermost layer (Fig. 7D). There was no statistically significant difference in

CXCL10 synthesis upon culture with islets encapsulated with PVPON as the outermost layer (Fig 7D). In addition to CXCL10, the pro-inflammatory chemokine CCL5 was similarly attenuated at levels of transcription and translation upon islet encapsulation with PVPON/TA multilayers containing TA (4–5) or PVPON (4–5.5) on the outermost layer (Figure 7E, F). (PVPON/TA) encapsulation of NOD.*Rag* islets did not mediate an increased regulatory T cell (Treg) response in this co-culture assay, as the levels of IL-10 and FoxP3 expression on CD4 T cells was not altered (data not shown). These results provide additional evidence that encapsulation of islets with (PVPON/TA) multilayers prior to transplantation has the potential to dampen pro-inflammatory Th1 cytokine and chemokine responses involved in islet graft rejection.

(PVPON/TA)-encapsulated islets are functional in vivo

To further examine the functional effects of (PVPON/TA) nanothin coatings, the ability of (PVPON/TA) encapsulated islets to reverse hyperglycemia in immunodeficient diabetic mice was examined. NOD.*scid* islets were encapsulated with (PVPON/TA) multilayers and transplanted in the epididymal fat pad of streptozotocin-treated diabetic NOD.*Rag* recipients (Figure 8A). Transplantation of 500 (PVPON/TA)-encapsulated islets efficiently restored diabetic NOD.*Rag* recipients to euglycemia by 2 days post-transfer. Reestablished islet function was also confirmed by performing an intraperitoneal glucose tolerance test as we previously described [26]. Transplantation of (PVPON/TA)-encapsulated islets efficiently restored glucose tolerance when challenged with a bolus dose of glucose (Figure 8B). Removal of the epididymal fat pad (denoted as * in mouse #6 and #9) containing the islet transplant resulted in mice returning to hyperglycemia (Figure 8A). Histological analysis of the epididymal fat pad demonstrated stable engraftment of transplanted islets and expression of insulin and glucagon by immunofluorescence with (PVPON/TA)-encapsulated islets (Figure 8C, 8D). These results provide evidence that encapsulation of islets with (PVPON/TA) multilayers can maintain islet function *in vivo* and restore euglycemia in diabetic mice.

DISCUSSION

These results demonstrate the importance of (PVPON/TA) multilayer biomaterials as a novel islet encapsulation nanothin coating to mediate immunosuppression by blunting pro-inflammatory chemokine expression, T cell trafficking, and maintaining islet function *in vivo*. In addition to a decrease in innate immune pro-inflammatory cytokines such as IL-12p70 and TNF- α [7], and the Th1 adaptive immune effector cytokine IFN- γ [6], our results demonstrate that autoreactive T cell migration is impaired with (PVPON/TA) multilayers. CXCL10 and CCL5 pro-inflammatory chemokine synthesis is decreased at the mRNA and protein levels after (PVPON/TA) capsule treatment with p(I:C)-stimulated macrophages. Functionally, the decrease in pro-inflammatory chemokine production impacted the ability of diabetogenic CD4 T cells to migrate across a trans-well membrane, further highlighting the additional immunosuppressive effects elicited by (PVPON/TA) multilayers including dissipating free radicals and suppressing pro-inflammatory cytokine synthesis [6, 7]. The significance of reducing these inflammatory molecules is that CXCL10 and CCL5 are important chemokines involved in the immunopathogenesis of T1D and islet

graft rejection [32, 47–51]. In the serum of T1D patients, CXCL10 levels are elevated, suggesting that CXCL10 is a probable marker for predicting T1D [33]. Increases in CCL5 serum levels in patients with T1D have been associated with disease progression [34]. Within the human and murine islet microenvironment, expression of CXCL10 and CCL5 are key contributors to pancreatic β -cell destruction in autoimmune diabetes [52]. These results provide additional evidence of the immunomodulatory potential of (PVPON/TA) biomaterials to dissipate ROS, reduce pro-inflammatory cytokines and chemokines, and also hinder T cell migration to sites of islet engraftment.

In support of our recent data demonstrating the importance of NOX-derived superoxide and oxidative stress on influencing pro-inflammatory M1 macrophage differentiation [30], we provide evidence that dissipation of free radicals with (PVPON/TA) coatings can also suppress CD40 and CD80 M1 macrophage activation markers. Expression of CD40 and CD80 are indicative of an M1 macrophage phenotype [53, 54], and the ability of 4–5 and 4–5.5 capsules to mediate this decreased pro-inflammatory macrophage response further highlights the importance of free radicals and oxidative stress on M1 macrophage differentiation [30, 31, 55]. Interestingly, the inability of 4–5 and 4–5.5 multilayers to blunt CD86 expression on p(I:C)-stimulated macrophages demonstrates that (PVPON/TA) nanothin coatings are not globally immunosuppressive and can still enable macrophages to function as antigen-presenting cells to stimulate T cells. The preferential decrease in CD40 and CD80 expression without compromising CD86 levels was also observed with LPS-stimulated RAW264.7 macrophages treated with triethylene glycol dimethacrylate, a monomer used in dental resins that can alter redox status [56–58]. Overall, the decrease in CD40 and CD80 levels supports the overarching hypothesis that the (PVPON/TA) multilayers are efficient in dampening pro-inflammatory M1 macrophage differentiation and activation.

Surprisingly, we did not observe a dramatic difference in T cell migration or CXCL10 synthesis and CD80 expression on p(I:C)-stimulated macrophages when tannic acid was located on the top (4–5) or inner (4–5.5) layer of the (PVPON) multilayers. However, differences were observed with *Ccl5* mRNA accumulation and with CCL5 protein levels between 4–5 and 4–5.5, as tannic acid in the inner layer was less efficient in decreasing *Ccl5* mRNA accumulation and protein synthesis in contrast to the outer layer. This observation would suggest that CCL5 expression is tightly regulated by tannic acid redox activity and oxidative stress. Previous studies have demonstrated a decrease in GSH antioxidant levels and a concomitant increase in CCL5 serum levels in patients with systemic lupus erythematosus, a systemic autoimmune disease [59]. An additional redox-dependent mechanism of CCL5 synthesis includes an NF- κ B-dependent response element in the promoter of the *Ccl5* gene [60] and interestingly, the presence of five tyrosine amino acids within CCL5 can undergo oxidative post-translational modification and the formation of CCL5 multimers that are essential for chemokine activity [61]. Therefore, under conditions of oxidative stress and inflammation, biologically active oligomers of CCL5 are generated to enhance T cell trafficking, but in the presence of an antioxidant such as tannic acid, the formation of CCL5 multimers is decreased and T cell chemotaxis is diminished. Conversely, tannic acid localization in the inner layer was able to significantly decrease CD40 expression in p(I:C)-stimulated macrophages more efficiently than the outer layer, which may suggest

that PVPON may inherently possess immunomodulatory effects. However, this is unlikely since we previously demonstrated that PVPON was unable to dissipate free radicals in the absence of tannic acid [6]. Alternatively, the antioxidant activity of tannic acid on the outer layer is not ideal to suppress CD40 expression and may be more responsive to suppressive effects when shielded by a PVPON layer. Future studies will involve altering the concentration of tannic acid on (PVPON/TA) multilayers to further define the ideal immunosuppressive effect on innate and adaptive immune responses.

In addition to decreasing diabetogenic CD4 T cell migration and macrophage activation, we mechanistically demonstrated that encapsulation of murine islets with (PVPON/TA) was immunoprotective and efficient in preventing Th1 effector responses involved in destroying pancreatic islets [6]. Paralleling our previous report demonstrating that encapsulation with (PVPON/TA) did not compromise, but enhanced islet function [7], we anticipate that islets encapsulated in (PVPON/TA) will facilitate the restoration of euglycemia and mediate immunosuppression. Furthermore, the inherent ability of polyphenolic compounds such as tannic acid to impart immunoregulatory responses is highly novel and may be efficacious in combination with lower doses of immunotherapeutics including tacrolimus, rapamycin, anti-thymocyte globulin, anti-CD25 (daclizumab), and anti-CD20 (rituximab) for stable islet transplant engraftment and protection from immune destruction [2, 62–64].

Finally, (PVPON/TA) encapsulation of islets did not compromise islet function *in vivo*, as euglycemia was quickly restored in diabetic mice after transplantation into the epididymal fat pad. These results demonstrate the feasibility of islet encapsulation with (PVPON/TA) nanothin coatings to maintain euglycemia *in vivo* and to modulate pro-inflammatory immune responses involved in islet graft destruction. Current studies are underway to determine the efficacy of (PVPON/TA)-encapsulated islets to delay both alloimmune and autoimmune responses after transplantation into spontaneously diabetic NOD or STZ-induced diabetic C57BL/6 mice.

CONCLUSIONS

It is apparent that for successful islet engraftment and tolerance induction, there is a dire need for novel immunotherapies that can decrease innate immune-derived signals and mitigate adaptive immune responses involved in graft rejection. Our results demonstrate the feasibility of (PVPON/TA) nanothin coatings to mediate immunosuppression by blunting inflammatory chemokine expression, T cell trafficking, pro-inflammatory M1 macrophage differentiation, and maintaining *in vivo* islet function. These results are significant since therapies that show promise in diminishing T cell recruitment are lacking and novel (PVPON/TA) nanothin coatings demonstrate potential as another strategy for immunosuppression. Future studies will determine the efficacy of (PVPON/TA) multilayer-encapsulated islets to prevent autoimmune diabetes in mouse models of allo- and xenotransplantation. We anticipate that islet encapsulation with (PVPON/TA) multilayers will have a profound immunomodulatory effect on allo- and xenoreactive immune cells, prevent pancreatic β -cell destruction, and restore euglycemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

APC	antigen presenting cell
ROS	reactive oxygen species
T1D	Type 1 diabetes
MTT	thiazolyl blue tetrazolium bromide
NOD	Non-Obese Diabetic
TA	tannic acid
PVPON	poly(N-vinylpyrrolidone)
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
FSC	forward scatter
SSC	side scatter

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Figure 1. Schematic demonstrating the synthesis of (PVPON/TA) multilayer coatings Hollow multilayer shells of (PVPON/TA)_n ($n = 5$ and 5.5 ; and denotes the number of PVPON/TA bilayers in the multilayer) were produced via hydrogen-bonded assembly of PVPON and TA on surfaces of silica particles (SiO₂) of 4 μm, followed by dissolution of the silica core and dialysis of (PVPON/TA)_n shells at pH=7.4 (A). Scanning electron microscopy images of the dried (PVPON/TA)₅ polymeric shells (B, C).

Figure 2. (PVPON/TA) multilayers reduce macromolecule-centered free radical formation upon p(I:C) stimulation

Immunofluorescence identification of macrophages (F4/80 - Alexa Fluor 647) and DMPO adducts (Alexa Fluor 488) of 25 $\mu\text{g/mL}$ p(I:C)-stimulated NOD bone marrow-derived macrophages co-treated with 1mM DMPO and capsule 4-5.5 for 12 hours (A). The fluorescence intensity of DMPO adducts by immune cells were quantitated with ImageJ software (B). Data shown represent average of 3 experiments performed in triplicate with the following total number of counted cells per group (Control: n=599; DMPO: n=632; p(I:C): n=526; p(I:C) + DMPO: n=697; capsule 4-5.5: n=429; capsule 4-5.5 + DMPO: n=461;

p(I:C) + capsule 4-5.5: n=457; p(I:C) + capsule 4-5.5 + DMPO: n=652). The red bar corresponds to the mean fluorescence intensity of DMPO-adducts normalized to macrophages expressing F4/80. Images were magnified at 40X and digitally enlarged. *ns*, not significant; *** $p<0.0001$

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Figure 3. Pro-inflammatory chemokine synthesis and T cell migration is reduced in p(I:C)-stimulated macrophages co-treated with (PVPON/TA) multilayers
Ccl5 (A) and *Cxcl10* (B) mRNA accumulation from (PVPON/TA)-treated bone marrow-derived macrophages was examined by qRT-PCR after 48-hour stimulation with 25 μ g/mL of p(I:C). Results were normalized to *Emr1* and the unstimulated samples were set to 1 and used as the calibrator control. Supernatant of (PVPON/TA)-treated bone marrow-derived macrophages stimulated at 25 μ g/mL of p(I:C) was examined via ELISA. CCL5 (C) and CXCL10 (D) chemokine expression at 72 hours was normalized to the no antigen/no

capsules control group. Migration of CD4 T cells to the bottom chamber of a transwell plate containing bone marrow-derived macrophages primed with p(I:C) in the presence or absence of 4-5 and 4-5.5 (PVPON/TA)-containing capsules following 24 hour incubation (**E**). Graphed data represents cell counts via trypan blue exclusion from 4 individual wells. Graphed data are representative of 3 independent experiments done in at least triplicates. *ns*, not significant; ND, not detected; *** $p<0.0001$; ** $p<0.01$; * $p<0.05$.

Figure 4. Treatment of p(I:C)-stimulated macrophages with (PVPON/TA) multilayers dampens expression of the M1 activation marker CD40

Flow cytometric analysis of CD40 within p(I:C) stimulated BM-M ϕ s in the presence or absence of (PVPON/TA) capsules for 24 hours (A). Contour plots represent CD40 by F4/80 of live, F4/80⁺ macrophages. Geometric mean fluorescence intensity (gMFI) was calculated by gating on F4/80⁺ cells. Macrophages were gated using forward scatter and side scatter profiles, doublets were excluded, and live cells were gated using side scatter profiles using a fixable live/dead stain, immunophenotyped by F4/80. Pooled CD40 percentages represent 3

independent experiments, depicting the percentage of CD40 expressing cells gated on the live, F4/80⁺ population (**B**). *ns*, not significant; *** $p < 0.001$; * $p < 0.05$.

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Figure 5. (PVPON/TA) multilayers can elicit a decrease in the extracellular M1 macrophage marker CD80 with p(I:C)-stimulated macrophages

Flow cytometric analysis of CD80 within p(I:C)-stimulated BM-M ϕ s in the presence or absence of (PVPON/TA) capsules for 24 hours (A). Macrophages were gated using forward scatter and side scatter profiles, doublets were excluded, and live cells were gated using side scatter profiles using a fixable live/dead stain, immunophenotyped by F4/80, and CD80 cell surface marker activation shown via percentage and geometric mean fluorescence intensity (gMFI). Pooled CD80 percentages represent 3 independent experiments done in triplicates

depicting the percentage of CD80 expressing cells gated on the F4/80 population (**B**). *ns*, not significant; **** $p<0.01$.

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Figure 6. CD86 expression is not diminished when p(I:C)-stimulated macrophages are in the presence of (PVPON/TA) multilayers

Flow cytometric analysis of CD86 within p(I:C)-stimulated BM-M ϕ s in the presence or absence of (PVPON/TA) capsules for 24 hours (A). Macrophages were gated using forward scatter and side scatter profiles, doublets were excluded, and live cells were gated using side scatter profiles using a fixable live/dead stain (Invitrogen), immunophenotyped by F4/80, and CD86 cell surface marker activation is shown via percentage and geometric mean fluorescence intensity (gMFI). Pooled CD86 percentages represent 3 independent

experiments done in triplicates depicting the percentage of CD86 expressing cells gated on the F4/80 population (**B**). *ns*, not significant; ***p*<0.01.

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Figure 7. Islet encapsulation with (PVPON/TA) is immunoprotective

Ifng mRNA accumulation (A), IFN- γ synthesis (B), *Cxcl10* mRNA accumulation (C), CXCL10 production (D), *Ccl5* mRNA (E), and CCL5 protein production (F) within purified BDC-2.5 CD4 T cells cultured with LPS-primed NOD bone marrow-derived dendritic cells, and (PVPON/TA)-encapsulated islets with multilayer configurations of 4-5 and 4-5.5. qRT-PCR results were normalized to *Gapdh* and the unstimulated or islet-stimulated samples were set to 1 and used as the calibrator control. Graphed data represent 3 independent

experiments done in triplicates. *ns*, not significant; ND, not detected; *ns*, not significant; *** $p < 0.001$; ** $p < 0.01$.

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Figure 8. Transplantation of (PVPON/TA)-encapsulated islets can restore euglycemia to diabetic mice

Daily blood glucose readings of streptozotocin-treated NOD.*Rag* mice before and after islet transplantation in the epididymal fat pad with 500 NOD.*scid* islets encapsulated in (PVPON/TA) (A). IPGTT assay with non-transplanted diabetic (n=5), euglycemic mice transplanted with encapsulated islets (n=5), and non-diabetic controls (n=5) (B). H&E stain (C) and immunofluorescence for insulin, glucagon, and DAPI (D) of (PVPON/TA)-encapsulated islets in the epididymal fat pad at 10X (C) and 40X (D) magnification. TX – islet transplant. * - islet graft removed.

Table 1

Chemical composition, size, and a capsule top layer of the (PVPON/TA) multilayer capsules.

Sample	Capsule chemical composition	Capsule size, μm	Capsule top layer
4-5	(PVPON/TA) ₅	4 μm	TA
4-5.5	(PVPON/TA) ₅ PVPON	4 μm	PVPON
PVPON	(PVPON) ₅	4 μm	PVPON