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Layer-by-Layer Cerium Oxide Nanoparticle Coating for Antioxidant Protection of Encapsulated Beta Cells

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Abstract

In type 1 diabetes, the replacement of the destroyed beta cells could restore physiological glucose regulation and eliminate the need for exogenous insulin. Immunoisolation of these foreign cellular transplants via biomaterial encapsulation is widely used to prevent graft rejection. While highly effective in blocking direct cell-to-cell contact, non-specific inflammatory reactions to the implant lead to the overproduction of reactive oxygen species (ROS), which contribute to foreign body reaction and encapsulated cell loss. For antioxidant protection, cerium oxide nanoparticles (CONP) are a self-renewable, ubiquitous, free radical scavenger currently explored in several biomedical applications. Herein, 2 to 12 alternating layers of CONP/alginate were assembled onto alginate microbeads containing beta cells using a layer-by-layer (LbL) technique. The resulting nano-composite coatings demonstrated robust antioxidant activity. The degree of cytoprotection correlated with layer number, indicating tunable antioxidant protection. Coating of alginate beads with 12-layers of CONP/alginate provided complete protection to the entrapped beta cells from exposure to 100 μM H_2O_2 , with no significant changes in metabolic activity, oxidant capacity, or insulin secretion dynamics, when compared to untreated controls. The flexibility of this LbL method, as well as its nano-scale profile, provides a versatile approach for imparting antioxidant protection to numerous biomedical implants, including beta cell transplantation.

Graphical Abstract

A potent nano-scale layer-by-layer antioxidant coating is assembled via alternating layers of cerium oxide nanoparticles (CONP) and alginate. Coatings are placed on biomaterial surfaces, such as alginate microcapsules. This ultrathin coating efficiently scavenges hydrogen peroxide and protects the underlying encapsulated cells from reactive oxygen species (ROS) mediated damage.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

The high activity and versatility of this coating provides a useful approach for protecting transplanted cells and scavenging ambient ROS.

Keywords

alginate; diabetes; reactive oxygen species; anti-inflammatory

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease that results in the targeted killing of insulin-producing beta cells within the pancreatic islet. Treatment with exogenous insulin prevents the lethality of the disease, but it is unable to maintain physiological blood glucose control. This insufficient glycemic regulation leads to numerous long-term complications, including cardiovascular disease, retinopathy, and neuropathy, which greatly elevates the healthcare costs of disease management.^[1] Beta cell replacement therapies, such as clinical islet transplantation (CIT), have the potential to restore natural glucose-responsive-insulin-secretion and prevent diabetes complications. The current CIT protocol involves the transplantation of cadaveric islets via intraportal infusion into the liver, followed by a rigorous immunosuppressive regimen.^[2] While this approach has significantly reduced lethal hypoglycemic events and improved the quality of life of these patients, the durability of cellular function is poor.^[3] The precipitous decline in islet efficacy over time is attributed to four primary mechanisms: 1) non-specific inflammatory host reactions; 2) allograft rejection due to self/nonself discrimination; 3) T1D associated auto-reactivity; and 4) poor engraftment of the foreign islets.^[4]

Allograft rejection is primarily directed by the adaptive arm of the immune system. Thus, efforts to block allograft rejection are typically focused on the depletion of T cells or suppression of immune activation.^[3a] Systemic immunosuppressive treatment is problematic, however, as it elevates the risk for opportunistic infections and imparts undesirable side effects.^[5] Non-drug alternatives to mitigate direct antigen recognition include polymeric cellular encapsulation. In this approach, islets are encased in a semi-permeable barrier, commonly alginate, which permits nutrients and insulin exchange while blocking cell- and complement-mediated immune effectors.^[6] While successful in preventing direct T cell killing, the implantation of encapsulated islets instigates other detrimental host cell responses, such as generalized inflammation and foreign body response (FBR).^[6b, 7]

Oxidative stress, i.e., elevated reactive oxygen species (ROS), plays a key role in supporting the inflammatory and innate immune responses observed post-implantation of encapsulated cells.^[8] The various ROS derivatives elevated in the transplant environment (e.g., hydrogen peroxide, superoxide radicals, nitric oxide) can easily penetrate the implanted capsule and

impart ROS-mediated cellular damage to the transplanted cells.^[9] As beta cells are more susceptible to ROS damage due to their decreased antioxidant profile, the impact of local ROS can be profound.^[10] In addition to generalized inflammation, ROS serves as a key signaling agent in stimulating foreign body responses that lead to fibrotic encapsulation and in adaptive immune cell activation.^[6b, 7, 11]

In recognition of the role of ROS in graft destabilization and beta cell sensitivity to oxidative damage, the utilization of free radical scavenging/anti-oxidant agents for islet and beta cells transplants is common, with varying reports on efficacy.^[7, 10, 12] Approaches that localize the antioxidant agent to the encapsulating material have demonstrated increased protection; ^[12g, 12h] however, most are limited in their catalytic activity, ROS targets, potency (i.e. limited density of redox-sensitive sites per volume of polymer), and renewability.

Cerium oxide nanoparticles (CONP) are unique free radical scavengers with remarkable catalytic properties. Their antioxidant capacity, attributed to mobile lattice oxygen on the surface that switches between the Ce (IV) and the Ce (III) states, results in a theoretically inexhaustible and ubiquitous catalyst.^[12k, 13] ^[12i, 14] Further, their nano-scale and high surface activity impart potent reactivity per unit volume. With these desirable characteristics, CONP have been applied as ROS scavengers for various biomedical applications, such as neurodegenerative disorders (Alzheimer's disease, Huntington's disease, and Parkinson's disease), retinal diseases, and in cancer as a radioprotectant.^[15] While highly promising, the translational use of CONP is complicated when delivered as a free nanoparticle, as these may drain out of the transplant site and impart cytotoxicity.^[16]

In our laboratory, we have sought to harness the potential of CONP via incorporation and entrapment within biomaterials. This approach should provide retention of catalytic activity, control of localization, and mitigation of cytotoxicity. Our previous work dispersed dextran-coated CONPs within alginate hydrogels and demonstrated improved cytocompatibility and antioxidant protection.^[9b] To expand the versatility of CONP-based biomaterials, here we report the generation of nano-scale antioxidant coatings via layer-by-layer (LbL) assembly of CONP and alginate (Figure 1A). Coatings were formed onto various materials and ROS-scavenging activity was quantified and correlated to layer number. Further, we explored the capacity of this "nano-armor" to protect encapsulated cells from ROS-mediated damage while preserving capsule permeability and cellular function.

2. Results

2.1. Material Characterization of Layer-by-layer CONP Coatings on Biomaterial Platforms

As outlined in Figure 1A, coatings were fabricated via alternating layers of CONP and alginate. The uniformity and scale of layer-by-layer assembly was first characterized onto planar surfaces via ellipsometry. CONP was initially deposited, followed by alginate with triplicate washes between layers (Figure 1B). As shown, exposure of the wafer to alternating CONP and alginate solutions resulted in the formation of predictable multi-layers, with 12 layer giving rise to a 52.4 ± 1.5 nm thick film. Alternating between CONP and alginate was required, as incubations with alginate-only did not result in layer formation.

To explore the versatility of CONP-coatings onto 3D surfaces, both silica and alginate microbeads were coated with 2, 6, or 12 alternating layers of CONP/alginate. As shown in Figure 1C-D, confocal microscopy images illustrated ultrathin coatings formed onto the different spherical materials, with increasing fluorescence intensity correlating with increasing layer number.

The antioxidant activity of the resulting ultrathin CONP/alginate coatings was evaluated via decomposition of environmental hydrogen peroxide. CONP/alginate layers deposited onto alginate microbeads were selected for this study and incubated in increasing concentrations of H₂O₂ (i.e., 25, 50, and 100 µM). As summarized in Figure 2A, significant scavenging of ambient H₂O₂ was observed when CONP was present. Beads coated with 2-layers (CONP + alginate) scavenged between 12.5 – 20 µM H₂O₂ within the 2 h incubation period with significantly higher variability in scavenging levels during the highest H₂O₂ treatment. The capacity and consistency of H₂O₂ deactivation increased when more layers were added, with 6- and 12-layer coatings exhibiting more potent, albeit equivalent, H₂O₂ scavenging capabilities. Within 2 h, both 6- or 12-layer CONP/alginate beads completely deactivated 25 and 50 µM H₂O₂, resulting in H₂O₂ readings statistically equivalent to 0 µM controls ($p > 0.9959$ for all groups). At the highest H₂O₂ dosage of 100 µM, some residual H₂O₂ remained for the 6- and 12-layer groups, but almost 90% of the added peroxide was deactivated.

A unique and desirable feature of CONP is its capacity for durable scavenging. To evaluate this property, 6- and 12-layer coatings were repeatedly subjected to high concentrations of H₂O₂. After three consecutive 1 h incubations with 100 µM H₂O₂, both 6- and 12-layer CONP/alginate coatings exhibited equivalent and continued capacity to efficiently scavenge ambient ROS, when compared to uncoated controls (Figure 2B). The scavenging capacity for 6-layer coatings were moderately impacted in the 3rd exposure, with a significant decrease in scavenging activity when compared to the first exposure ($p = 0.008$). For 12-layer coatings, no significant difference between the three exposures was measured. Overall, the 6-layer and 12-layer coatings scavenged 174 ± 14 and 187 ± 13 µM of H₂O₂, respectively, with minimal indication of decline in antioxidant activity.

For additional characterization, CONP/alginate coatings were colorimetric oxidation of TMB was also evaluated. As expected, colorimetric oxidation of TMB increased as a function of layers, supporting the retention of CONP activity with increasing layers (Figure S1 & S2).

To further explore the stability of the resulting coatings, the activity of alginate beads coated with 12-layers of CONP/alginate was assessed for up to 30 days post-coating. As summarized in Figure S3, colorimetric oxidation of TMB remained stable over the entire testing window, with no decline in activity for any of the time points tested.

2.2. Antioxidant Protection of CONP/Alginate Coatings for Encapsulated Beta Cells

The capacity of CONP/alginate coatings to impart antioxidant protection to encapsulated beta cells was examined using MIN6 beta cells, a murine beta cell line. Beta cells, encapsulated within alginate microbeads (683.07 ± 72.27 µm diameter) were subsequently

coated with 2-, 6-, or 12-layers of CONP/alginate. To evaluate the capacity of these ultrathin coatings to protect beta cells from oxidative stress, cell-loaded capsules were incubated with increasing concentrations of H_2O_2 (i.e., 25, 50, and 100 μM). Measurement of ambient H_2O_2 at the end of the incubation period (Figure 3A) illustrated similar trends in the scavenging capacity of the coatings when compared to cell-free beads, with the 6- and 12-layer coatings scavenging equivalent levels of H_2O_2 . Of note, the overall level of H_2O_2 outside of the bead was significantly lower for these studies, when compared to cell-free beads. This is likely due to the uptake and processing of H_2O_2 by the cells, which resulted in additional ROS destabilization.

The ability of CONP coatings to protect from H_2O_2 -mediated beta cell toxicity was characterized via quantification of metabolic activity, with metabolic values normalized to untreated controls for each coating strategy. As summarized in Figure 3B, the exposure of uncoated alginate-encapsulated beta cells to increasing levels of H_2O_2 resulted in significant declines in metabolic activity. This effect statistically plateaued between 50 and 100 μM H_2O_2 , indicating a maximum cytotoxic impact during this treatment window. For 2-layer coatings, exposure to 25 μM H_2O_2 resulted in no statistical change in the metabolic activity of the underlying encapsulated beta cells; however, ROS toxicity was observed when levels were increased to 50 and 100 μM H_2O_2 , with a significant decrease in metabolic activity when compared to non- H_2O_2 treated controls. Increasing the coating to 6-layers provided complete protection up to 50 μM H_2O_2 , but this protection diminished at 100 μM H_2O_2 . At 12-layers of CONP/alginate, no significant changes in metabolic activity was measured at any of the H_2O_2 dosages tested, when compared to its non- H_2O_2 treated 12-layer group.

To further investigate the influence of ROS exposure on beta cells, their intracellular antioxidant capacity was characterized, pre- and post- H_2O_2 exposure, within uncoated or CONP/alginate coated microbeads. The antioxidant profile of the beta cells was characterized via quantification of both total intracellular glutathione, which includes reduced glutathione (GSH) and oxidized glutathione (GSSG), and GSSG alone. The impact of H_2O_2 and/or CONP/alginate coatings on the overall ratio of reduced to oxidized glutathione (i.e., GSH:GSSG) was also calculated. Of note, a shift from GSH to GSSG within a cell indicates oxidation and an overall reduction in antioxidant capacity. For MIN6 beta cells, the ratio of GSH:GSSG has been reported to be in the range of 7–20^[17]. As summarized in Figure 3C-D, the addition of CONP/alginate layers resulted in a slight and concurrent reduction of both total glutathione and oxidized glutathione (GSSG); however, this modest decline did not lead in an overall shift in antioxidant capacity, as the GSH:GSSG ratio for all coated groups were statistically equivalent to control beads ($p > 0.88$). The addition of H_2O_2 to the bathing media, however, resulted in a substantial shift in oxidation for uncoated control beads, with significantly decreased total glutathione levels and increased GSSG, resulting in a significant reduction in the cells' GSH:GSSG ratio. For beads coated with either 2- and 6-layer CONP/alginate, exposure to H_2O_2 also shifted the entrapped cells' antioxidant capacity ($p < 0.0001$); however, 12-layer CONP/alginate coated beads were unaffected by this ROS hit ($p > 0.99$).

In addition to evaluating the impact of CONP coatings on beta cell viability and oxidative profile, its potential impediments on the functional capacity of these beta cells was also

examined. As shown in Figure 4A, the addition of up to 12-layers of CONP/alginate onto the alginate bead did not impart any measurable changes in the cells' insulin responsiveness to a glucose challenge. When beads were serially incubated in low, high, and then low glucose, insulin was released and measured in a synchronized pattern. The overall stimulation index (ratio of insulin released during high glucose stimulation over low glucose stimulation) from these beta cells, as summarized in Figure 4A inset, was also equivalent for all groups. Of interest, when encapsulated cells were exposed to H₂O₂ (Figure 4B), an elevated insulin release during the initial basal glucose challenge for control beads and beads coated with 2- and 6-layers of CONP/alginate was observed. This dysfunctional response was not measured for beads coated with 12-layer CONP/alginate and exposed to H₂O₂, resulting in the preservation of its insulin stimulation index (Figure 4B, inset).

Following this observed protection from acute peroxide exposure, we subsequently sought to characterize the capacity of these CONP coatings to provide renewable protection against ROS attack. In this approach, we exposed the same set of control or 2-, 6-, and 12-layer CONP/alginate coated beads containing MIN6 cells to repeated insults of H₂O₂. Specifically, 50 μ M H₂O₂ was added to the media for the same set of MIN6 alginate beads every day for a total of 3 days. As summarized in Figure 5, following a single exposure to H₂O₂, a significant reduction in MIN6 metabolic activity was observed for uncoated control beads and beads coated with only 2-layers of CONP/alginate. Further declines in metabolic activity were measured for these two groups following additional exposure to H₂O₂ on days 2 and 3. The use of 6- or 12- layers resulted in more potent protection, with increased durability to subsequent peroxide treatments. For 6-layer CONP/alginate beads, diminished protection was observed after two treatments with H₂O₂. Alternatively, 12-layer of CONP/alginate resulted in complete protection from all three H₂O₂ exposures, with no significant change in metabolic activity from untreated controls.

3. Discussion

First-responder immune cells react to the implantation of foreign materials and associated insults by creating an inflammatory environment. This prompt non-specific inflammatory response results in the overproduction of ROS. For cell-based implants, localized ROS at the implant site not only imparts oxidative damage to the cells, it also promotes the foreign body response (FBR) and instructs additional adaptive immune pathways.^[18] For beta cell encapsulation, ambient ROS can induce additional toxicity, due to the cells inherent sensitivity to oxidative stress.^[12b, 13b, 19] Antioxidant reinforcement to the material can diminish inflammation and injury to the implant.^[18, 20] Several enzymatic and nonenzymatic antioxidants approaches have been explored to prevent graft rejection (e.g. grafted polymers/proteins, polyphenols, glutathione, uric acid, etc.).^[21] For cellular encapsulation, groups have also screened materials that contain inherent antioxidant properties or have been modified to scavenge ROS, e.g., poly(propylene sulfate),^[22] poly(thioketal urethane),^[23] and CONP encapsulated in alginate.^[9b]

While bulk antioxidant materials show benefits in implant function, the development of an ultrathin antioxidant surface coating provides several advantages, such serving as the primary interface with the host, imparting minimal volume contributions to the implant,

providing an additional feature of permeability control, and permitting high flexibility in the utilization of the base encapsulating material. In one approach, Ma's group generated a novel antioxidant surface via layer-by-layer assembly of dextran-functionalized coatings containing nanoparticles (NP) doped with curcumin^[24]. Curcumin released into the environment effectively scavenged both H₂O₂ and nitric oxide. While most of the drug was released from the NP within the first 12 h, adjustments in the NP chemistry can likely increase the durability of this approach. In another approach, Kharlampieva and Tse's groups have highlighted the impact of antioxidant coatings on the modulation of undesirable host cell responses.^[12g, 25] Incorporating the natural polyphenolic antioxidant tannic acid within their layer-by-layer approach, coatings scavenged localized ROS and inhibited downstream adaptive immune responses, such as T cell trafficking and activation.^[12g, 25b] While clearly outlining the potential of antioxidant coatings in deactivating inflammatory pathways, the ROS scavenging capacity of tannic acid is limited. Further, by-products of phenolic oxidation, such as quinones, can impart toxicity or lead to coating instability.^[26] Thus, the utilization of a more durable ROS scavenger may improve the longevity of these benefits.

Antioxidant nanoparticles are strong candidates for integration into coatings, as their high surface area-to-volume ratio results in elevated redox-modulation per unit volume. Several nanoparticle materials, e.g., yttrium, titanium, zinc and silver, have been reported to effectively scavenge ROS in biomedical applications;^[27] however, careful selection of nanoparticle composition must be made to mitigate cytotoxicity. For example, the instability of zinc oxide under aqueous conditions generates cytotoxicity,^[27a] while elevated concentrations of zinc and titanium nanoparticles induces inflammation when phagocytosed by endothelial cells.^[27b] While yttrium- and silver-based nanoparticles exhibit minimal cytotoxic effects, the scavenging capabilities and renewability of these agents are limited.^[28] ^[29] Manganese oxide nanoparticles also show low toxicity and high antioxidant capacity, but degrade in the presence of H₂O₂.^[30] Alternatively, CONP has the desirable features of low cost, self-renewability, ubiquitous ROS scavenging, and cytocompatibility.^[12j, 12k, 31] The redox properties of CONP, however, is influenced by factors in the microenvironment, e.g., low pH can promote oxidant activity (Fenton-like catalyst).^[32] Thus, the immobilization of CONP could help preserve its intended redox properties and mitigate cellular phagocytosis. Furthermore, surface immobilization of CONP to the implant provides the local presentation of the antioxidant, which could promote healthy host cell responses to the implant and protect the underlying cells from ROS damage.^[33]

A layer-by-layer technique, using alternating layers of CONP and alginate, was employed for the formation of our nanoscale coatings. Alginate, an anionic polysaccharide at physiological pH,^[34] is commonly complexed with positively charged materials for biomedical applications, such as protein stabilization, drug delivery, and surface coatings.^[35] Alginate-based multilayer coatings on surfaces can be achieved by alternating opposite charged materials, e.g., chitosan.^[35a, 35b] Herein, sodium alginate and CONP (ZP = +16 mV) formed stable layers via electrostatic interactions. It cannot be discounted, however, that other interactions may also contribute to layer buildup, such as carboxylate and hydroxyl adsorption on CONP.^[36] Spectroscopic ellipsometry, imaging, and activity measurements collectively confirmed discrete LbL coatings containing redox active

inorganic nanoparticles, with elevated coating layers correlating with increased thickness, fluorescence, and scavenging capacity, respectively. The versatility of the LbL system was established via coating of planar surfaces and spherical surfaces of varying material properties, i.e., silica and alginate. While the actual thickness of the coating on these spheroidal surfaces may vary from those collected from planar Si wafers, cross-sectional confocal images illustrate nano-scale coatings significantly smaller than 100 μm . Furthermore, the stability of the resulting coatings was high in buffered solutions, with no change in coating activity after 30 days. With discrete and stable coatings formed on all platforms, this electrostatic LbL approach exhibits broad applicability to coat various materials. Further, the nanoscale nature of the coatings imparted minimal impacts on nutritional and insulin diffusion barriers. As evidence, encapsulated cells maintained their viability and insulin release profiles following coating of up to 12-layers of CONP/alginate.

Retention of CONP activity was demonstrated following LbL formation, providing proof that sodium alginate interactions were not inhibitory to CONP's antioxidant properties. CONP/alginate coatings of 6-layers or more efficiently scavenged physiologically relevant cytotoxic H_2O_2 concentrations (i.e., 50–100 μM),^[37] with a scavenging rate similar to catalase.^[38] Exposure of CONP/alginate coatings to sequential insults of physiologically relevant H_2O_2 fully illustrated its effectiveness, with catalytic capacity higher than other bulk antioxidants hydrogels used for beta cell encapsulation.^[22, 38] It is also worth noting that this robust catalytic activity was achieved using an ultrathin coating format, highlighting its high potency per unit volume when compared to other bulk antioxidant materials.^[22–23, 37b] Furthermore, this LbL film deposition method offers the advantage of controllable film thickness, which permits modulation of antioxidant capacity. When combined with the self-regenerating redox activity of CONPs, proper adjustment of CONP density could offer a sustainable antioxidant protection tailored to the needs of the implant.

For beta cell transplantation, it has been well documented that declines in antioxidant levels (e.g., catalase) are contributing factors to ROS-induced beta cell death and loss of function in type 1 diabetes.^[13b, 19a] Countering these effects by increasing catalase expression subsequently improves their viability and functionality.^[12b, 13b, 19b, 39] In this study, environmental H_2O_2 insults resulted in substantial cytotoxicity of the beta cell, despite their encapsulation within a hydrogel. This clearly illustrates the need for antioxidant strategies to protect the immunoisolated beta cells from ROS damage. Coating the encapsulated cell platform with CONP/alginate layers, however, imparted significant cytoprotection. Of interest, there was no difference in overall ROS scavenging capabilities between 6- and 12-layer CONP/alginate beads, as characterized via quantification of H_2O_2 in the bathing solution. There were, however, significant differences between these two groups when cellular metabolic activity, oxidative profile, and insulin responsiveness were characterized. Specifically, 6-layer coatings exhibited decreased metabolic activity, a shift in antioxidant capacity, and alterations in insulin secretory responses following high H_2O_2 exposure. Cells encapsulated within 12-layer CONP/alginate beads, however, showed no change in these cellular assays. It is postulated that this difference is due to the inadequacy of 6 layers of CONP/alginate to effectively scavenge most of the H_2O_2 prior to its transport through the coating. Thus, while the added H_2O_2 was eventually scavenged by the CONP, the underlying cells experienced greater ROS exposure, manifesting in oxidative stress. This theory is

supported by the shift in GSH:GSSG ratio, which also correlates to ROS beta cell exposure.^[17] For glucose stimulated insulin release, the elevated insulin secretion during basal glucose concentrations measured for 0-, 2-, and 6-layer CONP/alginate beads indicates ROS-mediated dysfunction, as it has been documented that both H₂O₂ exposure and activation of beta cell inflammatory cytokines imparts this response.^[12c, 40] Therefore, cell protection can be distinct from the antioxidant's scavenging properties. For our approach, it was dependent on the number of layers and subsequent coating completeness.

With establishment of the potential of this LbL platform, future work is focused on translating this work *in vivo*, as well as to other biomaterial applications. Specifically, exploration of the capacity of these CONP-based coatings to protect the underlying cells from ROS damage, while also impairing FBR pathways to improve implant biocompatibility, will be explored. By mitigating local ROS, the durability of encapsulated beta cell transplants could be extended. In addition, the flexibility of this LbL platform permits translation to other implants other devices or implants that may experience significant ROS challenges, such as catheters and skin grafts.

4. Conclusion

Antioxidant CONP coatings provide a robust platform for potent and durable ROS scavenging. Employing an LbL approach in the generation of CONP-coatings allowed control of overall thickness, uniformity, and antioxidant capability. Resulting CONP/alginate coatings protected beta cells from ROS-mediated cellular dysfunction, with 12-layers of CONP/alginate coatings providing complete preservation of metabolic activity, glucose stimulated response, and antioxidant activity after physiologically toxic ROS exposure. These adaptable LbL antioxidant coatings have broad applications in both cellular implantation, as well as areas where mitigation of chronic inflammation is desired.

5. Experimental Section

5.1 Characterization of CONP:

Cerium oxide nanoparticles aqueous dispersion (particle size <5 nm, 20 wt% in H₂O, pH~ 4, ID: 796077) was purchased from Sigma Aldrich. Nanoparticle hydrodynamic diameter and surface electric charge were quantified using dynamic light scattering (DLS) and zeta potential (ZP) via Nicomp ZLS Z3000 (Particle Sizing Systems, FL). Freshly prepared CONP solution, diluted to a concentration of 3 mg/mL in 4-morpholinepropanesulfonic acid (MOPS)/KNO₃ buffer (10 mM each, pH 7.4), was loaded into a 1 cm rectangular plastic cell for measurement. DLS spectra or ZP measurements consisted of 10 runs of 30 s or 3 runs of 3 cycles with a 4.5 V/cm E-field, respectively. DLS spectra are based on the Gaussian number distribution. Final CONP characterization: 3–5 nm diameter and +16 mV surface charge, as summarized in Figure S1.

For quality control, CONP catalytic activity in solution was measured via TMB Oxidation assay. TMB (3,3',5,5'-tetramethylbenzidine) is a substrate that develops a blue color (absorbance at 380 and 650 nm wavelengths) when oxidized by CONP. Aqueous dispersion of CONP (1 mg/ml final concentration) was co-incubated with TMB substrate in a 96 well

(Costar, flat bottom, clear) plate for 30 min at room temperature (RT). After incubation, oxidation was quantified by absorbance reading at 650 nm.

Layer-by-layer (LbL) Film Deposition onto Idealized Planar Surface: A silicon wafer (~11×13 mm) was treated with 0.1 M NaOH for 1 min at 80 °C and washed three times with water. The resulting Si wafer was coated with 12 alternating layers of CONP dispersion and alginate (both solutions at 3 mg/ml in MOPS buffer; 30 sec per incubation at room temperature), starting with CONP as the base layer. Control wafers were exposed to alginate only. After each coating, excess CONP or alginate was washed three times with MOPS buffer, water, ethanol, and blow-dried with argon. Surface film thickness was measured via J. A. Woollam Co. alpha-SE spectroscopic ellipsometer, at a 70° angle and standard mode settings, using the transparent film data model. Three readings were collected for each data point.

Fabrication of Alginate Beads: Sodium alginate (NovaMatrix PRONOVA UP-MVG, Mw > 200 kDa, G/M ratio = 1.5) was dissolved at 1.6% wt./v in MOPS buffer (10 mM MOPS, 3 mM KCl, 125 mM NaCl, pH 7.4) and filtered sterilized through a 0.22 µm nylon membrane. Ba-alginate microbeads were fabricated as previously described,^[41] with minor modifications. Briefly, a parallel air flow nozzle fed alginate, drop-wise, into a BaCl₂-MOPS buffer (10 mM MOPS, 50 mM BaCl₂, 3 mM KCl, 50 mM NaCl, 0.2 mM Tween 20, pH 7.4). Following 10 min cross-linking incubation, beads were washed three times with MOPS buffer. Alginate microbeads were stored in MOPS buffer at 4 °C.

LbL Coatings onto Microbeads: Alginate microbeads were equilibrated in MOPS buffer and transferred to a 24 well plate (Corning, non-tissue culture) for coating. The buffer was replaced with freshly prepared CONP aqueous dispersion (3 mg/ml in MOPS buffer). Microbeads were agitated for 30 s to promote homogeneous CONP coatings. The CONP solution was then removed and the beads were washed with MOPS buffer three times. After the third wash, the buffer was replaced with an alginate solution (3 mg/ml in MOPS buffer). Microbeads were mixed thoroughly for 30 s and then washed three times with MOPS buffer. This entire procedure was repeated for subsequent layers, up to 6 complete cycles.

Fluorescent Coatings on Alginate and Glass Beads: Fluorescently labeled alginate (Alg-CF) was manufactured by dissolving 25 mg of alginate (Alg, PRONOVA UP MVG) in 2.5 mL purified water followed by addition of 5 mg of 2-(N-morpholino)ethanesulfonic acid (MES), 5 mg of N-hydroxysuccinimide (NHS), and 0.5 mg of 4'-(aminomethyl)fluorescein hydrochloride (CF, dissolved in 100 µL ethanol). N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC, 25 mg in 100 µL purified water, prepared fresh) was then added and stirred for 20 min. Slowly (rate of 7 µL/min) 0.25 M NaOH was added to a total of 200 µL. After 1 h stirring, 5 mL ethanol was added. The precipitate that formed was dissolved in 2 mL of 50 mM NaCl and precipitated again with 5 mL ethanol, rinsed twice with water/ethanol (2:4 parts) mix, then ethanol, and dried under reduced pressure. This yielded 18 mg of orange solid film testing negative for Kaiser's test. ATR-FTIR characteristic bands: 3258, 2932, 1778, 1596, 1406, and 1023 cm⁻¹. Glass beads (Sigma, d = 75 µm) or alginate microcapsules (manufactured as described above) were coated with

CONP and Alg-CF using the same layer-by-layer concentrations and incubation times described for Si wafers.

TMB Activity of Coated Microbeads: CONP/alginate coated (0, 2, 6, and 12 layers) microbeads were exposed to TMB solution and incubated for 30 min. The reacted TMB solution was transferred to a 96 well plate (Costar, clear bottom, flat bottom) to quantify TMB oxidation (absorbance wavelength 650 nm).

H₂O₂ scavenging assay on coated microbeads: Coated microbeads were separated into 48 well plates (n = 30 per well) and suspended in 0, 25, 50, or 100 μ M of H₂O₂ solution. After 2 h incubation at 37 °C, 50 μ L of the solution was transferred to a 96 well plate for quantification of H₂O₂ via absorbance assay (Enzo Life Sciences). Specifically, 100 μ L colorimetric dye was added and the mixture was incubated at RT for 30 min protected from air and light. Samples were read at 585 nm. For studies that examined repeated exposure to H₂O₂, microbeads (30 per well for each group; 0, 6, and 12 L CONP/alginate) were plated in a 48-well plate in MOPS buffer and exposed to 200 μ L of 100 μ M of H₂O₂ at 37 °C for only 1 h. This incubating solution was then removed for H₂O₂ quantification and the beads were again refreshed with another 200 μ L of 100 μ M of H₂O₂. Beads were incubated for 1 h and H₂O₂ was again measured. This process was repeated one more time for a total of three separate exposures to H₂O₂.

Encapsulation of cell in alginate microbeads: Mouse insulinoma (MIN6) cell line (AddexBio) were cultured in T150 flask as monolayers. Cells were fed (every 2–3 days) with DMEM (DMEM) cell medium supplemented with 15% fetal bovine serum (FBS, Hyclone, Gibco), 1% penicillin/ streptomycin, 1% L-glutamine, 2% sodium pyruvate, 0.9% 2-mercaptoethanol, and 2.5% HEPES buffer. Cells were passed weekly and collected by trypsin digestion from the flask (passage 26 to 45). For alginate encapsulation, trypsinized single cells were homogenously mixed within 1.6% alginate solution at a ratio of six million cells per mL. The cell/alginate mixture was formed into microbeads, as described above. Encapsulated MIN6 cells were incubated overnight in DMEM utilizing 100 mm petri dish. MIN6 cell-loaded microbeads were then washed with MOPS buffer to remove any serum from DMEM, transferred to a 24 well plate, and coated with alternating layers of CONP and alginate, as described above.

H₂O₂ cytotoxicity assay: MIN6 beads (30) were aliquoted into each well (48 well plate) and incubated in MOPS-Glucose (MOPS supplemented with glucose, 0.5 mg of glucose per mL) to prevent cell media antioxidants interfering with the assay. H₂O₂ (25, 50, and 100 μ M) was added to the MIN6 beads and incubated for 2 h at 37 °C. Beads were then washed and assessed via alamar blue assay by adding 10% alamar blue solution (10% alamar blue, 90% DMEM) and incubating the beads for 4 h at 37 °C prior to fluorescence intensity measurement (Em/Ex: 560/590). For the time course experiment, MIN6 beads, coated or uncoated, were incubated with H₂O₂ (25–100 μ M) and assessed for metabolic activity in the same manner as described above. After the first assessment, the same beads were then washed with cell media three times and incubated overnight in cell culture incubator. After overnight incubation, MIN6 beads were then exposed again to 50 μ M of H₂O₂ for 2 h,

washed, and the alamar blue assay was repeated. This was repeated for a total of three times over three days on the same MIN6 beads subsets.

Cellular Oxidant Profile: MIN6 beads (30) were aliquoted into each well (48 well plate) and incubated in MOPS-Glucose with 50 μM of H_2O_2 at 37 $^\circ\text{C}$ for 2 h. Duplicates groups for each treatment set was used, with one group used to measure total glutathione concentration and a second to measure oxidized glutathione (GSSG) concentration. Following H_2O_2 incubation, beads were washed and total glutathione and GSSG was extracted and quantified. Briefly, Promega GSH/GSSG-Glo™ Assay was used, following the manufacturer's protocol with minor modifications to accommodate for alginate encapsulated cells. MIN6 beads were swelled with 100 μL of cell culture grade water, followed by the addition of 25 μL total glutathione lysis reagent (1 μL of Luciferin-NT, 10 μL of passive lysis reagent + 14 μL of water). Oxidized glutathione lysis reagent (1 μL of Luciferin-NT, 10 μL of passive lysis reagent + 14 μL of water + 0.5 mM NEM 0.5 μL) was added the other set of MIN6 beads to neutralize reduced glutathione and extract only oxidized glutathione reagent. Plates were shaken at 500 RPM for 15 min at RT. The generation reagent (50 μL) was then added to each well and incubation for 30 min at RT, followed by the addition of the detection reagent (100 μL) was then added to each well and incubated for 15 min at RT. The resulting solution (100 μL) was then transferred to a 96-well plate and luminescence intensity was read via spectrophotometer.

Glucose Stimulated Insulin Response (GSIR): MIN6 beads (30 per well) were aliquoted into 48 well plates with or without H_2O_2 (50 μM) in MOPS-Glucose at 37 $^\circ\text{C}$ for 2 h. After 2 h incubation, MIN6 beads were washed three times with MOPS glucose, followed by a wash with low glucose Krebs' buffer (LGK; 1.67 mM Glucose). MIN6 beads were then incubated for 90 min in LGK. Samples were then collected, and the beads were washed three times with LGK. MIN6 beads were washed with LGK and incubated for 5 min. Beads were then incubated with high glucose Krebs buffer (HGK; 16.7 mM glucose) for 90 min, followed by sample collection. Beads were then washed in LGK, incubated for 5 mins, and then incubated in LGK for 90 min for final insulin collection. Collected samples were then analyzed for insulin content via ELISA (Mercodia).

Statistical Analysis: Cell-free studies were collected from 9 independent replicates from 3 separate experiments. For MIN6 cells studies, comparisons were made using the same alginate and cell bead preparation with n = 3 biological replicates conducted for each assay; the entire study was then repeated a total of 3 times to validate trends. All values were expressed as mean $\pm$ SD. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). Statistical tests used one- or two-way ANOVA with post hoc Tukey's multiple comparisons. Statistical significance was considered at $p < 0.05$ with designations of **** $p < 0.0001$, *** $p < 0.001$; ** $p < 0.01$; and * $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Schematic of formation of CONP and alginate layer-by-layer coatings onto material surfaces. Slides or beads were incubated in alternating CONP and alginate solutions, up to 6 bi-layer cycles, to form coatings for testing. B) Ellipsometry measurement of layer formation onto idealized planar surfaces with alternating solutions of CONP and alginate or alginate-only (control). C-D) Imaging of LbL coatings on materials using CF-labeled alginate: C) Confocal multi-slice projection images of CONP/alginate coatings, 0-, 2-, 6-, and 12-Layers, formed on silica beads. Scale bar = 100 μm . Far right panel: Single slice cross-section of 12-L coatings. Scale bar = 25 μm . D) Confocal multi-slice projection images of CONP/alginate coatings, from 0 to 12-layers, formed on alginate microbeads. Scale bar = 100 μm . Far right panel: Single slice cross-section of 12-L coatings. Scale bar = 100 μm .

Figure 2.

Quantification of antioxidant scavenging capacity of CONP/alginate coatings on cell-free alginate beads. Beads were coated with 0, 2, 6, or 12 alternating layers of CONP/alginate.

A) Quantification of ambient H₂O₂ levels following 2 hrs incubation with 25, 50, or 100 μM H₂O₂. γ = statistically equivalent to 0 μM H₂O₂ per t-test. B) Ambient H₂O₂ levels following three consecutive 1 hr exposures to 100 μM H₂O₂. γ = statistically different to 1st exposure values for the same group per one-way ANOVA with Tukey post-hoc.

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

Figure 3.

Impact of coatings and ROS exposure on the metabolic activity and antioxidant capacity of entrapped beta cells. A) Ambient H_2O_2 levels 2 hrs after incubation of alginate beads containing MIN6 beta cells coated with 0-, 2-, 6-, or 12-layers of CONP/alginate with 25, 50, or 100 μM H_2O_2 . B) Change in metabolic activity for beta cells within alginate beads coated with 0-, 2-, 6-, or 12-layers of CONP/alginate following incubation with 25, 50, or 100 μM H_2O_2 . Fold change is in comparison to respective H_2O_2 -free controls. Changes in antioxidant capacity of entrapped MIN6 cells following exposure to 50 μM H_2O_2 via quantification of total glutathione (C), total GSSG (D), and the ratio of GSH:GSSG (E). Two-way and one-way ANOVA with Tukey posthoc; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 4.

Impact of coatings and ROS exposure on glucose responsive insulin release (GSIR) from beta cells. A) Beta cells encapsulated within alginate and coated with 0-, 2-, 6-, or 12-layers of CONP/alginate were stimulated under basal glucose (5 mM, checkered bars), then high glucose (25 mM, solid bars), followed by a basal glucose (dotted bars). Inset: Stimulation index (ratio of high glucose stimulation over low glucose stimulation). B) GSIR of beta cells encapsulated within alginate and coated with 0-, 2-, 6-, or 12-layers of CONP/alginate following exposure to H_2O_2 . Inset: Stimulation index for H_2O_2 treated group. * $p < 0.05$; **** $p < 0.0001$

Figure 5. Impact of repeated peroxide exposure on the metabolic activity of encapsulated beta cells. MIN6, encapsulated within alginate and coated with 0-, 2-, 6-, or 12-layer CONP/alginate, were exposed to 50 μM H_2O_2 for 2 h every 24 h for 3 consecutive days. Metabolic activity of the entrapped beta cells was quantified 2 h after adding H_2O_2 *** $p < 0.001$; **** $p < 0.0001$ from non- H_2O_2 treated controls.