Statistical Summary

Immobilization of chymotrypsin on hierarchical nylon 6,6 nanofiber improves enzyme performance

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Full Length Article

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ABSTRACT

Immobilized enzymes enable advances in bioprocessing efficiency and bioactive packaging. Enzyme immobilization onto macroscale solid supports is often limited by low protein loading, inadequate access to substrate, and non-ideal orientation to the solid support; immobilization on nanomaterials has improved activity retention, protein loading, and enabled improved performance in extreme environments, yet has practical limitations including handling, recovery. This work describes the immobilization of chymotrypsin to nylon 6,6 in two formats: electrospun nanofibers and planar films. Protein loading, enzyme activity, and kinetics were compared to that of commercially available systems (free chymotrypsin and chymotrypsin immobilized on agarose beads). Electrospun nylon 6,6 nanofibers had an average fiber diameter of 161 ± 73 nm, improving protein loading compared to its planar macroscale counterpart. Chymotrypsin immobilized onto nylon nanofibers exhibited shifts in both working optimum pH and temperature with an increase from pH 7.8 to pH 9, and increased optimum temperature by 10°C compared to free enzyme. The nanofibers also enhanced thermostability compared to native enzyme, enzyme on planar films, and the commercial standard agarose beads with 35% activity retained after 12 hr at 50°C. This work demonstrates the potential of hierarchical nanomaterials in improving enzyme performance, leveraging benefits of both nano and macroscale supports.

Keywords: enzyme immobilization, electrospinning, nanofibers, nylon 6,6, chymotrypsin, hierarchical material

INTRODUCTION

Enzymes are highly specific biological catalysts, capable of improving specificity and therefore efficiency in a range of bioprocessing applications. Immobilized enzymes take advantage of various attachment and coating schemes in order to allow for recovery, regeneration, reuse, and appropriate dosing of enzymes. Enzyme immobilization has also been shown to increase the thermostability, pH stability, and overall storage stability of the enzyme [1-3]. However, immobilization often adversely impacts enzyme performance due to protein denaturation and disadvantageous protein orientation during attachment [4-6]. Methods to increase activity include achieving higher protein loading and increasing retention of enzyme activity after immobilization to the solid support.

The chemical and physical attributes of the solid support in an immobilized enzyme system influence the success of enzyme immobilization. When creating biocatalytic materials, the biocompatibility, mechanical resistance, and chemical structure of the solid support must be considered. One physical characteristic that influences both protein loading and retained enzyme activity is solid support size. Decreasing the dimension of the solid support to the nanoscale results in substantial increases in the total surface area per unit mass as well as results in unique physiochemical characteristics of the material. These unique properties of nanomaterials have enabled advances in their research for applications in electronics, sensors, medical devices, drug delivery, and food and agriculture [7-12]. Indeed, nanomaterials exhibit a significantly higher surface area to volume ratio than their macroscale counterparts, and are often able to maintain and even improve tensile, mechanical, and wetting properties of the bulk material [13-16]. Increased surface area enables increased enzyme immobilization and has been shown to influence activity retention of immobilized enzyme by affecting protein-protein and protein-

material interactions [17,18]. Immobilization to nanoscale materials has also been shown to increase enzyme thermostability and pH stability [18-20] and enables improved performance due to fewer diffusion limitations by creating an immediate environment that mimics that of free enzyme [1,18]. Indeed, much of the research on improving immobilized enzyme performance on nanomaterials has focused on nanoparticles. Klein *et al.* explored the effect of chitosan nanoparticle size on immobilized β -galactosidase for lactose hydrolysis. β -galactosidase on nanoparticles was found to have higher activity retention, but lower thermostability [21]. In one piece of work, performance of β -galactosidase on magnetic core nanoparticles improved with reducing nanoparticle diameter, yet with decreasing diameter came a corresponding increase in recovery time to commercially impractical timeframes (17 hours for 18 nm nanoparticles) [22]. Further, the size of the nanoparticles makes it difficult to verify that 100% of all material has been recovered during studies on reuse. There has therefore been a motivation to explore immobilization of enzymes on hierarchically structured materials in which both nano and macroscale size domains enable the performance retention of nanomaterials but the improved handling and recovery of macroscale supports.

Fibers are an example of a high surface area, scalable material that maintains ease of handling as a woven mat. Electrospinning produces nanoscale fibers as non-woven fiber mats by the application of a high voltage to liquid polymer solutions as it passes through a tight capillary. Electrospinning is an emerging method for nanomaterial production that is applicable to many liquid polymer solutions, and can be scaled-up. The polymer solution builds positive charge at its surface, and is drawn to a grounded collector by the formation of a Taylor Cone [23]. The drawn polymer dries by the whipping action and movement incurred during collection. By using the same charge build-up and drying principles, needle-less electrospinning uses a charged rotating

wire to produce large (up to 1.6 m wide, roll-to-roll) non-woven nanofiber sheets commercially (Scheme 1) [24-28]. Polymer nanofibers represent a commercially scalable hierarchical material, with nanofibers of dimensions ranging from <100 to 800 nm, and webs of material formed on a much larger (centimeters to meters) scale.

Depending on their bulk material, nanofibers can be readily functionalized and have served as enzyme supports in biosensors, drug delivery, active packaging and tissue regeneration applications [29-32]. Ge *et al.* tested the antimicrobial ability of glucose oxidase immobilized onto polymer composite nanofibers in order to increase shelf-life [33]. A study conducted by Campbell *et al.* evaluated enzyme-enzyme interactions and nonspecific attachments of peroxidases and glucose oxidase immobilized to carbon nano-supports. They found maximizing activity on a nano-support was dependent on enzyme-material compatibility and solid support characteristics such as curvature and chemical make-up [34]. While previous works have demonstrated improvements in enzyme performance after immobilization on nanofibers, there remains an opportunity to explicitly demonstrate the influence of immobilization on nanoscale and macro-scale materials of the same composition on enzyme performance.

In this work, chymotrypsin, an enzyme used in the food industry for beverage clarification and protein digestion, was covalently immobilized onto amine functionalized nylon 6,6 nanofibers and planar films via glutaraldehyde. Enzyme performance was characterized by protein loading, activity retention, determination of kinetic parameters, and both pH and thermostability. Performance was further compared to that of free chymotrypsin and a commercially available immobilized chymotrypsin in agarose beads.

EXPERIMENTAL

Materials

Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, potassium phosphate monobasic, potassium phosphate dibasic, sodium dodecyl sulfate, formic acid (88%), and dimethylformamide were purchased from Fisher Scientific (Fairlawn, N.J., U.S.A.). Glutaraldehyde (25%) was purchased from Alfa Aesar (Ward Hill, Mass., U.S.A.). Type II lyophilized α -chymotrypsin (amino acid sequence from BRENDA *Bos taurus* UniProt P00767 pBLAST, E.C. 3.4.21.1) from bovine pancreas (40 units/mg), nylon 6,6, N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (NSPN), L-lysine (>98%), and picrylsulfonic acid solution (TNBS) (5% wt./vol) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). The Pierce BCA Protein Kit was purchased from Thermo Scientific (Waltham, MA., U.S.A.). Chymotrypsin immobilized by cross-linking on agarose beads (Bead-CT) were purchased from ProteoChem (Loves Park, IL).

Methods

Immobilization of Chymotrypsin on Nylon 6,6 Planar Films (Film-CT) and Nanofibers (NF-CT)

To characterize the influence of support topography on immobilized enzyme performance, chymotrypsin was immobilized onto amine-functionalized nylon 6,6 via glutaraldehyde cross-linking in the formats of both planar films (Film-CT) and electrospun nanofibers (NF-CT) as depicted in Scheme 1. Chymotrypsin was purified by passing rehydrated protein through a 0.22 μ m PTFE syringe filter, and subsequent separation using centrifugal filtration (MWCO 10,000). Purified α -chymotrypsin was rehydrated in 0.1M, pH 7.8 potassium phosphate buffer, and stored at 4°C until further use. Purified chymotrypsin concentration in buffer was quantified using the BCA assay in preparation for immobilization. Nylon 6,6 films (2 x 2 cm) were washed prior to surface modification in isopropanol, acetone, and water. Cleaned

films were subjected to surface acid hydrolysis in 3M HCl for 30 min, followed by rinsing for 5 min in deionized water. Washed films were stored dry at room temperature for further analysis. Both sides of the films were exposed to 30 min UV-ozone treatment, at 3 cm from the light source (JeLite, FL) to generate primary amine groups on the film surface. Film surfaces were exposed to 28 mW/cm² UV light at 254 nm. Films were then reacted in 5% (v/v) glutaraldehyde in 0.1M, pH 7.8 potassium phosphate buffer for 1 hr prior to chymotrypsin conjugation for 1 hr in 1000 µg/mL α-chymotrypsin (0.1M, pH 7.8 potassium phosphate buffer). All assay solutions were prepared at volumes of 1 mL/cm² material.

Nanofiber mats were prepared by electrospinning a solution of nylon 6,6 in formic acid (12 % w/v). Nanofiber mats were produced by needle-less electrospinning in an Elmarco Nanospider (NS Lab 1WS500) running between 45 and 47 kV to an aluminum foil covered flat collector 14 cm from the polymer source (Scheme 1). Positively charged taught wires move through a trough filled with polymer solution, forming droplet nodes along the wire surface. Nanofibers are drawn to a grounded collector directly above the rotating wires, generating a 45 × 20 cm mat of nanofibers. Nanofiber mats were collected after 1 hr of electrospinning, and cut into 1.5 cm diameter circular nanofiber mats by a circular die. Nanofiber mats were removed from the collector foil and subjected to 20 min UV-ozone treatment to introduce primary and secondary amines to the fiber surface [35,36]. Amine functionalized nanofiber mats were then reacted in 5% (v/v) glutaraldehyde solution in 0.1 M, pH 7.8 potassium phosphate for 1 hr, followed by enzyme conjugation in 1000 µg/mL purified α-chymotrypsin (0.1M, pH 7.8 potassium phosphate buffer) for 1 hr. All conjugation solutions were prepared at 1 mL/cm² nanofiber mat.

Protein Quantification on Nylon 6,6 supports

The amide bonds of nylon 6,6 interfere with the BCA protein assay reagents due to their reducing potential, therefore the TNBS primary amine assay was used to indirectly quantify protein content of chymotrypsin immobilized on planar and nanofiber nylon 6,6 supports, with glutaraldehyde activated supports as controls. NF-CT mats were incubated at 37°C in 0.5 mL of 0.1M, pH 8.5 sodium bicarbonate buffer with 0.01% (v/v) TNBS while shaking. The reaction was stopped after 2 hrs with 0.25 mL 10% SDS, and absorbance was read at 410 nm. Film-CT was characterized using the same method, but with increased assay volume to completely cover the samples. All assay substrate concentrations and volume ratios in relation to each other remained the same. Films were submerged in 2 mL buffer, 1 mL TNBS, and 1 mL SDS (4 mL total volume) to accommodate the film shape. The primary amine concentration determined by TNBS was converted to estimated total protein content using values of available primary amines per enzyme and enzyme molecular mass provided by the BRENDA database (BRENDA *Bos taurus* UniProt P00767 pBLAST, E.C. 3.4.21.1). The protein content calculated from TNBS was used to determine the activity retention of immobilized enzyme per unit mass.

Purification of Commercial Chymotrypsin on Agarose Beads (ProteoChem Bead-CT)

Chymotrypsin immobilized on agarose beads were purified before use by a modified method provided by the supplier. Equal amounts of ProteoChem Bead-CT and 0.1M, pH 7.8 potassium phosphate buffer were mixed and centrifuged at 6000 rpm (2000 x g). The resulting supernatant was removed and replaced with additional buffer. ProteoChem Bead-CT beads were washed three times with buffer and stored at 4°C in buffer for further use. The total protein on ProteoChem Bead-CT was determined by the BCA assay, where 2mL of working reagent was reacted for 30 min at 37 °C with 0.1 mL of sample (purified support in storage buffer), followed

by reading absorbance at 562 nm and comparison to a standard curve of BSA in potassium phosphate buffer.

Chymotrypsin Activity

Activities of free enzyme, NF-CT, Film-CT, and ProteoChem Bead-CT were measured using the synthetic substrate, NSPN, by a modification of the method outlined by DeMar and Kaspar [37,38]. A solution of NSPN was prepared to 20 mg/mL (32 mM) in DMF and stored at 4°C until use. Immobilized chymotrypsin samples were submerged in 10 mM, pH 7.8 sodium phosphate buffer to which 30 μ L of 7 mg/mL NSPN, diluted from stock in 10 mM, pH 7.8 sodium phosphate buffer, was added. The final activity assay volume was 2 mL. Samples were incubated at 25°C, and absorbances at 420 nm were measured at regularly timed intervals through 60 min to quantify hydrolysis of p-nitroanilide from the synthetic substrate. Activity in μ mol/min/g protein was calculated using only the linear change in absorbance. All activities were normalized to their respective maximum activities as activity per gram of protein for comparison. The activity of free chymotrypsin was measured similarly at a total volume of 2 mL, where 1 μ g protein was added to the assay.

Temperature and pH activity profiles and optima were determined by varying either temperature (every 5°C from 20°C to 60°C, constant pH value of 7.8) or pH (3-10, constant temperature of 25 °C). Buffers for pH activity profiles were prepared at 10 mM according to the target buffering pH ranges including sodium citrate (pH 3-5), potassium phosphate (pH 7-8), sodium phosphate (pH 7.8), and sodium carbonate (pH 9-10). The thermostability for immobilized chymotrypsin was determined by evaluating enzyme activity retention after 12 hrs incubation at 30, 40, 50, and 60°C in 10mM, pH 7.8 sodium phosphate buffer.

Michaelis-Menten Kinetic constants were determined at the optimum temperature and pH established for free enzyme (pH 7.8, 25°C) using the synthetic substrate, NSPN, at concentrations of 1, 2, 4, 6, 8, and 10 µg/mL prepared from the 20 µg/mL stock solution. Measurements were taken at regular intervals over 30 min at 420 nm.

Scanning Electron Microscopy (SEM)

Scanning electron micrographs were captured on a benchtop SEM (JEOL Neoscope JCM 6000) operating at 10 kV (Nikon Instruments, Inc. Melville, NY). To prevent surface charging, samples were coated with 20 nm of gold utilizing argon gas by a Cressington Sputter Coater 108auto (Watford, UK) prior to microscopy. Nanofiber diameter was determined by image analysis of electron micrographs, using ImageJ (NIH). A total of 75 diameter values were measured, representing 25 diameters from each of 3 independent micrographs, acquired under the same magnification.

Surface Area (Quantachrome Autosorb iQ)

Total surface area of the nanofiber mats was calculated using Brunauer-Emmett-Teller Theory [39-41]. Up to 10 mg of nanofiber mat were outgassed in multiple steps; 90 min at 35°C, 90 min at 60°C, 180 min at 90°C, and finally 110°C for 240 min to drive off residual solvent without heat damage to the nanofiber mat. Adsorption and desorption isotherms were created using liquid and gaseous nitrogen, with helium backfill on a Quantachrome Autosorb iQ (Florida, U.S.A.). A multipoint analysis was composed of up to 20 relative pressures between 0 and 0.2 p/p₀ and Brunauer-Emmett-Teller calculations were applied by Quantachrome software.

Statistical Analysis

Unless otherwise noted, all determinations were performed in quadruplicate, and values are representative of independent experiments performed on two separate days. One-Way

Analysis of Variance (ANOVA) with Tukey's pairwise comparison post-test ($p < 0.05$) and Michaelis-Menten kinetic constants were calculated using GraphPad Prism (v. 5.04, GraphPad Software, La Jolla, CA).

RESULTS

Surface Area and Morphology of Solid Supports

Electron microscopy and gas sorption surface area quantification were performed to characterize the morphology of immobilized enzyme systems. Electron micrographs revealed a smooth, uniform surface for clean nylon 6,6 film. Nylon 6,6 nanofibers were produced in a non-woven mat of 0.177 ± 0.012 mm thickness with smooth and cylindrical morphology, with an average diameter of 164 ± 73 nm (Fig. 1). Surface modification and enzyme immobilization caused some loss of distinctive fiber shape due to UV functionalization, glutaraldehyde application, and chymotrypsin coating, with fiber junctions appearing to join the mat into a cohesive network. However, the nanofiber mats did not become planar. ProteoChem Bead-CT exhibited a typical spherical morphology with a smooth, uniform surface (Fig. 2). Surface area of ProteoChem Bead-CT was variable due to the range of bead diameters, with up to $3468 \text{ cm}^2/\text{g}$ surface area compared to $23.05 \text{ cm}^2/\text{g}$ of the planar film. In contrast, nanofiber mats presented up to $81.23 \text{ m}^2/\text{g}$ total surface area by gas sorption, an increase in available surface area of $\sim 40,000$ times that of the planar film, and ~ 250 times that of the commercial agarose bead.

Protein Loading on Macro and Nanoscale Supports

Protein loading on nylon supports was quantified by the TNBS primary amine assay, using numbers of primary amines per enzyme and enzyme molecular weight reported in the BRENDA database (*Bos taurus* UniProt P00767 pBLAST, E.C. 3.4.21.1). Protein content was used to compare enzyme activity retention per mass protein, as well as to demonstrate the

influence on material morphology on protein loading capacity. Film-CT presented 0.005 μg protein/mg solid support, while NF-CT had 1.63 μg protein / mg solid support. This 326 fold increase in protein per unit mass of solid support was not directly proportional to the increase in surface area achieved by introducing the nanoscale as would be expected from surface analysis data. The assay develops color when TNBS loses a sulfonic acid group and attaches onto primary amines, producing a chromatic response. The colorimetric assay is quantified by analysis of color available in the reaction solution. However, TNBS is hydrophilic and may not have fully disassociated from the immobilized enzyme material which may account for lower than expected protein increase at the nanoscale [42-44]. As expected, the ProteoChem Bead-CT had the most conjugated protein at 18.7 $\mu\text{g}/\text{mg}$ solid support. The observed differences in order of magnitude of protein content compared to the nylon 6,6 supports are due to differences in material synthesis. The reported nylon 6,6 immobilized enzyme system is prepared by immobilization of chymotrypsin on the surface of a solid support, whereas ProteoChem Bead-CT is prepared by cross-linking enzyme throughout the bulk of agarose beads.

Activity Retention of Immobilized Chymotrypsin

The influence of solid support size and morphology on retention of enzyme activity after immobilization was determined by quantifying activity with a synthetic substrate (NSPN) at optimum parameters outlined by the supplier for free enzyme (25°, pH 7.8) (Figure 3). Data are reported as percentage activity retention, by comparison to activity of free enzyme under the same conditions. ProteoChem Bead-CT exhibited an activity retention of 29.2% compared to free chymotrypsin. To enable parallel comparison to chymotrypsin on nylon immobilized enzyme systems, all activity retention assays were performed at 25°C. The optimum temperature for ProteoChem Bead-CT is reported to be 35°C, likely influencing the observed retention of

activity. Nevertheless, results from the temperature activity profile assays (reported below in Figure 5) suggest that the general trend of enzyme activity retention between the three systems (NF-CT>Bead-CT>Film-CT) are expected to be the same regardless of temperature at which activity assays are performed. Immobilization of chymotrypsin on planar nylon films (Film-CT) resulted in a 96.5% reduction in activity (3.50% retained activity). Such a significant loss is similar to other reports of enzyme activity retention after immobilization on a planar polymer support. Wong *et al.* found lactase lost activity with increased protein mass through Layer-by-Layer deposition [45]. Rodrigues *et al.* proposed that loss of cellulase and alcohol dehydrogenase activity on Langmuir-Blodgett films was due to enzyme packing patterns and the surface environment, not denaturation [46-48]. In contrast, despite use of the same solid support (nylon 6,6) and the same bioconjugation chemistries (amine to amine conjugation by glutaraldehyde cross-linker), NF-CT retained 55.6% of free chymotrypsin activity. These results support our hypothesis that immobilization on nano-scale solid supports improves retention of enzyme activity.

Immobilization Shifts pH and Temperature Activity Profiles

To characterize the influence of enzyme immobilization on performance at varying pH values and temperatures, pH and temperature activity profiles were created by varying pH at constant temperature (25°C, Fig. 4) or varying temperature at constant pH (pH 7.8, Figure 5) as noted in the Methods section. Data are presented as relative activity, normalized to maximum activity at the optimum pH determined for each immobilized enzyme system. All immobilized enzyme systems experienced shifts in optimum pH compared to free enzyme. Free chymotrypsin presented the sharpest activity profile, with the highest activity at the established pH 7.8 and relative activities below 0.5 at all other pH values. ProteoChem Bead-CT retained relative

activities above 0.5 at pH 6 and above with its optimum matching that of free chymotrypsin at pH 7.8 and pH 8.0. Immobilization of chymotrypsin on the modified nylon supports resulted in a left-skewed pH activity profile, suggesting that the reported bioconjugation chemistry (amine to amine via glutaraldehyde) shifted the working range of chymotrypsin to more basic conditions. Film-CT presented a pH optimum at pH 8.0 with significant activity retention at pH 9.0-10 and below 50% activity below pH 7.0. The observed increase in pH optimum was more pronounced for NF-CT, with an optimum pH between pH 9.0 and pH 10. These results are in agreement with prior reports where pH optima of immobilized enzymes shift towards higher, basic, values. For example, chymotrypsin immobilized with trypsin onto microspheres had its optimal pH shifted to pH 8.2, where Li *et al.* attributed the shift to amine group (-NH₂) losses during covalent coupling [49]. Lozano *et al.* noted enzymes experienced pH sensitivity closest to the isoelectric point due to protonation and deprotonation events occurring at lysine and arginine amino acid residues which changed the charge of the enzyme. The enzyme internally reduced folding energies needed to maintain native conformations which correlate structure to function, and therefore the pH optimum [50]. Temperature activity profiles of immobilized enzyme systems were compared to that of free chymotrypsin by plotting relative activity (normalized to maximum activity values for each system) against temperature (Fig. 5). It is interesting to note that despite the manufacturer supplied temperature optimum of 25°C, our results indicated that free chymotrypsin had an optimal temperature of 40°C, likely a result of differences in synthetic substrate. Both optimum and activity profiles of ProteoChem Bead-CT were similar to free enzyme, in support of ProteoChem recommended optima. Enzyme immobilization has been reported to shift temperature optima as well as enable overall activity retention over a broader range of working temperatures [19,20]. Both Film-CT and NF-CT systems yielded similar

temperature optima of 45-50 °C, with NF-CT exhibiting a slight increase in temperature optimum. Likewise, temperature activity profiles were similar for Film-CT and NF-CT systems, with at or above 0.5 relative activity values at temperatures of 30-50 °C. These results suggest that immobilization onto nylon supports by glutaraldehyde cross-linking improves the temperature working range for activity retention after immobilization, most notably for NF-CT, for which the percent activity retention was significantly higher than for Film-CT. Although free chymotrypsin has higher absolute activity over the immobilized systems, the shifts in the relative retained activities of the nanoscale immobilized system suggests the potential for solid support systems to tailor enzyme performance in specific applications.

In addition to altering optimum working temperatures, enzyme immobilization can influence, stabilizing and often enhancing, thermostability by modification of its tertiary structure [50-52]. Such resistance to denaturation under extreme conditions such as extended exposure to elevated temperatures supports the utilization of immobilized enzyme systems in industrial bioprocessing applications. Here, the thermostabilities of free and immobilized chymotrypsin systems were determined by quantifying relative activity (by normalization to maximum activity for each system) at time points up to 12 hrs during incubated at 30, 40, 50, and 60°C (Fig. 6). Free Chymotrypsin exhibited poor thermostability, with only 16.67% activity retention after 12 hrs at 30 °C, and 99.99% activity loss at 60°C (Fig. 6a.). ProteoChem Bead-CT also exhibited poor thermostability, with less than 10% retained activity after 4 hours incubation at any of the temperatures tested. Other reports note denaturation as a cause for thermal instability, but cross-linked alginate beads may influence autoproteolysis in solution [53,54]. In contrast, Film-CT retained 68.03% activity after 2 hrs at 30°C and maintained 23.41% after 12 hrs (Fig. 6c.). At further elevated temperatures of 40, 50, and 60°C, Film-CT did not retain

observable activity after incubation. It is worth noting that while Film-CT had an apparent improvement in thermostability at 30 °C compared to Bead-CT, the overall loss of activity (reported in Fig. 3) was higher for Film-CT, so the apparent improvement in thermostability is likely not of practical significance. In contrast, NF-CT retained over 30% activity after 12 hrs at 30, 40, and 50°C. After 2 hrs at 30°C, NF-CT retained 94.22% activity, and 88.05% after 12 hrs of incubation. NF-CT maintained 64.84% activity at 40°C and 30.25% at 50°C after 12 hrs (Fig. 6d.). Only at 60°C did NF-CT lose activity (98.65%) similar to that of free chymotrypsin, ProteoChem Bead-CT, and Film-CT. The observed higher standard deviation for NF-CT values was likely a result of the variability of fiber diameters.

These results suggest that the activity retention of the macroscale solid support, Film-CT, performed similarly to the industry standard, ProteoChem Bead-CT, and did not retain more activity after incubation than free chymotrypsin. As noted previously, the nanofiber mat support for NF-CT had about 40,000 times more surface area due to its nanoscale than the planar film. Both macroscale supports lost enzymatic activity similarly to that of free chymotrypsin, most lost after 2 hrs at all temperatures. Chymotrypsin immobilized on the nanoscale material retained more activity at higher temperatures.

Comparing Michaelis-Menten Kinetics

Kinetic constants for enzyme activity act as a characteristic comparison between systems that transcends absolute product determination as a result of total protein loading between solid support surface areas. Michaelis-Menten kinetic constants were calculated for each immobilized chymotrypsin system by determining the amount of product produced over time for a range of synthetic substrate concentrations. First, it must be assumed the enzyme system is saturated with substrate and that there is only one active site available for catalysis since the constants are in

effect comparing the maximum achievable velocity from the highest saturation of substrate. The amount of product released over time, or enzyme velocity, was graphed versus substrate concentration to calculate Michaelis-Menten constants as described in the Methods section. All constants were compared to those determined for free chymotrypsin and to each other.

There were no significant differences in maximum velocity, $V_{\max}$, for each immobilized enzyme system compared each other and to free enzyme. $V_{\max}$ represented the rate at which enzyme creates product when saturated with substrate. Velocity was proportional to substrate concentration until $V_{\max}$ was reached as was expected for Michaelis-Menten enzyme kinetics. $V_{\max}$ was used to derive the constant, K_m , that represented the inverse relationship to substrate affinity. High K_m is an indication of an enzyme's low affinity (willingness to attach and hold onto) to a particular substrate, and low K_m is an indication of high affinity to a substrate at a set temperature and pH. Substrate affinity is dependent on the substrate type itself as well as the immobilization method and assay environment. In this case, ProteoChem Bead-CT had a K_m value that was significantly higher than the other systems, indicating lower affinity for the synthetic substrate was experienced during catalysis (Table 1). This apparent rise in K_m can be attributed to diffusional limitations, and not necessarily lower affinity for the synthetic substrate based on the different solid support morphology where enzyme is cross-linked throughout the agarose bead and not just at the surface. Low affinity (high K_m) shows an enzyme may be unwilling to form an enzyme-substrate complex to produce product. In contrast low K_m , or high substrate affinity, can also hinder catalytic efficiency such that the enzyme-substrate complex necessary for biocatalysis will not disassociate quickly enough to create product efficiently. Only the K_m of Bead-CT was significantly different from other treatments, and was up to two magnitudes higher than that of free chymotrypsin. This suggested Bead-CT samples had a lower

affinity for the synthetic substrate. Agarose beads were hydrophilic and experienced no difficulties during assay mixing, but the tendency for Bead-CT to fall out of solution during activity assays may have attributed to overall diffusion difficulties for forming an enzyme-substrate complex. The lower affinity of Bead-CT may have helped to drive its competitive velocity next to the other immobilized enzyme systems.

The turnover number, k_{cat} , relates V_{max} to the enzyme concentration in the system. This value represents the amount of substrate each active site was able to convert to product over time, and was therefore dependent on enzyme concentration. The apparent turnover number can be interpreted as the velocity of each active site. Increasing enzyme concentration increases the number of active sites available for catalysis, and therefore is normalized for true comparison between systems. The k_{cat} values for all immobilized chymotrypsin systems were significantly different from that of free chymotrypsin. NF-CT had the highest k_{cat} relative to free chymotrypsin and Film-CT had the lowest k_{cat} relative to free chymotrypsin by a power of 10 (Table 1). Therefore NF-CT was able to create more product over time when compared to macro-scale supports which was supported by the large differences in protein content per unit mass of solid support. As k_{cat} was dependent on enzyme concentration, increases in the surface area due to reduced size (and increased protein) may have driven the constants of all of the treatments to larger than that of Film-CT.

The catalytic efficiency, k_{cat}/K_m , tells of substrate specificity in combination with the speed at which the enzyme and substrate can form a complex for catalysis, and is limited by diffusion. Catalytic efficiency compares the ability of an enzyme and substrate to interact, but not the speed at which product is formed. Therefore, k_{cat}/K_m speaks to the number of collisions a substrate and enzyme experience. All k_{cat}/K_m values were significantly different from free

chymotrypsin. Again, NF-CT had the highest k_{cat}/K_m when compared to free chymotrypsin and Film-CT had the lowest k_{cat}/K_m , which indicated the support size and morphology affected the collision frequency and diffusion of substrate to immobilized enzyme (Table 1). This may be in part to enzyme packing on the various solid supports that is influenced by curvature and size.

These results are in support of prior works which demonstrate improved performance of enzymes after immobilization on nanoscale solid supports due to high interconnectivity and decreased mass transfer hindrance in the microenvironment [55,56]. The observed increased catalytic efficiency may be due to the microenvironment created or maintained around the enzyme during immobilization. The smaller support may increase protein loading, but may also make the enzyme believe it is in an environment similar to free enzyme benefitting the turnover and catalytic efficiency of the immobilized enzyme. This influences overall substrate affinity and can reduce diffusion limitations taken into account during catalytic efficiency calculations.

CONCLUSIONS

In this work, chymotrypsin was immobilized onto nylon 6,6 supports in two size formats: as macroscale planar films and as electrospun nanofiber mats. Performance, pH and temperature activity profiles, and kinetics were characterized and compared to that of free and commercially available immobilized chymotrypsin. Nylon 6,6 nanofibers exhibited about 40,000 times more surface area per gram than planar film and about 250 times more surface area agarose beads. More potential sites for surface immobilization enabled an increase in enzyme activity retention. Previous works have shown immobilization can shift an enzyme's pH and temperature working ranges of an enzyme. The pH activity profile of chymotrypsin immobilized onto nylon supports was left skewed to higher pH optima values, and maintained more activity over a wider pH range. Immobilization onto the nylon nanofiber resulted in increased thermostability with higher

retained activity over a wider temperature range, and an increased temperature optimum of 50°C when compared to free chymotrypsin and chymotrypsin immobilized to macro-scale planar films and ProteoChem Bead-CT. NF-CT had an increased pH optimum, with the highest absolute activity at pH 9, and maintained 97% of that activity at pH 10. Chymotrypsin immobilized to macro-scale supports had similar pH activity profile as free enzyme. The determination of Michaelis-Menten kinetic constants showed NF-CT had similar maximum velocity and turnover rate compared to free chymotrypsin. The catalytic efficiency was higher for NF-CT than Film-CT and ProteoChem Bead-CT indicating chymotrypsin immobilized to the nanoscale supports experienced more diffusion related collisions with substrate that produced product than chymotrypsin immobilized to the other macro-scale materials. These results suggest that while the chymotrypsin was bound to a solid support, the nanoscale material may create a micro-environment that mimics free enzyme. Immobilized chymotrypsin was able to maintain the most activity and expand its working range while attached to nanofibers. Immobilization to nanoscale polymer materials effectively increases the surface area to volume ratio of a solid support, and may allow enzymes to experience reduced lateral interaction with neighboring proteins, in effect creating a microenvironment that is similar to that of free native enzyme. Utilization of hierarchically assembled solid supports for enzyme immobilization, as demonstrated here, may leverage benefits of both nano- and macroscale size regimes. Nanofibers improve enzyme performance in extreme environments similar to that observed after immobilization on nanoparticles, yet assembly into a larger, macroscale mat enables handling and recovery to facility use in food and bioprocessing applications.

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REFERENCES

- [1] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microb Technol* 2007;40:1451-1463.
- [2] Sheldon RA. Enzyme Immobilization: The Quest for Optimum Performance. *Adv Synth Catal* 2007;349:1289-1307.
- [3] Brady D, Jordaan J. Advances in enzyme immobilisation. *Biotechnol Lett* 2009;31:1639-1650.
- [4] Garcia- Galan C, Berenguer- Murcia Á, Fernandez- Lafuente R, Rodrigues RC. Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis & Catalysis* 2011;353:2885-2904.
- [5] Barbosa O, Torres R, Ortiz C, Fernandez-Lafuente R. Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. *Process Biochemistry* 2012;47:1220-1227.
- [6] Santos, Jose Cleiton S dos, Barbosa O, Ortiz C, Berenguer- Murcia A, Rodrigues RC, Fernandez- Lafuente R. Importance of the Support Properties for Immobilization or Purification of Enzymes. *ChemCatChem* 2015;7:2413-2432.
- [7] Mnyusiwalla A, Daar AS, Singer PA. 'Mind the gap': science and ethics in nanotechnology. *Nanotechnology* 2003;14:R9.
- [8] Vaddiraju S, Tomazos I, Burgess DJ, Jain FC, Papadimitrakopoulos F. Emerging synergy between nanotechnology and implantable biosensors: a review. *Biosensors and Bioelectronics* 2010;25:1553-1565.
- [9] Jianrong C, Yuqing M, Nongyue H, Xiaohua W, Sijiao L. Nanotechnology and biosensors. *Biotechnol Adv* 2004;22:505-518.
- [10] Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nature Reviews Cancer* 2005;5:161-171.
- [11] Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. *ACS nano* 2009;3:16-20.
- [12] Weiss J, Takhistov P, McClements DJ. Functional Materials in Food Nanotechnology. *J Food Sci* 2006;71:R107-R116.
- [13] Arinstein A, Burman M, Gendelman O, Zussman E. Effect of supramolecular structure on polymer nanofibre elasticity. *Nature nanotechnology* 2007;2:59-62.

- [14] Zussman E, Burman M, Yarin A, Khalfin R, Cohen Y. Tensile deformation of electrospun nylon- 6, 6 nanofibers. *Journal of Polymer Science Part B: Polymer Physics* 2006;44:1482-1489.
- [15] Wong S, Baji A, Leng S. Effect of fiber diameter on tensile properties of electrospun poly (ϵ -caprolactone). *Polymer* 2008;49:4713-4722.
- [16] Stachewicz U, Barber AH. Enhanced wetting behavior at electrospun polyamide nanofiber surfaces. *Langmuir* 2011;27:3024-3029.
- [17] Talbert J, Goddard J. Enzymes on material surfaces. *Colloids Surf , B* 2012;93:8-19.
- [18] Talbert JN, Goddard JM. Influence of nanoparticle diameter on conjugated enzyme activity. *Food Bioprod Process* 2013;91:693-699.
- [19] Ansari SA, Husain Q. Potential applications of enzymes immobilized on/in nano materials: A review. *Biotechnol Adv* 2012;30:512-523.
- [20] Cipolatti EP, Silva MJA, Klein M, Feddern V, Feltes MMC, Oliveira JV, Ninow JL, de Oliveira D. Current status and trends in enzymatic nanoimmobilization. *J Molec Catal B* 2014;99:56-67.
- [21] Klein MP, Nunes MR, Rodrigues RC, Benvenuti EV, Costa TM, Hertz PF, Ninow JL. Effect of the support size on the properties of β -galactosidase immobilized on chitosan: advantages and disadvantages of macro and nanoparticles. *Biomacromolecules* 2012;13:2456-2464.
- [22] Talbert JN, Goddard JM. Characterization of Lactase-Conjugated Magnetic Nanoparticles. *Process Biochem* 2013;48:656-662.
- [23] Andradý AL. Science and technology of polymer nanofibers. Hoboken, N.J: Wiley-Interscience; 2008.
- [24] Oldham CJ, Gong B, Spagnola JC, Jur JS, Senecal KJ, Godfrey TA, Parsons GN. Encapsulation and chemical resistance of electrospun nylon nanofibers coated using integrated atomic and molecular layer deposition. *J Electrochem Soc* 2011;158:D549-D556.
- [25] Niu H, Wang X, Lin T. Needleless electrospinning: influences of fibre generator geometry. *Journal of the Textile Institute* 2012;103:787-794.
- [26] Niu H, Lin T. Fiber generators in needleless electrospinning. *Journal of nanomaterials* 2012;2012:12.
- [27] Han W, Nurwaha D, Li C, Wang X. Free surface electrospun fibers: The combined effect of processing parameters. *Polymer Engineering & Science* 2014;54:189-197.

- [28] Shuakat MN, Lin T. Recent Developments in Electrospinning of Nanofiber Yarns. *Journal of nanoscience and nanotechnology* 2014;14:1389-1408.
- [29] Manesh K, Kim HT, Santhosh P, Gopalan A, Lee K. A novel glucose biosensor based on immobilization of glucose oxidase into multiwall carbon nanotubes–polyelectrolyte-loaded electrospun nanofibrous membrane. *Biosensors and Bioelectronics* 2008;23:771-779.
- [30] Yoo HS, Kim TG, Park TG. Surface-functionalized electrospun nanofibers for tissue engineering and drug delivery. *Adv Drug Deliv Rev* 2009;61:1033-1042.
- [31] Pérez-Esteve E, Bernardos A, Martínez-Máñez R, M Barat J. Nanotechnology in the development of novel functional foods or their package. An overview based in patent analysis. *Recent patents on food, nutrition & agriculture* 2013;5:35-43.
- [32] Hosseinkhani H, Hosseinkhani M, Khademhosseini A, Kobayashi H. Bone regeneration through controlled release of bone morphogenetic protein-2 from 3-D tissue engineered nano-scaffold. *J Controlled Release* 2007;117:380-386.
- [33] Ge L, Zhao Y, Mo T, Li J, Li P. Immobilization of glucose oxidase in electrospun nanofibrous membranes for food preservation. *Food Control* 2012;26:188-193.
- [34] Campbell AS, Dong C, Meng F, Hardinger J, Perhinschi G, Wu N, Dinu CZ. Enzyme catalytic efficiency: A function of bio–nano interface reactions. *ACS applied materials & interfaces* 2014;6:5393-5403.
- [35] Skordoulis CD, Makropoulou M, Serafetinides AA. Ablation of nylon-6,6 with UV and IR lasers. *Appl Surf Sci* 1995;86:239-244.
- [36] Pantchev I, Farquet P, Surbeck H, Meyer T. Surface modified Nylon 6,6 and application for adsorption and detection of uranium in potable water. *React Funct Polym* 2007;67:127-135.
- [37] DelMar E, Largman C, Brodrick J, Geokas M. A sensitive new substrate for chymotrypsin. *Anal Biochem* 1979;99:316-320.
- [38] Kaspar P, Moller G, Wahlefeld A. New photometric assay for chymotrypsin in stool. *Clin Chem* 1984;30:1753-1757.
- [39] Ahn C, Ye Y, Ratnakumar B, Witham C, Bowman Jr R, Fultz B. Hydrogen desorption and adsorption measurements on graphite nanofibers. *Appl Phys Lett* 1998;73:3378-3380.
- [40] Peigney A, Laurent C, Flahaut E, Bacsá R, Rousset A. Specific surface area of carbon nanotubes and bundles of carbon nanotubes. *Carbon* 2001;39:507-514.
- [41] Ji L, Zhang X. Fabrication of porous carbon nanofibers and their application as anode materials for rechargeable lithium-ion batteries. *Nanotechnology* 2009;20:155705.

- [42] Kakade M, Liener IE. Determination of available lysine in proteins. *Anal Biochem* 1969;27:273-280.
- [43] Weber C, Coester C, Kreuter J, Langer K. Desolvation process and surface characterisation of protein nanoparticles. *Int J Pharm* 2000;194:91-102.
- [44] Hermanson GT. *Bioconjugate Techniques*. Burlington : Elsevier,; 2008.
- [45] Wong DE, Talbert JN, Goddard JM. Layer by Layer Assembly of a Biocatalytic Packaging Film: Lactase covalently Bound to Low- Density Polyethylene. *J Food Sci* 2013;78:853-860.
- [46] Rodrigues D, Camilo FF, Caseli L. Cellulase and alcohol dehydrogenase immobilized in Langmuir and Langmuir–Blodgett films and their molecular-level effects upon contact with cellulose and ethanol. *Langmuir* 2014;30:1855-1863.
- [47] Caseli L, Furriel RPM, de Andrade JF, Leone FA, Zaniquelli MED. Surface density as a significant parameter for the enzymatic activity of two forms of alkaline phosphatase immobilized on phospholipid Langmuir–Blodgett films. *J Colloid Interface Sci* 2004;275:123-130.
- [48] Caseli L, Oliveira RG, Masui DC, Furriel RP, Leone FA, Maggio B, Zaniquelli MED. Effect of molecular surface packing on the enzymatic activity modulation of an anchored protein on phospholipid Langmuir monolayers. *Langmuir* 2005;21:4090-4095.
- [49] Li D, Ding H, Zhou T. Covalent immobilization of mixed proteases, trypsin and chymotrypsin, onto modified polyvinyl chloride microspheres. *J Agric Food Chem* 2013;61:10447-10453.
- [50] Lozano P, Diego T, Iborra JL. Dynamic Structure/Function Relationships in the α - Chymotrypsin Deactivation Process by Heat and pH. *European Journal of Biochemistry* 1997;248:80-85.
- [51] Torchilin V, Maksimenko A, Smirnov V, Berezin I, Klivanov A, Martinek K. The principles of enzyme stabilization. III. The effect of the length of intra-molecular cross-linkages on thermostability of enzymes. *Biochimica et Biophysica Acta (BBA)-Enzymology* 1978;522:277-283.
- [52] Torchilin V, Maksimenko A, Smirnov V, Berezin I, Klivanov A, Martinek K. The principles of enzyme stabilization IV. Modification of ‘key’ functional groups in the tertiary structure of proteins. *Biochimica et Biophysica Acta (BBA)-Enzymology* 1979;567:1-11.
- [53] Mohapatra SC, Hsu JT. Immobilization of α - chymotrypsin for use in batch and continuous reactors. *Journal of Chemical Technology and Biotechnology* 2000;75:519-525.
- [54] Ortega N, Perez-Mateos M, Pilar MC, Busto MD. Neutrase immobilization on alginate–glutaraldehyde beads by covalent attachment. *J Agric Food Chem* 2008;57:109-115.

[55] Iyer PV, Ananthanarayan L. Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochemistry* 2008;43:1019-1032.

[56] Misson M, Dai S, Jin B, Chen BH, Zhang H. Manipulation of nanofiber-based β -galactosidase nanoenvironment for enhancement of galacto-oligosaccharide production. *J Biotechnol* 2016;222:56-64.

FIGURES

Graphical Abstract: Utilizing the benefits of hierarchical scaling of electrospun nylon 6,6 nanofibers for chymotrypsin immobilization.

Scheme 1: Schematic of material production and material functionalization. Immobilized chymotrypsin on electrospun nylon 6,6 nanofiber mats, planar nylon 6,6 films (NF-CT, Film-CT)., and agarose beads were compared. Micrographs are of electrospun and functionalized electrospun nanofiber mats at 1000 $\times$ magnification. Additional image of unit courtesy of Elmarco.

Figure 1: Diameter distribution of electrospun nylon 6,6 nanofiber mats. Diameter data was determined by ImageJ analysis of SEM images at 5000x magnification. Twenty-five measurements were collected from each of three images, $n = 75$. The average diameter was calculated as 164 ± 73 nm.

Figure 2: Morphology of immobilized chymotrypsin systems [a) ProteoChem Bead-CT, b) planar nylon 6,6 film, c) electrospun nylon 6,6 nanofibers] as determined by scanning electron micrographs (1000× magnification) where scale bars represent 20 µm. Inset of functionalized nanofiber material (NF-CT) with scale bar representing 2 µm at 10000× magnification. Reported values of surface area per mass solid support were determined by BET theory on nitrogen adsorption isotherms.

Figure 3: Percent activity retention compared to free chymotrypsin at 25°C and pH 7.8. Values represent means $\pm$ standard deviations of n=8 determinations (quadruplicate determinations on each of two independent days).

Figure 4: pH activity profiles of free and immobilized chymotrypsin. Activities have been normalized to maximum activity values of each immobilized enzyme system. Values represent means $\pm$ standard deviations of $n=8$ determinations (four sample determinations on each of two independent days).

Figure 5: Temperature activity profiles of free and immobilized chymotrypsin. Activities have been normalized to the maximum activity value of each immobilized enzyme system. Values represent means $\pm$ standard deviations of $n=8$ determinations (four sample determinations on each of two independent days).

Figure 6: Thermostability of a. free chymotrypsin, b. Bead-CT, c. Film-CT, and d. NF-CT. Activities have been normalized to the maximum activity of each enzyme system observed, and were assayed against controls of solid support and glutaraldehyde. Values represent means $\pm$ standard deviation of $n=4$ determinations and are representative of experiments performed on two independent trials. Models of inactivation (one-phase exponential decay) have been fitted to the values.

Table 1: Michaelis-Menten kinetic constants of free and immobilized chymotrypsin determined at 25°C and pH 7.8 with NSPN concentrations ranging from 1 – 20 mg/mL. Different superscript letters indicate significant differences at $p < 0.05$.

	Relative Activity (%)	V_{max} (mM/s)	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/mM s)
Free Chymotrypsin	100	$5.92\text{e-}4 \pm 7.0\text{e-}5^a$	4.10 ± 1.5^a	$1.48\text{e}4 \pm 5.3\text{e}2^a$	3611 ± 130^a
ProteoChem Bead-CT	29.2	$4.01\text{e-}4 \pm 1.6\text{e-}3^a$	190 ± 48^b	$2.00\text{e}3 \pm 11^b$	342.4 ± 0.014^b
Film-CT	3.50	$6.57\text{e-}6 \pm 2.0\text{e-}6^b$	16.2 ± 6.5^a	1.99 ± 0.56^c	0.1230 ± 0.333^c
NF-CT	55.6	$8.00\text{e-}4 \pm 1.3\text{e-}4^a$	5.84 ± 2.4^a	$2.90\text{e}3 \pm 70^d$	496.2 ± 28.5^d