Engineering the Multienzymatic Activity of Cerium Oxide Nanoparticle Coatings for the Antioxidant Protection of Implants

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Imbalance of oxidants is a universal contributor to the failure of implanted devices and tissues. A sustained oxidative environment leads to cytotoxicity, prolonged inflammation, and ultimately host rejection of implanted devices/grafts. The incorporation of antioxidant materials can inhibit this redox/inflammatory cycle and enhance implant efficacy. Cerium oxide nanoparticle (CONP) is a highly promising agent that exhibits potent, ubiquitous, and self-renewable antioxidant properties. Integrating CONP as surface coatings provides ease in translating antioxidant properties to various implants/grafts. Herein, the formation of CONP coatings, generated via the sequential deposition of CONP and alginate, and the impact of coating properties, pH, and polymer molecular weight, on their resulting redox profile are described. Investigation of CONP deposition, layer formation, and coating uniformity/thickness on their resulting oxidant scavenging activity identify key parameters for customizing global antioxidant properties. Results found lower molecular weight alginate and pH shift CONP activity to a higher H$_2$O$_2$ to O$_2$-scavenging capability. The antioxidant properties measured for these various coatings translate to distinct antioxidant protection to the underlying encapsulated cells. Information gained from this work can be leveraged to tailor coatings toward specific oxidant-scavenging applications and prolong the function of medical devices and cellular implants.

1. Introduction

A promising antioxidant for local oxidative stress scavenging is cerium oxide nanoparticles. While initially used for nonmedical applications (e.g., corrosion prevention, optical devices, UV absorbers), cerium oxide nanoparticles have been recently explored for biomedical applications as a therapy for redox imbalances in inflammatory diseases, such as cancer, diabetes, wound healing, neurodegenerative, and cardiovascular conditions.[1,2] Cerium oxide exists in two states: cerium sesquioxide (Ce$_2$O$_3$) in the trivalent (Ce$^{3+}$) state; and cerium dioxide (CeO$_2$) in the tetravalent (Ce$^{4+}$) state.[3] Cerium oxide uniquely undergoes continuous valence state cycling, permitting self-renewal after interacting with different reactive species. The capacity to rapidly cycle between valence states provides multienzymatic antioxidant activity. Specifically, a shift from Ce$^{3+}$ to Ce$^{4+}$ results in superoxide dismutase (SOD)-mimetic activity, which scavenges superoxide (O$_2^-$), while a shift from Ce$^{4+}$ to Ce$^{3+}$ provides catalase-mimetic activity, which deactivates H$_2$O$_2$.[4] This theoretically inexhaustible redox capability sets cerium oxide apart other metal oxides such as transition metals (e.g., iron, zinc, and titanium) and other lanthanides (yttrium).[5,6] In the nanoparticle form, cerium oxide nanoparticles (CONP) exhibit elevated catalytic capabilities, as its high surface-area-to-volume leads to increased oxygen vacancies and a higher ratio of Ce$^{3+}$ to Ce$^{4+}$.[7] The CONP valence ratio can be further manipulated by intrinsic factors, such as the synthesis method, aging, surface stabilizers, and size.[8-11] However, extrinsic factors such as temperature, pH, and interactions with specific molecules can also influence the Ce$^{3+}$/Ce$^{4+}$ ratio.[8,12,13] CONP have been explored with these appealing properties as a treatment for a wide range of inflammatory diseases. These antioxidant nanoparticles can be engineered to target specific diseased tissues and cells and modulate the redox activity according to its microenvironment.[8] For biomedical implant applications, however, the infusion of nanoparticles into the implant site presents challenges in retention and potential clinical toxicity.[14] To bypass these issues, several groups have immobilized nanoparticles within various biomaterials to incorporate superior redox properties of CONP and circumvent toxicity concerns.[15] Leveraging established methods for

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integrating nanoparticles into nanoscale coatings via layer-by-layer (LbL).

CONP was recently immobilized into a conformal coating on the implant surface (Figure 1A,B). Functional LbL antioxidant coatings were generated by alternating layers of alginate and CONP onto metallic, ceramic, and polymeric biomaterial surfaces of varying size and shape (Figure 1A,B). Alginate was used as the interconnecting layer due to its antifouling properties and its ease of layer formation with CONP.

Resulting CONP-alginate layers exhibited antioxidant capabilities and downstream cytoprotection, demonstrating this approach’s potential.

To further investigate the utility and tunability of this LbL approach, we sought to investigate critical material parameters that may direct CONP deposition, layer uniformity, and overall antioxidant activity. Herein, we investigated two primary coating parameters: alginate molecular weight and CONP pH (Figure 2A). Different molecular weight alginates were utilized to investigate the potential role of coordination complexes between Ce$^{3+}$ and carboxylate anions ($\text{COO}^-$) on CONP-alginate layer formation and uniformity. We also explored the impact of Ce$^{3+}$/COO$^-$ interactions on subsequent layer redox activity, as modulation of Ce$^{3+}$ availability may shift this antioxidant particle

Figure 1. Formation of CONP-based coatings. A) Ultrathin coatings generated from sequential incubation in CONP and alginate can be formed onto various biomaterial surfaces. B) Investigation of coating glass, hydrogels, and metal alloys via LbL coating with CONP and alginate.

Figure 2. Layer-by-layer coatings of CONP and alginate onto idealized silicon wafers was dependent on material properties. A) Schematic of the six distinct groups tested, screening acidic and neutral pH of CONP in suspension and three alginate molecular weights. B) The effect of these factors on coating thickness and C–E) rate of layering onto planar silica wafers was evaluated via ellipsometry. Two-way and one-way ANOVA with Tukey’s post-hoc test: $^{****p < 0.0001}$, $^{***p < 0.001}$, $^{**p < 0.01}$, and $^*p < 0.05$, where * represents differences between groups and γ compares groups with same alginate MW but different CONP pH.
toward a catalase-mimetic activity.\(^{[12,19]}\) CONP pH was also adjusted from a standard acidic to a net neutral suspension. The pH of the CONP solution can serve multiple roles in coating properties, with the potential to alter not only alginate interactions but also CONP-redox activity.\(^{[20]}\)

Herein, we conducted a comprehensive study on the effect of CONP pH and alginate molecular weight on layer formation and resulting antioxidant activity. First, the development and resulting thickness and uniformity of coatings were evaluated. Next, the CONP concentration, Ce\(^{3+}/\text{Ce}^{4+}\) ratio, and activity of resulting layers were uniquely characterized and contextualized. Finally, the implications of these features in the generation of antioxidant coatings capable of altering deleterious host responses to implants were discussed.

2. Results

2.1. Characterization of the Impact of pH and Alginate Molecular Weight on CONP/Alginate Coatings on Biomaterial Platforms

To investigate the role of CONP and alginate interactions on the layer properties, we manipulated two primary parameters: alginate molecular weight and CONP pH. To modulate alginate molecular weight (MW), high guluronic alginate sources, specifically MVG (MW > 200 kDa), LVG (MW = 200–75 kDa), and vLVG, (MW < 75 kDa), were used. CONP pH was adjusted from its classic acidic suspension (pH 4, designated CONP\(_4\)) to a net neutral suspension (pH = 7.4; designated as CONP\(_{7,4}\)) using sucrose to stabilize the nanoparticles and prevent immediate pH-driven precipitation.\(^{[13,21]}\) Other agents, such as poly(acrylic acid), dextran, sucrose, and citric acid, were screened as potential stabilizers, but sucrose provided simplicity in generation and did not impact CONP activity (unlike other methods). DLS characterization of both CONP\(_4\) and CONP\(_{7,4}\) measured minimal changes in particle size: 6.8 ± 2.5 and 8 ± 4.5 nm, respectively. Although the use of saccharides for CONP stabilization has been previously reported (e.g., glucose and dextran), this is the first report of the use of sucrose, to the best of our knowledge.\(^{[13,21]}\)

The thickness of CONP/alginate layers formed on an idealized planar silica surface was tracked to characterize the impact of CONP pH and alginate MW on layer formation. Six groups were tested: A) CONP\(_4\) and MVG; B) CONP\(_4\) and LVG; C) CONP\(_4\) and vLVG; D) CONP\(_{7,4}\) and MVG; E) CONP\(_{7,4}\) and LVG; and F) CONP\(_{7,4}\) and vLVG (Figure 2A). Each layer was distinctly deposited onto a dry silicon wafer, with excess material rinsed away before the next layer’s addition. Figure 2B shows that coating thickness was measured after every CONP/Alginate bilayer via ellipsometry. Examination of total coating thickness after 12-layers found alginate molecular weight, but not CONP pH, to impact overall layer thickness (p < 0.0001 and 0.121, respectively; two-way ANOVA). A positive correlation was found between alginate molecular weight and coating thickness (r = 0.98 and 0.99 for CONP\(_4\) and CONP\(_{7,4}\), respectively). Results implicate alginate molecular weight as the dominant parameter in layering thickness when coating planar silica surfaces.

After characterizing the total coating thickness at 12 layers, the rate of layer formation at different coating stages was evaluated. Globally, CONP pH impacted the efficiency of layer deposition. Specifically, for the first layers, acidic CONP formed thicker layers more efficiently than neutral CONP (p < 0.0001) with a 40, 37.7, and 20.2% increase in coating thickness for alginate MVG, LVG, and vLVG, respectively, when compared to their CONP\(_{7,4}\) analog (Figure 2C). The phenomenon was reversed for the next four layers for MVG and LVG combinations, where the CONP\(_{7,4}\) accumulated 42.9 (p = 0.002) and 90% (p = 0.0002) faster than CONP\(_4\), respectively. No significant difference in material accumulation between acidic and physiological pH CONP was measured for alginate vLVG for layers four to eight (Figure 2D). For the final four layers, while alginate MVG exhibited no impact in CONP pH, the rate of layer thickness was higher at CONP\(_{7,4}\) than CONP\(_4\) when coating with alginate LVG (108.67%) and vLVG (73.1%). Figure 2E. Ellipsometry results indicate that on a planar, idealized surface, acidic CONP accelerated material deposition during early stages and plateaued at later stages. In contrast, the material deposition was more discrete and linear at physiological CONP. Moreover, the higher MW alginate showed accelerated material deposition at earlier stages.

While ellipsometry is widely used to measure coating thickness at the nanometer scale accurately, measurements must be done under anhydrous conditions. In addition, Lbl coatings can be impacted by the material surface charge, hydrophobicity, topography, and even geometry. To examine the versatility of these coatings to be applied to various surfaces and examine coatings under hydrated conditions, coatings were applied to alginate hydrogel beads. Bright-field microscopy images of alginate microbeads coated with alginate and CONP illustrated cerium within each coating formulation (Figure 3A). Interestingly, coatings showed alginate precipitates at the surface of beads coated when the higher molecular weight alginate (i.e., MVG) was used. In contrast, coatings formed using alginate vLVG exhibited a decreased presence of precipitates.

To further investigate coating morphology, the alginate used in the CONP/alginate coatings was fluorescently tagged and resulting 6- and 12-layer-coated hydrogel beads were visualized by confocal imaging (Figure 3B,D). Image analysis of 3D image projections of coated beads characterized alginate coating uniformity via percent area covered (Figure 3C), while alginate coating thickness was measured from cross-sectional images (Figure 3E).

Visualization of the alginate-carboxyfluorescein (CF) coatings formed using the CONP-alginate layers validated the previously observed differences in alginate precipitation and highlighted differences in alginate deposition. Analysis of alginate coverage implicated differences in coating uniformity associated with alginate molecular weight. The longer chain alginate coatings (MVG) resulted in complete coverage of the hydrogel bead after only six layers, with no difference in percent area covered between 6 and 12 layers (CONP\(_4\); p > 0.9999, CONP\(_{7,4}\); p = 0.61); this indicates that the higher molecular weight alginate results in more efficient microbead coverage. For the lower molecular weights, a more dramatic change between 6 and 12 layers was observed. For example, coatings formed using alginate vLVG-CF exhibited a 60% increase in coverage between layers 6 and 12, regardless of the pH. For the lower molecular weight alginates (LVG and vLVG), the neutral CONP enhanced coating uniformity. Specifically, coatings formed using the intermediate alginate
Figure 3. CONP/alginate LbL coatings onto hydrogels were dependent on coating material properties. A) Bright-field images of alginate microbeads allowed for visual evaluation of overall coating morphology via optical changes in bead transparency. B) 3D confocal projection images of alginate (labeled via carboxyfluorescein) deposited onto alginate microbeads following CONP/alginate coating permitted image quantification of %area coverage of alginate-CF. C) to investigate the effect of MW and CONP pH on overall coating uniformity. D) Cross-sectional confocal images of alginate-CF within CONP/alginate coatings at the center of the microbead permitted image quantification of overall coating thickness E) for each formulation. Scale = 200 μm. Two-way and one-way ANOVA with Tukey’s post-hoc test: ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05, where * represents differences between groups, Δ compares 6- and 12-layer coatings, and γ compares groups with same alginate MW but varying CONP pH.
molecular weight (i.e., LVG) and CONP7.4 exhibited no significant change in coating uniformity between 6 and 12 layers. In contrast, LVG + CONP coated beads exhibited a significant 39.1% increase in percent coverage between 6 and 12 layers ($p < 0.001$). Further investigation of this data showed that the MW of alginate influences the surface coverage (6-layers, $p < 0.0001$; 12-layer coatings, $p = 0.018$). However, the pH during coating fabrication can also influence the uniformity of the coatings (6-layers, $p = 0.0204$; 12-layer coatings, $p = 0.0066$), and the effect of these two factors are dependent on each other (MW $\times$ pH: 6-layers, $p < 0.0001$; 12-layer coatings, $p = 0.0002$). Investigation of individual comparisons show that at acidic pH, surface coverage increases as the MW of alginate increases (6-layers, $p < 0.0001$; 12-layer coatings, $p = 0.031$). At physiological pH, the initial six layers follow this trend ($p < 0.0001$), but 12-layer coatings show higher surface coverage for the lowest MW alginate (vLVG) when compared to LVG ($p = 0.0001$), and insignificantly different to alginate MVG ($p = 0.306$). Individual comparisons of these results are illustrated in Figure 3B and C and statistical differences are further described in Table 1.

To further understand the differences in alginate deposition via CONP/alginate coatings onto hydrogels, a cross-sectional image, approximately halfway through the microbead, was captured to calculate each coating’s ring thickness (Figure 3D,E). After six layers, the effect of alginate molecular weight on layer thickness was only noted for coatings formed using acidic pH. Specifically, while MVG, LVG, and vLVG coatings formed using CONP7.4 exhibited equivalent thickness after six layers, replacement with CONP4 resulted in a positive correlation between coating thickness and alginate molecular weight, with CONP4/MVG resulting in 40% and 52.2% thicker coatings than alginate CONP4/LVG and CONP4/vLVG, respectively. After 12 layers, the effect of CONP pH on the ring thickness followed a similar trend, with the acidic CONP decreasing coating thickness with decreasing MW ($p < 0.001$). Specifically, coatings formed using vLVG exhibited significantly thinner coatings than those formed using MVG and LVG (45.9% and 32.5% decrease, respectively). Furthermore, only coatings formed using alginate vLVG showed a significant change in coating thickness when the CONP pH was adjusted to 7.4, with a 67% increase in thickness ($p < 0.0001$). Overall, evaluating alginate deposition in CONP/alginate coatings onto hydrogel microbeads implicates that acidic CONP results in thinner coatings for the lower molecular weight alginates (LVG and vLVG). The assessment method, however, cannot conclude if these thinner coatings result from tighter CONP-alginate interactions or decreased alginate deposition.

Understanding the effect of these coating formulations on alginate uniformity and thickness can be useful; however, these results can be independent of the amount of cerium captured within each coating formulation, as the amount of alginate deposited is not a direct indicator of cerium concentration within the coatings. To capture this information, coated beads were processed and analyzed via induced coupled plasma-mass spectrometry (ICP-MS). Interestingly, the concentration of cerium did not directly correlate with alginate coating thickness and uniformity. While alginate deposition analysis showed minimal differences between 6- and 12-layer coated beads compared to LVG, the cerium concentration increased twofold for 12-layer versus 6-layer beads for coatings formed using MVG and LVG (Figure 4A). The impact of layer number on cerium content was more pronounced for acidic CONP, with all alginate types exhibiting a twofold to threefold increase in cerium content when the layer number was increased from 6 to 12. The difference in fold-change between acidic and physiological pH (Figure 4B) correlated with ellipsometry results, which showed that the rate of layer formation at acidic pH was higher at early coating stages. In contrast, at physiological pH, there was less variation in rate between layers.

To further understand these results, the cerium concentration per coating was normalized to the coating thickness, as assessed via fluorescent alginate imaging. Results show that the ratio of cerium to coating thickness increased as the number of CONP/alginate bilayers was doubled (Figure 4C). Globally, these results identify the CONP4 and MVG or LVG coatings as superior in CONP deposition after 12 layers, when compared to the other tested combinations. Alternatively, the use of CONP7.4 and vLVG results in the smallest amount of CONP deposition.

### 2.2. Effect of Alginate MW and CONP pH Multienzymatic Activity of Coatings

The goal of developing CONP-based coatings was to provide antioxidant protection at the biomaterial interface. CONP has the potential to impart a spectrum of antioxidant activity, as it can cycle continuously between its tetra- ($\text{Ce}^{4+}$) and trivalent ($\text{Ce}^{3+}$) states (Figure 1C). The $\text{Ce}^{4+}$ state exhibits a more catalase-like activity, while the $\text{Ce}^{3+}$ state mimics superoxide dismutase (SOD). Since the molecular weight of alginate and the pH of CONP during fabrication affected the uniformity and thickness of the coatings, it was hypothesized that these parameters could also impact global oxidant activity. If changing these parameters alter the ratio of available $\text{Ce}^{4+}/\text{Ce}^{3+}$ in the coatings, different coatings could be then be used to target diverse oxidative stress conditions in which either $\text{H}_2\text{O}_2$ or SO is predominant.

To understand the effect of alginate MW and CONP pH in the $\text{Ce}^{4+}/\text{Ce}^{3+}$ ratio, cyclic voltammetry (CV) was used. First, stainless steel (SS) electrodes were coated with the different formulations of CONP and alginate. These coated working electrodes were then analyzed via CV, which permits evaluation of

| Table 1. Effect of MW of alginate on percent surface coverage: mean difference. |
|--------------------------------------|
|**CONP**| **MVG vs. LVG** | **MVG vs. vLVG** | **LVG vs. vLVG** | **CONP7.4** |
|**MVG vs. LVG**| 35.79% ($p = 0.0001$) | 51.82% ($p < 0.0001$) | 16.04% ($p = 0.0137$) |
|**6 layers**| 35.79% ($p = 0.0001$) | 51.82% ($p < 0.0001$) | 16.04% ($p = 0.0137$) |
|**12 layers**| 35.32% ($p = 0.0234$) | 13.29% ($p = 0.0240$) | 6.265% ($p = 0.4286$) |

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the reduction and oxidation potential of the coating (see example traces in Figure 5A,B). The evaluation of the maximum anodic peak on a CV graph provides insight into the electrode’s reductive capacity, while the maximum cathodic CV peak characterizes its oxidative capacity (Figure S1, Supporting Information). Translating this to CONP, an increase in the reductive capacity indicates an elevated Ce⁴⁺/Ce³⁺ ratio and an elevated catalase-mimetic activity. Alternatively, an increase in oxidative capacity translates to a decreased Ce⁴⁺/Ce³⁺ ratio and an elevated SOD-mimetic activity. Thus, evaluating alterations in the coated wire’s anodic and cathodic properties provides insight into the CONP/alginate coating’s preferential redox activity.

All CONP/alginate coating formulations resulted in an increased peak anodic current, when compared to uncoated control electrodes (see, e.g., 12-layer traces in Figure 5A,B). Increasing layers from 6 to 12 further elevated peak anodic levels for all coating combinations, except those formed using MVG alginate. Examination of the peak cathode levels revealed a differing trend, where the presence of the CONP/alginate coating either decreased or unaltered this value compared to uncoated control wires. The quantification of the max anodic and cathodic currents of 12-layer coatings measured via CV is summarized in Figure S1, Supporting Information. The measured anodic max current was significantly impacted by both alginate type and CONP pH (p < 0.0001 for both parameters; two-way ANOVA), with coatings formed using LVG alginate, imparting the most consistent and significant effect. Coatings formed using LVG were not impacted by pH (p = 0.81), while coatings formed using vLVG were dramatically affected (56.1 vs. 15.5 μAmps for CONP4 versus CONP7.4; p < 0.0001). Despite these differences, results collectively indicate that CONP/alginate coatings increase the metal wire’s reductive capacity, thereby exhibiting elevated catalase-mimetic activity. Examination of the cathodic properties shows that CONP/alginate imparted minimal effects on oxidative
capacity. Only the CONP/4/MVG and CONP/7.4/vLVG coatings significantly decreased the maximum cathodic current compared to uncoated wire. This decrease in oxidative activity indicates that these specific coatings may exhibit decreased SOD-mimetic activity. A caveat of this analysis was that these max peak current changes were dependent on several factors, including the cerium concentration deposited onto the wires.

Since a higher cerium concentration can skew the max peak current, a ratio of the max anodic current to the max cathodic current was calculated (Figure S1, Supporting Information).[22,23] For MGV-based coatings, the Ce4+/Ce3+ ratio was unaltered when the number of layers was increased from six to twelve \((p = 0.0619)\), regardless of the CONP pH used. In contrast, for the lower molecular weight alginites of LVG and vLVG, the addition of six new layers increased the Ce4+/Ce3+ ratio, independent of the CONP pH \((p < 0.0001)\) for all. A focused analysis of the 12-layer alginate coatings found both CONP pH and alginate molecular weight significantly impacting the Ce4+/Ce3+ ratio \((p = 0.005\) and < 0.0001, respectively), with MGV exhibiting the lowest Ce4+/Ce3+ ratio when compared to coatings formed using the lower MW alginates. Coatings formed using vLVG were most impacted by CONP pH, with a significant decrease in catalase-mimetic activity when CONP pH was increased from 4 to 7.4 \((p = 0.0037)\). Overall, the normalization of the anodic to the cathodic peaks showed a positive correlation between decreasing alginate molecular weight and the Ce4+/Ce3+ ratio \((p < 0.0001)\). There was also a positive correlation between the acidity of CONP during coating fabrication and the Ce4+/Ce3+ ratio \((p < 0.0054)\). However, pH only impacted the Ce4+/Ce3+ ratio for lower MW alginate, due to the interaction between these two factors \((p < 0.0008)\). Overall, analysis of the synergistic effect of CONP pH and alginate MW on the coatings’ multienzymatic activity indicates that an array of coating formulations can induce in a wide range of antioxidant properties.

2.3. Characterization of Scavenging Capabilities in Hydrogels

CV indications of skewed multienzymatic activity of CONP/Alginate coatings was validated by incubating coatings with distinct environmental oxidants. For evaluation of SOD-mimetic activity, superoxide (SO) scavenging was measured. For catalase-mimetic activity, TMB and H2O2 oxidation, was measured. Tests were conducted for CONP/Alginate coatings formed onto alginate microbeads.

For SO generation, xanthine and xanthine oxidase was added to the incubation media. All CONP coating formulations demonstrated SOD-mimetic activity with significant SO scavenging, when compared to uncoated controls \((p < 0.0001)\); however, there was no difference in SO consumption between any of the coating groups \((p = 0.097)\) (Figure 6A,B).

The catalase-mimetic activity was assessed in coated alginate beads via oxidation of TMB substrate, as TMB exhibits a color change when CONP is reduced from Ce4+ to Ce3+. For all CONP coating formulations, TMB absorbance values in the incubating solution were elevated, compared to uncoated controls, indicating increased TMB oxidation (Figure S2, Supporting Information). Additionally, increasing the layers from six to twelve further increased ambient TMB oxidation for all groups, except for CONP/7.4/MVG. Coatings, however, also exhibited a blue color following TMB exposure, indicating binding of the oxidized TMB within the CONP/alginate layers (Figure S2, Supporting Information). Since the assay only measured color changes in the incubating solution, the full catalase-like activity of the coatings was not fully captured by this method.

Figure 5. The redox activity of different formulations on coated stainless-steel wires establishes variance of activity with CONP/alginate coating properties. Representative traces of reduction/oxidation curves for wires coated using A) acidic and B) neutral pH CONP assessed via cyclic voltammetry (CV). Dashed lines: Max peak cathodic and anodic currents (as indicated) from uncoated wire. C) Since variations in cerium concentration deposited onto the wires.

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As catalase activity accelerates H₂O₂ oxidation into oxygen, the CONP coated microbeads' capacity to deactivate H₂O₂ was quantified.[24] In addition, to explore the full potential of CONP scavenging capabilities, the same coated beads were exposed to a total of three distinct and sequential challenges of 100 μM H₂O₂. On the first H₂O₂ challenge, 12-layer beads coated with CONP₄ showed similar activity, although LVG coatings were slightly (14.9%) more active than vLVG (p = 0.0002). At physiological pH, alginate LVG contained the moderately higher scavenging capability (11–14%) compared to both MVG (p = 0.003) and vLVG (p = 0.0006). After two additional H₂O₂ bolus challenges, the scavenging capabilities between alginate LVG was still superior to vLVG, regardless of pH (CONP₄ and CONP₇.₄ p = 0.04 and 0.017, respectively). There was no difference between MVG and the lower MW alginate coatings at either or physiological pH. Globally, acidic conditions did not impact the H₂O₂-scavenging capabilities of 12-layer coatings (p = 0.198). Examination of six-layer coatings indicated exhaustion of H₂O₂-scavenging capabilities for coatings formulated using physiological pH, with the greatest decline in activity measured for CONP₇.₄/vLVG coatings (see Figure 7A,B).

As reported earlier, the CONP content among coating formulations varied, and the scavenging capability is dependent on the amount of cerium. To incorporate this parameter into assessment of enzymatic activity, the H₂O₂ scavenging capacity for each coating formulation was normalized to its measured cerium content (Figure 7C,D). As shown, 12-layer coatings formed using the lower MW alginate (vLVG) were superior in catalase-mimetic antioxidant activity at both acidic (p = 0.0007 and < 0.0001 for LVG and MVG, respectively) and at physiological pH (p < 0.0001 for both LVG and MVG). Furthermore, an increase in CONP pH resulted in increased H₂O₂-scavenging per cerium content for both 6- and 12-layer beads (p < 0.0001). Specifically, 12-layer coatings showed that CONP₇.₄/MVG and CONP₇.₄/vLVG formulations have a higher H₂O₂-scavenging capability than these coatings formed using acidic pH CONP (p = 0.0177 and p = 0.0002, respectively). These results confirm that an increase
in catalase-mimetic activity can be achieved by decreasing the MW of alginate and increasing the pH of CONP during fabrication.

Overall, the H$_2$O$_2$-scavenging studies validate the CV results that a decrease in MW of alginate and an increase in CONP pH increases the coatings’ catalase-/SOD-activity ratio. The disparity in MW between MVG and vLVG allowed us to understand the effect of MW on CONP redox orientation.

2.4. Examination of H$_2$O$_2$/SO Protection of Coatings on Encapsulated MIN6 cells

Potent antioxidant coatings can protect tissue grafts from local oxidative stress generated by the host in response to the implantation procedure. In one approach, antioxidant coatings can serve to protect transplanted insulin-producing β-cells from the deleterious effects of extracellular reactive oxygen species generated at the implant site. To assess the ability of CONP/alginate coatings to protect β-cells from oxidative stress, MIN6 β-cells were encapsulated within alginate microbeads coated with or different formulations of CONP and alginate.

To understand the baseline impact of CONP/Alginate coatings on beta-cell metabolism, metabolic activity was assessed postcoating. For this work, coatings generated using alginate MVG and vLVG were studied, given that they exhibited the most distinct differences in coating properties and activity. For all formulations tested, six-layer coatings did not impact cellular metabolic activity ($p = 0.3037$; Figure S3A, Supporting Information). However, at the 12-layer dosage, selected formulations decreased cellular responses. Specifically, CONP$_{4}$/MVG coatings imparted the most impact, with a decrease of 18.9% in metabolic activity when compared to the uncoated control ($p < 0.001$; Figure 8A). Decreasing the MW of alginate mitigated the effect, while replacing the CONP to net neutral reduced the negative impact of the MVG coating to ≈5%. Combining both a neutral CONP and a lower molecular weight alginate (vLVG) did not synergize to decrease the coating’s effect in cell metabolism further, as the results were insignificantly different to CONP$_{7.4}$/MVG and CONP$_{4}$/vLVG coatings ($p > 0.999$ and 0.271, respectively). Globally, while alternations could decrease the cell impact to only ≈5%, both parameters of CONP pH and alginate molecular weight had a significant impact on the base metabolic activity of the encapsulated cells ($p < 0.0001$ and 0.0002, respectively; two-way ANOVA).

To further investigate the coating effect on beta cell health and function, insulin secretion in response to a high glucose challenge (i.e., 16.7 mM) was quantified. There was no significant difference between 0-L controls and 12-L coated beads, regardless of formulation (Figure 8B, $p = 0.0619$). Results implicate that coatings did not impact insulin secretion, both in terms of cell function and protein diffusion out of the coated microbeads.

Additional investigation into the antioxidant profile of the encapsulated cells was also conducted via measurement of both reduced and oxidized glutathione (GSH and GSSG, respectively) via a flow cytometer.

**Figure 8.** The impact of CONP/alginate coatings on the baseline beta cell metabolic activity was dependent on coating material properties. A) The baseline metabolic activity and B) insulin secretory response to a glucose challenge of encapsulated cells following coating with 12 layers of the designated coating formulations. C) The effect of 12-L coatings on oxidative cell health was measured via reduced:oxidized glutathione (GSH:GSSG) ratio, D) total glutathione, and E) oxidized glutathione. Gray dashed line: untreated controls. Two-way and one-way ANOVA with Tukey’s post-hoc test: ****$p < 0.0001$, **$p < 0.001$, *$p < 0.01$, and *$p < 0.05$, where * represents differences between groups, Δ compares 12-L coatings to 0-L beads, and γ compares groups with same alginate MW and varying pH of CONP.
total intracellular glutathione, which includes both reduced glutathione (GSH) and oxidized glutathione (GSSG).\textsuperscript{25} In addition, GSSG levels were quantified. Globally, the GSH:GSSG ratio provides a comparable index of oxidative cell health, with reduced ratios implicating increased oxidative stress.\textsuperscript{26} As summarized in Figure 8C, coatings formed using acidic CONP imparted no change (\(p = 0.999\) and 0.468 for coatings formed using MVG or vLVG respectively). Coatings using CONP stabilized at physiological pH exhibited increased oxidative cell health, as indicated by an increased GSH:GSSG ratio (MVG, \(p < 0.001\); vLVG, \(p = 0.0015\)). The beneficial impacts of coatings formed using vLVG appear to be driven by elevated total glutathione levels, while coatings formed using CONP\(_4\) coatings caused a decrease in total glutathione levels (Figure 8D). These impacts correlate with metabolic activity results. Of interest, all CONP-based coatings conveyed a significant decrease in the presence of oxidative glutathione when compared to uncoated controls (Figure 8E).

Despite the modest decrease in baseline metabolic activity for selected coatings, a robust antioxidant protection would impart a net positive impact on prolonging the survival of encapsulated cell therapies. To examine the potency of the various coating formulations on protecting the underlying cells from oxidative injury, coated microbeads were incubated with either H\(_2\)O\(_2\) or SO. Following the oxidative challenge, downstream impacts on metabolic activity were assessed and compared to unchallenged controls. The exposure of uncoated microbeads to H\(_2\)O\(_2\) or SO conveyed significant impacts on the metabolic activity of the encapsulated cells, with a 33.3% or 34.2% decrease in cellular metabolic activity, respectively. Coating encapsulated cell microbeads with six layers of CONP and alginate did not impart substantial protection, regardless of the formulation (Figure S3B, Supporting Information). Increasing the layer number to 12 layers showed enhanced protection, depending on the coating formulation (Figure 9A). Specifically, beads coated with CONP\(_4\)/MVG fully protected against both H\(_2\)O\(_2\)- and SO-mediated loss in metabolic activity, with values insignificantly different to the non-challenged controls (\(p = 0.635\) and 0.704, respectively). Alternatively, coatings with CONP\(_4\)/vLVG were only able to fully protect against an H\(_2\)O\(_2\) insult (\(p = 0.814\) and \(p < 0.0001\) vs untreated controls and H\(_2\)O\(_2\)-treated controls, respectively), with no measurable impact on superoxide protection (\(p < 0.0001\) and \(p = 0.845\) vs untreated controls and SO-treated controls, respectively).

Examination of the effect of CONP pH on cytoprotection from external oxidants, coated formed using alternating layers of CONP\(_{7.4}\) and alginate MVG or vLVG were examined. Even though the CONP\(_{7.4}\)/MVG exhibited an approximately twofold increase in CONP content over the CONP\(_{7.4}\)/vLVG formulation, their protective impact was generally similar. When exposed to an exogenous H\(_2\)O\(_2\) challenge, both MVG and vLVG coatings formed using CONP\(_{7.4}\) imparted a protective effect, mitigating declines in metabolic activity from 33% to only 7.34% and 6.76%, respectively (\(p < 0.001\) for both when compared to treated controls). Resulting metabolic activities for CONP\(_{7.4}\)/MVG and CONP\(_{7.4}\)/vLVG coated cells were insignificantly different (\(p = 0.999\)) although they technically imparted different statistical benefits when compared to untreated controls (\(p = 0.06\) and

Figure 9. Protection of encapsulated beta cells from exogenous ROS insults was dependent on coating material properties. The impact of oxidative stress from either H\(_2\)O\(_2\) or SO on the underlying cells was evaluated and summarized as fold change from untreated controls (gray dashed line). A) Metabolic activity and B) insulin secretory response to a glucose challenge of encapsulated cells following treatment with H\(_2\)O\(_2\) or Xa/XO (SO generating). Uncoated or microbeads coated with 12-layers of the designated coating formulations were tested. C) The effect of 12-L coatings on protecting cells was also measured via reduced:oxidized glutathione (GSH:GSSG) ratio, D) total glutathione, and E) oxidized glutathione. Gray dashed line: untreated controls. Two-way and one-way ANOVA with Tukey’s post-hoc test: \(*\*\*p < 0.0001, \***p < 0.001, \**p < 0.01, \*p < 0.05, \) where * represents differences between groups, \(\Delta\) compares 12-L coatings to 0-L beads, and \(\gamma\) compares groups with same alginate MW and varying pH of CONP.
For Xa/XO challenged microbeads, the protective nature of the coatings was less pronounced with a modest, but significant, shift from 34.1% (untreated controls) to 25.3 and 21.2% (p < 0.0001) for MVG and vLVG coated beads, respectively; this level of protection was not comparable to untreated controls (p < 0.0001 for both coating formulations). Even though different coating formulations had varying protective effects when assessed via metabolic activity, insulin secretion in response to glucose was minimally altered (Figure 9B). It should be noted, however, that characterizing beta cell functional health under acute oxidative stress via insulin release is complicated, as oxidative insults can stimulate insulin secretion.[27]

To investigate coating impacts on oxidative cell health, intracellular glutathione levels (total and oxidative) was quantified for cells within coated or uncoated beads exposed to an ROS challenge (Figure 9C–E). In concordance with metabolic activity results, uncoated beads showed a significant decrease in GSH:GSSG ratio following H$_2$O$_2$ (p = 0.0032) and Xa/XO (p = 0.0017) challenge, implicating a decrease in intracellular antioxidant protection. Interestingly, this altered ratio was not due to changes in total glutathione concentrations (p = 0.455 and 0.957 for H$_2$O$_2$ and Xa/XO when compared to untreated controls). Instead, there was a significant elevation in the presence of intracellular oxidized glutathione in both H$_2$O$_2$ and Xa/XO treated uncoated controls (p < 0.0001 for both).

For encapsulated cells coated with CONP-based layers, changes in their antioxidant potential was dependent on the coating composition. CONP$_4$/MVG coated cells treated to H$_2$O$_2$ exhibited an unaltered GSH:GSSG ratio, when compared to unchallenged coated controls (p = 0.538); however, this formulation did not prevent a drop in GSH:GSSG ratio when challenged with Xa/XO (p = 0.0011). This alteration was associated with a decrease in total glutathione (p = 0.0035). Coatings formed using CONP$_4$/vLVG exhibited declines in GSH:GSSG ratio following exposure to either H$_2$O$_2$ and Xa/XO (p < 0.0001). For H$_2$O$_2$ exposed CONP$_4$/vLVG coatings, these intracellular antioxidant alterations were conveyed by elevated levels of oxidized glutathione (p = 0.042 compared to untreated), as total glutathione concentrations remained unchanged (p = 0.206 compared to untreated). For Xa/XO exposed CONP$_4$/vLVG coatings, changes were caused by lower levels in both total and oxidized glutathione (p = 0.014 and < 0.0001, respectively). Finally, none of the coating formulations using CONP$_{7,4}$ resulted in preservation of their untreated intracellular antioxidant levels, regardless of the type of ROS exposure (p < 0.0001).

3. Discussion

An imbalance in oxidants can initiate an endless loop between inflammation and oxidative stress. Research groups have addressed this challenge by engineering methods to supplement biomaterial implants with antioxidant properties. These approaches include redox-sensitive biomaterials such as poly(thiolketal) urethane (PTK-UR), that can serve as an oxidant sponge and deliver therapeutic agents as the material degrades.[28,29] Alternative approaches are based on forming hydrogen-bonded polyphenols onto biomaterials and tissue grafts to protect from oxidative-stress-mediated cytotoxicity.[30] In addition to protecting tissue grafts from oxidative damage, these materials can further mitigate inflammation and create a balance between inflammatory and tolerogenic phenotypes.[12–14] Although these antioxidant approaches show potential, the materials used in these studies exhibit limited redox capabilities and self-renewing potential.

For longer term studies, metal oxide nanoparticles such as cerium oxide, manganese, zinc, titanium, and yttrium are more appropriate, as their catalytic activity per unit volume is substantially higher and more durable than that of polyphenols and redox-sensitive polymers.[6,31] Of these catalytic nanoparticles, cerium oxide exhibits superior self-renewability potential and multi-enzymatic activity.[11,19,32] While CONP is a promising antioxidant/anti-inflammatory therapeutic for a wide range of biomedical applications, systemic administration of nanoparticles still raises concerns regarding toxicity and clearance.[14]

Therefore, our group has engineered an LbL technique to immobilize redox-active nanoparticles by alternatively depositing CONP and alginate onto the surface of biomaterials. In previous work, resulting LbL coatings exhibited redox activity that protected encapsulated murine beta-cells from H$_2$O$_2$-/SO$_2$-mediated decreases in metabolism, oxidative stress, and functionality.[17]

In the current work, CONP/Alginate coating combination was further investigated to exploit its full potential. Based on previous literature, it was hypothesized that factors such as molecular weight (MW) of the intermediate anionic layer and the net pH of the CONP could impact the thickness, uniformity, cerium content, and redox activity of the coatings through CONP/alginate interactions.[10,33–37] Potential interactions between CONP and alginate that can drive the formation of specific nano- and micro-complexes include: i) charge, ii) acidic precipitation of alginate, iii) CONP precipitation onto biomaterials surfaces, iv) hydrogen bonding, v) chelating ligands, and vi) covalent complexes.[38] Manipulation of CONP pH and alginate MW can dictate the primary type of interactions driving CONP/alginate complexes, which can play key roles in controlling coating thickness, uniformity, Ce$^{4+}$/Ce$^{3+}$ ratio, and, subsequently, overall enzymatic activity.

Layer-by-layer deposition for different formulations of CONP and alginate resulted in varying thickness and layering rates. As reported in previous LbL studies, polyelectrolyte complexes formed using higher MW polymers typically leads to thicker coatings.[15] For coatings formed using acidic CONP, a positive correlation between high molecular weight alginate and coating thickness was measured via both ellipsometry and fluorescent imaging quantification. The investigation into the efficacy of layer formation was another parameter of interest. Although many LbL coatings grow linearly, several material combinations, such as poly(t-lysine) (PLL)/alginate and PLL/hyaluronic acid, deposit layers in more of an exponential-like manner.[19] Similarly, CONP/alginate coatings formed using acidic CONP exhibited a more exponential growth behavior, followed by linear growth. A steep increase in thickness at the early stages, followed by stabilization at later stages of layering, is a characteristic pattern of several polyelectrolytes and nanoparticle coatings.[40] This is postulated to be due to surface aggregation of the nanoparticles.[41] Furthermore, higher MW polymers yield a steeper exponential growth phase than lower MW polymers, which
explains the higher growth rate of alginate MVG and LVG compared to vLVG at early stages.[131]

Previous reports have also shown that the coating thickness of weak polyelectrolyte complexes can be controlled by changing the pH during fabrication.[135,136] A comparison of coatings formed using a net neutral CONP validated these correlations, as these layers exhibited a more discreet and linear layer deposition that was not significantly impacted by alginate MW. These trends correlate with previous studies showing that polycations exhibit the highest increase in layer thickness at pH 4–6.[135] Even though the exponential growth behavior of CONP/alginate complexes was accelerated at acidic pH, the self-precipitation of alginate may be another contributing factor to an early increase in thickness.[20] Overall, under both dry or hydrated conditions, the lower MW alginate exhibited more disparate differences in coating thickness and uniformity between early and later stages of coatings.

Independent of coating thickness and coverage, the MW of alginate and CONP pH influenced the amount of CONP entrapped within the coatings. As expected, the concentration of cerium deposited in the coatings increased between 6 and 12 layers, despite no significant change in coating uniformity on beads. In addition, the concentration of cerium deposited on hydrogel microbeads was positively correlated to alginate MW, with decreasing CONP presence as the alginate MW decreased. CONP pH also played a key role, with the acidic formulation resulting in enhanced CONP coating presence. Normalization of CONP to coating thickness indicated elevated CONP capture efficiency when the neutral CONP formulation and lowest MW alginate was used. Examination of these global trends, indicates that alginate MW and CONP pH impart changes in the interactions between these two materials, resulting in variability in CONP capture efficiency and global layer formation.

Investigation into the established crosslinking behavior of alginate can provide insight into potential CONP-alginate interactions. In alginate cationic crosslinking, cations preferentially align along the longest alginate junctions before creating new junctions.[42,43] Under this assumption, higher MW alginites should exhibit faster-crosslinking kinetics due to decreased loose-end fraction, which would allow for elevated accommodation of the cationic CONP between adjacent alginate chains.[43] Moreover, high MW chains are more flexible than lower MW alginate, which are more prone to shrink and contract, thereby contributing to intrachain dimerization or precipitation.[43,44] Acidic conditions can further exacerbate this precipitation since, the alginate chain is composed of mannuronic and guluronic acid monomers, which have pK values of 3.38 and 3.65, respectively.[20] Thus, based on published reports, it is postulated that the higher MW alginites were more prone to precipitation, which was intensified by the presence of the acidic CONP.

To investigate if these distinct CONP-alginate interactions induced variability in antioxidant features, CV electrochemical analysis was employed.[42] CV analysis of coatings showed anodic and cathodic peaks, indicating that all CONP coatings exhibited fully reversible oxidation and reduction. Examination of differences in the peak magnitude from the cathode and anode regions during electrochemical stimulation indicated differences in the reduction and oxidation potential of the different coating formulations. As the magnitude of these peaks can be influenced by the concentration of cerium deposited onto the SS electrode, the ratio of anodic/cathodic current, and hence the Ce⁴⁺/Ce³⁺ ratio of the coatings, was investigated and correlated to its overall antioxidant behavior.

The molecular weight of the alginate substantially altered the Ce⁴⁺/Ce³⁺ ratio of the embedded CONP. Specifically, formulations using alginate vLVG exhibited the highest Ce⁴⁺/Ce³⁺ ratio, while coatings formed using alginate MVG exhibited the lowest Ce⁴⁺/Ce³⁺ ratio. The mechanism driving this observed correlation is not completely clear; however, it is postulated that these shifts are due to the type of interactions between alginate and CONP. Due to the decreased bias of lower MW alginate to self-precipitation, there is enhanced potential for coordinated cerium-alginate interactions, specifically complexes between Ce³⁺ in the CONP and carboxylate anions (i.e., COO⁻) in alginate.[12] As the hard and soft acid and base (HSAB) theory explains, Ce³⁺, a hard acid, can bind to soft bases such as phosphates or hydroxides over sulfates and hydroxides.[12] Strong bases, such as phosphate or carboxylate anions, can bind to Ce⁴⁺, which can block reversible switching to Ce⁴⁺.[45] The inhibition of Ce⁴⁺ by carboxylate anions interactions shifts the overall activity of CONP toward Ce⁴⁺, which it already favors due to its xenon-like configuration.[19] Therefore, an enhanced presence of coordinated CONP-alginate complex would result in a coating biased toward a catalase-mimetic activity. Contrarily, the highest MW alginate, MVG, likely exhibited the lowest Ce⁴⁺/Ce³⁺ ratio due to a shift from coordinated CONP-alginate complexation to self-participation, which would preserve the baseline Ce⁴⁺/Ce³⁺ ratio of the CONP. This formulation would also result in enhanced CONP deposition, as validated in the ICP-MS measurements. Coatings formed using the alginate LVG appear to follow a more complex pattern, as its MW range is between the other alginites (MVG > LVG > vLVG) and contains both long and short strands. Due to this characteristic, alginate LVG likely interacts with CONP through both precipitation and coordinated complexes, depending on the MW of each alginate strand. While longer chains will accommodate cations along the length of a chain before it crosslinks with a new chain,[42,43] the short chains will interact with Ce³⁺ in a discrete manner similar to vLVG, which would shift the redox activity to a SOD-mimetic activity. Resulting coatings formed using LVG would exhibit more CONP deposition, due to enhanced self-precipitation, but also elevated CONP-alginate complexes. Thus, LVG coatings exhibit a global redox potential in between the two other alginate types.

In addition to alginate MW, the effect of pH also plays a role in the Ce⁴⁺/Ce³⁺ ratio of coatings. Several groups have reported changes in redox activity due to pH.[10,42,17,46] There were no measurable differences in redox activity between CONP₄/MVG and CONP₇,₄/MVG, although there was a 1.6-fold decrease in CONP integration. These results further implicate self-precipitation as the dominant interaction, whereby the pH does not alter CONP activity, but lower pH elevates CONP self-precipitation during coating. For the lowest alginate MW (vLVG), the acidic CONP elevated the Ce⁴⁺/Ce³⁺ ratio of the resulting coatings compared to neutral CONP coatings. This shift has been observed in previous publications, in which an acidic...
environment leads to an increase in catalase-mimetic (Ce$^{4+}$) activity.

Once the effects of MW and pH during fabrication were fully characterized, each formulation’s properties were assessed in their ability to mitigate different sources of oxidative stress in the microenvironment. Even 6-layer CONP$_{7.4}$/vLVG, which consumed the least amount of H$_2$O$_2$ (1.043 μM min$^{-1}$), has an equivalent scavenging rate to catechol enzyme (1 μM min$^{-1}$). For the surface area of nanoscale CONP coatings, the H$_2$O$_2$-scavenging rate is comparable to bulk antioxidants such as PTK-UR and to nanoscale antioxidants such as polyphenols.

The catalase-mimetic activity of different coating formulations was tested via TMB oxidation assay, showing equivalent activity between MVG and vLVG. Even though these two coating formulations have different cerium content, the catalase-mimetic activity of vLVG coatings was higher. However, this assay is surface-based, and the change of absorbance in the liquid might not represent the full TMB oxidation. For that reason, catalase-mimetic activity was measured via exposure to three sequential challenges with cytotoxic levels of H$_2$O$_2$ consumption. The H$_2$O$_2$ consumption assay showed similar results to TMB oxidation, where MVG and vLVG had similar scavenging capability. However, coating formulations with alginate vLVG contain less cerium than those of MVG. Normalization of H$_2$O$_2$ to the total cerium content shows that lower MW alginate shifts the activity of the coatings toward a higher catalase/SOD ratio. These results validate the theory explained above that CONP interacts with lower MW alginate primarily via Ce$^{3+}$/COO$^-$ coordination complexes rather than precipitation. These interactions lead to Ce$^{3+}$ inhibition (responsible for SOD activity), in which case the primary redox modulator is Ce$^{4+}$ (responsible for catalase activity).

Although hydrogen peroxide consumption results concur with the various techniques used to assess the Ce$^{4+}$/Ce$^{3+}$ ratio, it is important to point out the caveats of the H$_2$O$_2$ consumption assay: 1) The scavenging capabilities of the coatings were not fully exhausted, and the long-term capacity of these coating are yet to be investigated; and 2) beads were incubated with H$_2$O$_2$ for 1 h before measuring scavenging capabilities, which is enough time for these molecules to diffuse through the bead. Even though this data is an accurate indicator of the scavenging rate of H$_2$O$_2$, these results might not result in cell protection if H$_2$O$_2$ diffuses freely through the coatings.

As a final validation of the tunability of the antioxidant coatings in terms of redox activity, the ability of the different coating formulations to protect encapsulated cells was assessed by exposing coated and non-coated encapsulated cells to SO or H$_2$O$_2$. Each formulation showed specificity in protection for either SO or H$_2$O$_2$, depending on coating uniformity, cerium content, and Ce$^{4+}$/Ce$^{3+}$ ratio. Beads coated with CONP$_4$/MVG were the most efficient formulation at protecting cells from both SO and H$_2$O$_2$. While CV results found the CONP$_4$/MVG formulation exhibited a preference to the SOD-mimetic side of the redox spectrum, it is suspected that the high concentration of cerium captured in this coating supported the equal protection against H$_2$O$_2$ and SO. Interestingly, CONP$_4$/vLVG exhibited a unique preferential protection against only a H$_2$O$_2$ challenge. While coatings formed using alginate vLVG contained almost a twofold lower cerium concentration than coatings formed using alginate MVG, the global shift of these vLVG coatings toward a higher Ce$^{4+}$/Ce$^{3+}$ ratio allowed the CONP$_4$/vLVG coating to be as effective as CONP$_4$/MVG coatings in catalase-mimetic activity. However, this preferential catalase activity was insufficient in preventing an H$_2$O$_2$-induced decrease in the GSH:GSSG ratio. In addition, coatings formed using CONP$_{7.4}$ were not as effective in protecting encapsulated cells as their acidic pH counterparts, indicating that their decreased CONP presence reduced their global enzymatic activity.

To expand on coating modularity, manipulations in layer formation, and in material selection can be explored. For example, alternating or block layering of alginate and CONP$_4$ and CONP$_{7.4}$ may result in different layer thicknesses, as well as antioxidant activity, thereby creating coating that excel in broader ROS protection. In addition, other materials besides alginate can be investigated, such as poly(lactic-co-glycolic acid), gelatin, or cellulose acetate. Furthermore, the encapsulation of CONP with polymers (e.g., polystyrene sulfonate) or surface functionalization could expand its capacity to layer or link to numerous other polymers.

Overall, investigation into the effects of CONP and alginate parameters on antioxidant coatings and activity revealed the capacity to modulate antioxidant properties, as well as coating features. The ease in which CONP/alginate coatings were formed on silica, metal, and hydrogels further illustrates the utility of this approach for broad translation to numerous medical implants. For example, this method can potentially mitigate fibrosis and oxidant-mediated electrical/mechanical malfunction of continuous glucose monitors (CGM), cardiovascular stents/valves, and orthopedic prostheses. The scavenging of oxidants in the local environment could not only suppress foreign body responses, but potentially protect transplanted cells from the deleterious effects of ROS. Future work will seek to translate these coatings to in vivo applications to examine the impact of this redox modularity on host responses that lead to fibrous encapsulation, elevated adaptive immune response, and damage to the implanted cells. Finally, in addition to antioxidant properties, these CONP-coatings can be explored for their capacity to induce angiogenesis or provide antibacterial protection.

4. Conclusion

Alternating layers of CONP and sodium alginate yielded antioxidant, multienzymatic coatings that can be formed on metallic, ceramic, and polymeric surfaces. The uniformity, thickness, and the ratio of SOD/Catalase-mimetic activity can be manipulated through changes in the molecular weight of alginate and the pH of CONP dispersion. In conclusion, the mechanism driving the coating dictates the activity of CONP in coatings. The two major mechanisms driving CONP/alginate interactions are precipitation and coordination complexes. Higher MW alginate is more susceptible to precipitation, and coordination complexes between Ce and carboxylate anions in alginate are the prominent binding mechanism in lower MW coatings. The primary mechanism in each coating formulation dictates its Ce$^{4+}$/Ce$^{3+}$ ratio. Coatings with lower MW has a higher catalase-mimetic activity due to Ce$^{3+}$ inhibition and increased...
availability of Ce⁴⁺. These controllable antioxidant coatings have a broad range of applications, from increasing the electroconductivity of inert surfaces to protecting tissue grafts from oxidative stress and the potential to mitigate FBR to biomaterial implants.

5. Experimental Section

**Materials:** The cerium(IV) oxide nanoparticles dispersion (CONP, 20% wt/wt in H₂O, low pH, d < 5 nm) was purchased from Alfa Aesar (Cat# 47232). Three PRONOVA UP grade sodium alginites (Alg) with a G/M ratio of 1.5 were purchased from NovaMatrix and consisted of the following: 1) MVG with MW > 200 kDa, 2) LGV with MW 75-200 kDa, and 3) vLVG with MW < 75 kDa. Sucrose (Cat# S7903) and all other chemicals were reagent grade or higher purity and purchased from Sigma Aldrich. Sodium alginates (Alg) with a G/M ratio of 1.5 were purchased from NovaMatrix and consisted of the following: 1) MVG with MW > 200 kDa, 2) LVG with MW 75-200 kDa, and 3) vLVG with MW < 75 kDa. Sucrose (Cat# S7903) and all other chemicals were reagent grade or higher purity and purchased from Sigma Aldrich.

**Preparation of CONP at pH 7.4:** The CONP dispersion, as received from the manufacturer, had a pH of 4. To adjust to pH 7.4, the 20% CONP dispersion was diluted in MOPS-MV buffer (10 mM sucrose, 10 mM MOPS, 125 mM NaCl, 3 mM KCl, pH 7.4) to a final concentration of 3 mg mL⁻¹ and pH adjusted to 7.4 with 1 M NaOH. Nanoparticle size was acquired following the DLS methods explained in Section 5.1. However, nanoparticles were diluted in a 40 mM sucrose-MOPS/KNO₃ buffered solution.

**Ellipsometry:** A silicon wafer (Si wafer, single side polished, no dopant, 2 in. diameter × 0.5 mm thick) was cut into 11 × 13 mm² pieces. Before coating, the Si wafer was first cleaned with acetone (3 ×) and plasma treated for 5 min at high-intensity settings using a Plasma Cleaner (Harrick Plasma, PDC-001-HP). The Si wafer was placed in a glass test tube and incubated with a dispersion of CONP (3 mg mL⁻¹) for 30 s, followed by washes of MOPS-buffer (3 ×) and -ionized water (1 ×). The Si wafer was then incubated with a solution of sodium alginate (3 mg mL⁻¹) for 30 s, followed by washes of MOPS-buffer (3 ×), -ionized water (1 ×), ethanol (3 ×), and dried with a stream of argon. The Si wafer thickness was measured every bilayer using a J. A. Woollam Co. alpha-SE spectroscopic ellipsometer, at an angle of 70° and the potential to mitigate FBR to biomaterial implants.

**Microbead Microscopy:** Bright-field images of coated alginite beads were collected using a Zeiss AxiosObserver Microscope. For fluorescent imaging, fluorescently labeled alginite (LVLG, LVG or MVG) was synthesized by dissolving 25 mg of alginite (LVLG, LVG, or MVG) in 2.5 mL of purified water, followed by the addition of 5 mg of 2-(N-morpholino)ethanesulfonic acid, 5 mg of Na-hydroxysuccinimide, and 0.5 mg of 4'-[aminomethyl fluorescein] (CF, dissolved in 100 μL of ethanol). Then, N-(3-dimethylamino propyl)-N'-ethyl carbodiimide hydrochloride was dissolved in 100 μL of purified water, followed by 20 min stirring. After stirring, 200 μL of 0.25 kV NaCl was added at a rate of 7 μL min⁻¹, and the solution was stirred for 1 h. After 1 h of stirring, the solution was precipitated with 5 mL of ethanol, resuspended in 2 mL of 50 mM NaCl, and precipitated again in 5 mL of ethanol. The resulting alginite-CF was rinsed two times with a mix of 2 mL of water and 4 mL of ethanol, then 3 × with ethanol, and dried under reduced pressure. Beads were coated following the same protocol from Section 2.5 but using fluorescent alginite. Fluorescent coatings were quantified via ImageJ by setting a Region of Interest (ROI) around the bead circumference and measuring the percent area covered by Alginite-CF. To measure the coating thickness, a cross-sectional image of the coated beads was exported to ImageJ. ROIs were drawn around the outer and inner diameter of the coating. The area of each circle was obtained using the measurement function in ImageJ. The area of each circle was used to calculate the diameter, and the difference between the inner and outer circle was calculated to obtain the thickness of each coating. The sample size for all fluorescent quantification was ≥10.

**ICP-MS:** A total of 50 alginite microbeads were handpicked (n = 4 per group) and digested in 15 mL polypropylene tubes with 0.5 mL nitric acid (Optima grade, Fisher Chemical, Fair Lawn, NJ) at 100 °C for 2 h on a dust-protected hotplate. The digests were subsequently diluted to 15 mL volumes with ultrapure water (EMD Millipore, Burlington, MA). The CONP dispersion, as received from the manufacturer, had a pH of 4. To adjust to pH 7.4, the 20% CONP dispersion was diluted in MOPS-MV buffer (10 mM sucrose, 10 mM MOPS, 125 mM NaCl, 3 mM KCl, pH 7.4) to a final concentration of 3 mg mL⁻¹ and pH adjusted to 7.4 with 1 M NaOH. Nanoparticle size was acquired following the DLS methods explained in Section 5.1. However, nanoparticles were diluted in a 40 mM sucrose-MOPS/KNO₃ buffered solution.

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**CV:** CV technique was performed as previously described, with some modifications.[53] Briefly, a few centimeters of stainless steel wires (127 μm diameter, A-M Systems Inc., WA) were coated with different formulations of CONP and alginite. The coated stainless-steel wire was submerged 1 cm into a MOPS buffer solution at room temperature and connected to an Autolab potentiostat PGSTAT12 (EcoChemie, Utrecht, The Netherlands) using a three-electrode configuration. CV measurements consisted of 0.5 V s⁻¹ linear sweeps from -0.6 to 0.8 V. A 100 μL of TMB substrate. The plate was shaken at 500 rpm for 30 min covered from light. After CONP oxidized the TMB substrate, 150 μL of solution (n = 3) was transferred to a clear flat bottom 96 well plate for absorbance quantification (650 nm) utilizing a Molecular Devices SpectraMax MS reader.

**Hydrogen Peroxide Consumption Assay:** A total of 50 microbeads were incubated with 500 μL of a solution containing 320 μM of Xanthine, 93 mM of Xanthine Oxidase from bovine milk (X1875), and 83.33 μM of Cytochrome C. Beads were incubated for 15 min covered from light before transferring 150 μL (n = 3) to a 96-well flat-bottom plate to read absorbance at 550 nm. The rate of superoxide consumption was calculated using the following equation:

\[
\text{O}_2^- = \frac{\Delta A}{t}
\]

where ΔA is the difference between reduced and oxidized cytochrome C, V is the total volume per well (150 μL), K is a constant 21 × 10⁻³ cm⁻¹ M⁻¹, l is the path length, and t is the total time of incubation.

**Protection of Encapsulated β-Cells Assay:** Mouse INS-1E Cells (MIN6) cells were encapsulated in 1.6% (w/v) alginite MVG at a cell loading density of six million cells per mL of alginite. Encapsulation of cells was done under sterile conditions following the same procedure and settings as in Section 2.4. After encapsulation, MIN6 cell-containing beads were left to rest for at least 1 h before coating with different formulations of CONP and alginite. Coating of encapsulated MIN6 cells followed the same procedure as in Section 2.5. However, additional washes before the coating process were needed to ensure cell media was removed entirely to prevent any agents in the cell media from interfering with the coating process. Cell-containing microbeads (n = 30 per well) rested in cell media overnight before assessing protection from oxidative stress challenge.

**Insulin Secretion:** MIN6 cells response to high glucose (16.67 mM) concentration was assessed following previously published methods.[17] Briefly, control and coated beads (n = 30 per well) were exposed to a
Krebs buffer solution of with 16.67 mM of high glucose for 90 min at 37 C. Supernatant was then collected for insulin content analysis via ELISA (Merckodia).

Reduced: Oxidized Glutathione Ratio: A total of 30 beads per well were exposed to either hydrogen peroxide or xanthine/xanthine oxidase system for 2 h at 37 C. After ROS challenge, beads were washed to stop oxidative damage and groups were prepared for glutathione quantification. The total glutathione (N = 3) and oxidized glutathione (N = 3) were measured from coated and control MIN6 cells-containing beads following previous protocols.[1] Briefly, the solution in the wells were replaced with 100 μL of cell-culture-grade water, followed by the addition of 25 μL of either total glutathione lysis reagent or oxidized glutathione lysis reagent. Plates were shaken at 500 rpm for 15 min at RT. After cell lysis, 50 μL of generation reagent was added to each well and plates were incubated for 30 min at RT. Then, 100 μL of detection reagent was added to each well and plates were covered from light and incubated for 15 min at RT. Finally, 100 μL was transferred from each well (n = 2) to a 96 well plate for luminescent detection.

Statistical Analysis: Ellipsometry and CV studies were collected from three scans per group. Alginate bead data was collected from independent replicates (n = 6–9) from distinctly separate studies (N = 3) to validate trends. Cell protection data were collected from independent replicates (n = 3) from distinctly separate studies (N ≥ 2) to validate trends. All values were expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism 8.0 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.

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

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data are available from the corresponding author upon reasonable request.

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