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Influence of Hierarchical Interfacial Assembly on Lipase Stability and Performance in Deep Eutectic Solvent

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INFLUENCE OF HIERARCHICAL INTERFACIAL ASSEMBLY ON LIPASE
STABILITY AND PERFORMANCE IN DEEP EUTECTIC SOLVENT

A Thesis Presented

by

STEPHANIE M. ANDLER

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ABSTRACT

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MAY 2016

STEPHANIE M. ANDLER, B.S., UNIVERSITY OF ILLINOIS

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Julie M. Goddard

Hierarchical systems that integrate nano- and macroscale structural elements can offer enhanced stability over traditional immobilization methods. Microparticles were synthesized using interfacial assembly of lipase with (CLMP-N) and without (CLMP) nanoparticles into a crosslinked polymeric core, to determine the impact of the highly ordered system on lipase stability in extreme environments. Kinetic analysis revealed the macrostructure significantly increases the turnover rate (k_{cat}) following immobilization. The macrostructure also stabilized lipase at neutral and basic pH values, while the nanoparticles influenced stability under acidic pH conditions. A greener solvent, choline chloride and urea, was applied to produce sugar ester surfactants. Microparticles exhibited decreases in the turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) following exposure, but retained over 60% and 20% activity after exposure at 50 ℃ and 60 ℃, respectively. CLMP and CLMP-N outperformed the commercially available lipase per unit protein in the production of sugar esters. The utilization of greener solvent systems with hierarchical immobilized enzyme systems has the potential to improve processing efficiency and sustainability for the production of value-added agricultural products.

Keywords: immobilization, ionic liquid, bioprocessing, nano, lipase, sugar ester

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DES: Deep Eutectic Solvent

ChCl:U: Choline Chloride: Urea

CLMP: Cross-Linked Microparticle

CLMP-N: Cross-Liked Microparticle-Nanoparticles

N435: Novozym 435

CHAPTER 1

INFLUENCE OF HIERARCHICAL INTERFACIAL ASSEMBLY ON LIPASE

STABILITY

1.1.   Introduction

Lipases in bioprocessing can catalyze the production of value-added agricultural products such as food-grade emulsifiers (e.g. sugar esters) that can be produced from agricultural waste streams.[1–3] Traditionally, organic solvents have been used for these biotransformations.[4–11] To alleviate health and environmental concerns associated with organic solvents, researchers point to the use of so-called 'greener solvents' which have low volatility, low toxicity, and high biodegradability.[12] Deep eutectic solvents are advantageous to organic solvents for lipase mediated biotransformations, as a result of their ability to solubilize both polar and nonpolar esterification reactants.[2,13–19] Mixtures of choline chloride and urea (ChCl:U) form a low-cost deep eutectic solvent with a combined freezing point of 12 ºC.[14] The inherent high viscosity of ChCl:U hinders the industrial implementation in enzymatic processes; however, viscosity can be decreased by increasing reaction temperature,[14] which in turn can denature enzymes. Researchers have explored enzyme immobilization techniques (e.g. physical adsorption, entrapment, crosslinking, and covalent attachment) to improve enzyme stability.[20,21] However, challenges remain with traditional immobilization methods including loss of activity, restriction of active site accessibility, and loss of protein from the surface.

Nanomaterials (e.g. nanoparticles, nanofibers, nanorods, nanoporous silica) are *Candida*tes for enzyme stabilization due to their high surface area, resulting in increased protein loading, as well as their enhancement of enzyme activity and stability.[22–24]

Nanoparticles can impact the microenvironment of enzymes by decreasing both lateral protein-protein interactions, as well as protein interactions with the surface, resulting in increased enzyme activity.[25–28] Additionally, nanomaterial surface chemistry can be altered using capping agents, such as dopamine, to increase the affinity for enzyme immobilization.[29,30] A main drawback of nanoimmobilization is the ability to efficiently recover and recycle the enzyme, even when magnetic properties are imparted to nanomaterials.[28,31] Specifically, recovery from viscous solvents, such as deep eutectic solvents, could be challenging to achieve. Larger structures can facilitate recovery of the enzyme through standard methods (e.g. centrifugation), however the immobilized enzymes need to retain activity after repeated use.[32] Commercially available lipase is adsorbed on acrylic resin beads (Novozym 435), permitting recovery, but is also susceptible to protein leaching.[33] Therefore, immobilization techniques incorporating both macroscale and nanoscale features, may improve the commercial feasibility of immobilized enzyme systems.

We have previously reported a hierarchical assembly technique for enzyme immobilization in which lipase-nanoparticle conjugates interfacially assemble around a macroscale polymeric core resulting in cross-linked microparticles (CLMPs) (Figure 1).[33,34] This technique harnessed the magnetic and interfacial properties of the nanoparticles and the facile recovery of the macroscale core.[33,35,36] These hierarchically assembled microparticles exhibited stability against protein leaching, and displayed enhanced stability to pH and temperature extremes, compared to both native lipase and Novozym 435.[33] There remains a need to demonstrate the performance of these hierarchical structures under commercially relevant conditions; specifically, limited

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research exists on the use of deep eutectic solvents for the production of sugar esters.[15,37] Further, understanding how nanoparticles and core material influence enzyme stability can help develop industrially feasible immobilized enzyme systems.
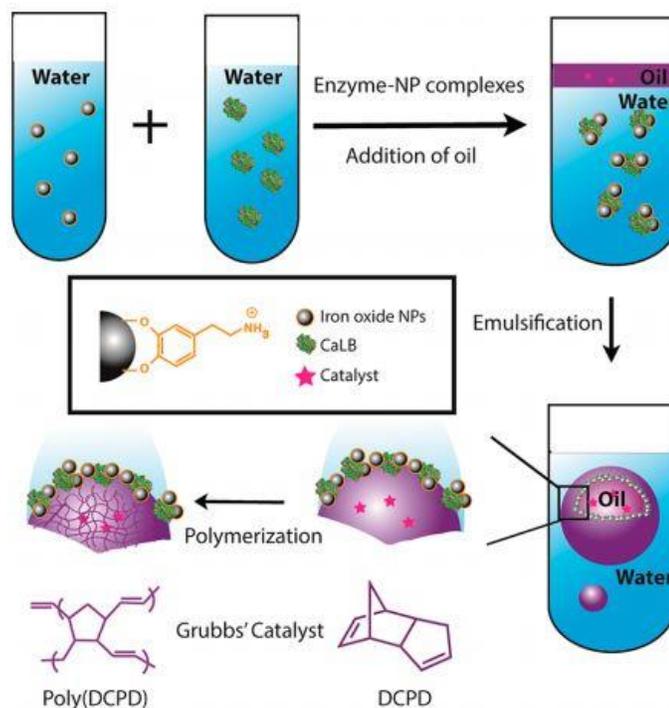
Figure 1. Schematic of hierarchical interfacial assembly of microparticles. Reprinted with permission from J.N. Talbert, L.-S. Wang, B. Duncan, Y. Jeong, S.M. Andler, V.M. Rotello, et al., Immobilization and Stabilization of Lipase (CaLB) through Hierarchical Interfacial Assembly, Biomacromolecules. 15 (2014) 3915–3922. doi:10.1021/bm500970b. Copyright 2016. American Chemical Society.

The aim of this research was to characterize the performance of hierarchically assembled lipase nanocomposites in deep eutectic solvents as well as their efficacy in producing sugar ester surfactants. Synthesis of lipase CLMPs with and without nanoparticles enabled quantitative determination of the influence of nanostructures and macrostructures on the stability of lipase in denaturing conditions, including greener solvents. Hierarchically ordered composites were prepared as described previously,[33] with slight modification to create microparticles in the absence of nanoparticles, to

analyze kinetic parameters pH stability, and performance in ChCl:U. This series of experiments aimed to determine the extent to which the nanoparticles and core material stabilized the microenvironment and overall stability of lipase in a commercially relevant application.

1.2. Materials and Methods

1.2.1. Materials

Lipase CV-CALBY (*Candida antarctica* lipase B) was purchased from Chiralvision (Leiden, Netherlands). Resorufin butyrate was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Vinyl laurate was purchased from MP Biomedicals (Santa Ana, CA, USA). Diethylene glycol, sodium hydroxide, iron (III) chloride hexahydrate, hydroxytyramine hydrochloride (dopamine), choline chloride, glycerol, sand (40-100 mesh), and syringe filters (0.22µm) were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Iron(II) chloride tetrahydrate, dicyclopentadiene, ruthenium-based 1st generation Grubbs' catalyst, trichlorobenzene, urea, lauric acid, glucose, 4Å molecular sieves, Amicon Ultra 0.5 ml centrifugal filter devices (10K MWCO), and lipase acrylic resin from *Candida antarctica* (Novozym 435) were purchased from MilliporeSigma (Darmstadt, Germany).

1.2.2. Enzyme Purification

A 10% solution of lipase (wt/vol) in MES buffer was passed through a 0.22 µm syringe filter attached to a luer-lock syringe, then distributed into 0.5 mL 10K MWCO microcentrifuge tubes. The tubes were centrifuged for 14,000 x g for 15 minutes, followed by retentate dilution to approximately 1 mg protein/ml. Protein content was initially determined using the bicinchoninic acid (BCA) assay, read using a wavelength

of 562 nm against a standard curve of bovine serum albumin.[38] A standard curve of lipase was then constructed at 280 nm and used for regular analysis of purified lipase. Lipase was diluted in 10 mM MES buffer, pH 7, to 111 µg protein/ml and stored in the refrigerator until further use.

1.2.3. Fabrication of CLMP and CLMP-N

CLMP-N and CLMP were fabricated as described previously, with minor modification for CLMP fabrication (Figure 1).[33,34] Dopamine capped iron oxide nanoparticles were prepared via a one-pot procedure as described previously.[30,33,34] The aqueous phase of both CLMP-N and CLMP consisted of 55.5 µg/mL purified lipase, with the addition of 100 µg/mL dopamine-capped iron oxide nanoparticles for CLMP-N, which associated with the enzyme for 5 minutes prior to amalgamation. The oil phase, containing 0.42 M toluene, 6 mM Grubb's Catalyst, 6.67 M dicyclopentadiene, and 0.4 M trichlorobenzene, was added 10% (vol/vol) to the aqueous phase (90% vol/vol) and emulsified for 30 seconds (4200 cpm, Wykle Research). After incubation at room temperature (ca. 22 ℃) for 5 hours, CLMP-N and CLMP were centrifuged (2000 x g, 30 seconds, 3.8 cm tube height), washed three times with nanopure water, and stored in 10 mM MES buffer, pH 7, at 4 ℃ for further testing.

1.2.4. Microscopy

Scanning electron micrographs were obtained by the Rotello Group, using a FEI Magellan 400 Field-Emission Scanning Electron Microscope with a 1.0 kV acceleration voltage. Transmission electron micrographs were obtained using a JEOL 2000-FX 200 kV on CF-400-Cu carbon film square mesh copper grid (Electron Microscopy Science;

Hatfield, PA). Micrographs are representative of five images acquired at random locations from each of two independent samples.

1.2.5. Enzyme Kinetics

CLMP, CLMP-N, and purified native enzyme kinetics were analyzed according to previous work with slight modification.[33,34] Briefly, all samples were tested at a final protein concentration of approximately 1.67 µg/mL, with resorufin butyrate substrate concentrations ranging from 1-200 µM in 10 mM MES buffer, pH 7. Sample reading was performed at 50 ºC under constant (medium) shaking, and fluorescence values were read every 30 seconds for 12 minutes at 528/20 nm excitation and 590/20 nm emission wavelengths (Biotek Synergy). Rates were determined from an empirically calculated extinction coefficient derived from a standard curve of resorufin sodium salt. Michaelis-Menten enzyme kinetics were used to extrapolate the Michaelis constant (K_m) and maximum velocity (V_{max}) from a linear regression plot of substrate concentration versus velocity (v. 5.04, Graphpad Software, Inc., La Jolla, CA, U.S.A.). Assuming a molecular weight of 33,000 Da for CaLB, the turnover number (k_{cat}) was calculated by dividing V_{max} by the enzyme concentration.

1.2.6. pH Stability

CLMP, CLMP-N, and purified native lipase were diluted in 10 mM buffers adjusted to pH 3, 7, and 10 (sodium citrate, sodium phosphate, sodium carbonate buffers, respectively) buffers to an estimated protein concentration of 15 µg/mL. Samples were incubated at 30 ºC under constant rotation for 24 hours then diluted to approximately 1.67 µg/mL protein in 0.1 M MES buffer, pH 7. Activity measurements were performed using the synthetic substrate resorufin butyrate (10 µM in 10 mM MES buffer, pH 7).[33]

Fluorescence values were obtained every 30 seconds for 12 minutes, and subsequent rates were determined as described previously. Activity retention was calculated in relation to the original CLMP/CLMP-N/Native activities that were not exposed to pH changes.

1.2.7. Stability in Deep Eutectic Solvents (DES)

CLMP and CLMP-N were dispersed in ChCl:U at a protein concentration of approximately 15 µg/mL. Samples were tested at 2, 4, 6, 12, 24, 36, and 48 hours at either 50 ℃ or 60 ℃, and washed with nanopure water before centrifugal recovery (2000 x g, 2 minutes, 3.8 cm tube height). Final dilution to 0.167 µg/mL in 10 mM MES buffer pH 7 ensured negligible interactions of residual DES with resorufin butyrate. Activity measurements were conducted and analyzed as described previously. Activity retained was calculated as relative to the original CLMP/CLMP-N that was not exposed to elevated temperatures and DES.

For modeling, nonlinear regression, accounting for immobilized enzyme systems, was used based on a series-type deactivation model (Equation 1 and Equation 2).[33,39,40]

$$Eq\ 1:\ E \xrightarrow{k_1} E_I \xrightarrow{k_2} E_D$$
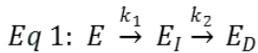
$$Eq\ 2:\ activity = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] \exp(-k_1 t) - \left[\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_1}{k_2 - k_1}\right] \exp(-k_2 t)$$

Where: t = time, E = native enzyme specific activity, E_I = enzyme intermediate specific activity, E_D = denatured enzyme activity, α_1 = E_I/E relative specific activity, α_2 = E_D/E relative specific activity, k_1 = E → E_I rate constant, and k_2 = E_I → E_D rate constant.

1.2.8. Influence of DES Exposure on Apparent Kinetic Parameters

CLMP and CLMP-N were dispersed in ChCl:U, at an estimated protein concentration of 15 µg protein/mL, and incubated at 50 ℃ or 60 ℃. After 12 hours,

samples were washed with nanopure water and recovered through centrifugation (2000 x g, 2 minutes, 3.8 cm tube height). Samples were diluted with 10 mM MES buffer, pH 7 and tested for kinetics using resorufin butyrate as a substrate, as described previously, with minor modifications. Substrate concentrations tested ranged from 5-100 μM to facilitate sample handling. Unexposed CLMP and CLMP-N were tested for apparent kinetic parameters simultaneously for comparison.

1.2.9. Glucose Ester Synthesis in DES

Lipase can participate in two esterification reactions used to produce sugar esters: direct esterification and transesterification (Figure 2). The byproducts from direct esterification and transesterification are water and ethenol (which likely tautomerizes to acetaldehyde), respectively.[16] Lipase is more active towards the transesterification reaction; however, the water byproduct of direct esterification is more desirable for greener processing.
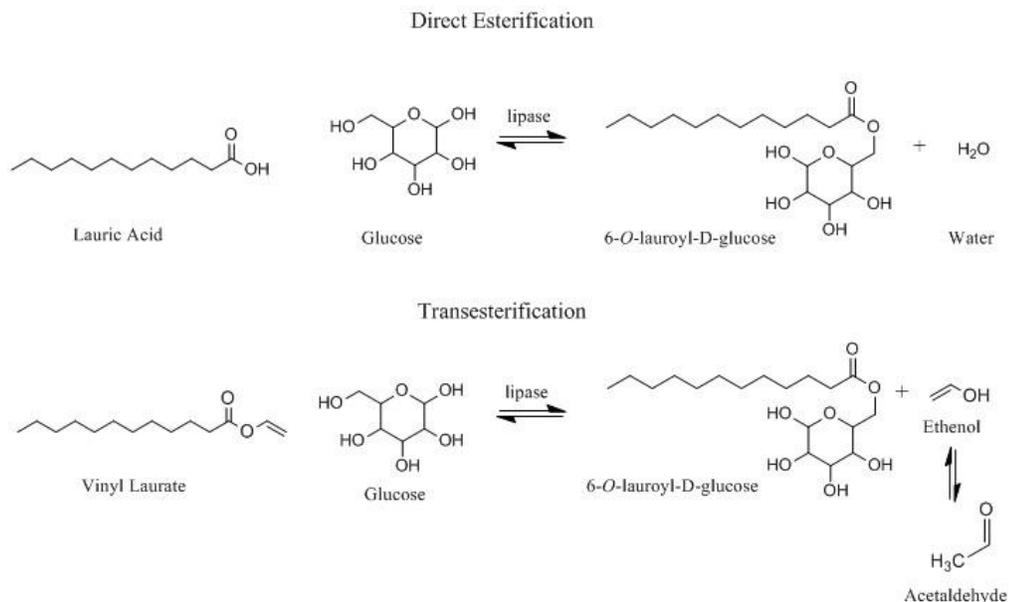
Figure 2. Proposed esterification reactions for glucose ester synthesis.

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For direct esterification, ChCl:U was dried over molecular sieves for 48 hours prior to dissolution of reactants. Glucose and lauric acid (1:1 molar ratio, 222 mM) were suspended in ChCl:U, as described previously, with the addition molecular sieves,[41] and rotated at 50 ℃ for 12 hours. The reaction began by adding 50 mg Novozym 435 to 0.5 mL of the reaction solution, with approximately 150 mg molecular sieves. For CLMP/CLMP-N, 1 mL of microparticles was centrifuged for 30 seconds (2,000 x g, 3.8 cm tube height) and the buffer was removed. To start the reaction, 0.5 mL of the reaction solution was added to the collected CLMPs (estimated 50 µg protein/tube), along with approximately 150 mg molecular sieves. After samples reacted under rotation for 12 hours at 50 ℃, 0.5 mL of deionized water was added to each sample and centrifuged for 2 minutes (2,000 x g, 3.8 cm tube height). To determine residual glucose, 0.5 mL of the aqueous phase was reacted with 0.5 mL DNS reagent and incubated for 5 minutes in a hot water bath (100 ℃).[42] Samples were diluted, cooled on ice for 3 minutes, and read spectrophotometrically at 540 nm. Sample absorbances were compared to a standard curve of glucose, originating from the initial reaction solution and exposed to identical dilution and centrifugation. Percent conversion was determined by the loss of glucose in solution, then normalized to the amount of protein present.

For transesterification, 222 mM glucose and 222 mM vinyl laurate were suspended in ChCl:U and rotated at 50 ℃ for 12 hours prior to reaction with lipase. To prepare CLMP/CLMP-N samples, 1 mL of microparticles was centrifuged for 30 seconds (2,000 x g, 3.8 cm tube height). The storage buffer was removed and 0.5 mL of the reaction solution was added to CLMP/CLMP-N, as well as to 50 mg Novozym 435 to

start the reaction. After 12 hours rotation at 50 ºC, samples were analyzed for glucose content and percent conversion, as described previously.

1.2.10. Statistical Analysis

Unless otherwise reported, analyses were performed in quadruplicate and results are representative of experiments repeated on two independent days. Statistical analysis was performed using Graphpad Prisim software (v. 5.04, Graphpad Software, La Jolla, CA). Curve fitting analysis, unpaired t-test, and one-way analysis of variance (ANOVA) with Tukey's multiple comparison test identified statistical differences among samples at $p < 0.05$.

1.3.   Results and Discussion

1.3.1. Particle Characterization

SEM and TEM micrographs revealed a rough microparticle surface for CLMP-N, (Figure 3). These clusters at the surface are hypothesized to be the electrostatically coupled lipase and iron oxide nanoparticles. CLMP showed a smoother microparticle surface, likely because of the absence of iron oxide nanoparticles. The large size of the particles (average 10 µM), facilitates rapid separation of the particles through simple centrifugation, as opposed to nanomaterials, which can take hours to magnetically separate.[33,43]
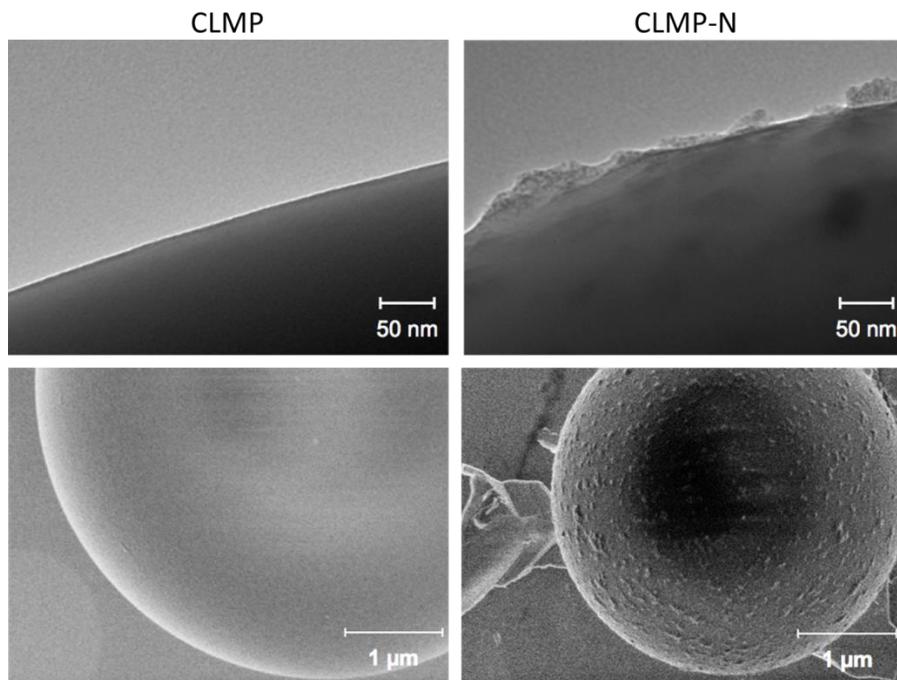
Figure 3. Scanning electron micrographs (bottom) and transmission electron micrographs (top) of CLMP (left) and CLMP-N (right). Micrographs are representative of five images acquired at random locations from each of two independent samples. Images courtesy Li-Sheng Wang.

1.3.2. Enzyme Kinetics

Michaelis constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) were characterized for native lipase, CLMP-N, and CLMP to quantify the influence of the hierarchical assembly technique on enzyme kinetics (Figure 4). Upon immobilization of native lipase into CLMP, K_m increases form 13.84 μM to 23.68 μM (Table 1). Compared to CLMP, the presence of nanoparticles in CLMP-N results in a decrease in the apparent K_m to 21.06 μM. These data suggest the cross-linked core imparts some mass transfer restrictions to the immobilized lipase.

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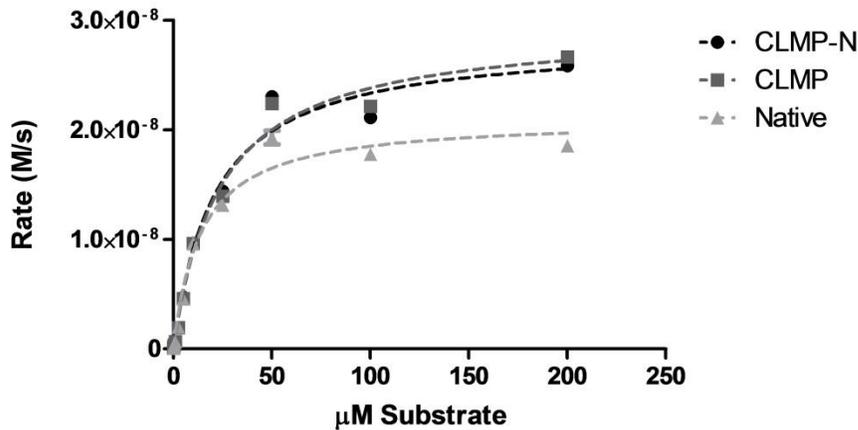
Figure 4. Apparent kinetics of CLMP-N, CLMP, and native lipase using resorufin butyrate as the substrate at 50 ºC in 10 mM MES buffer, pH 7. Values represent average ± standard deviation of n=4 determinations. Data are representative of experiments performed on two independent days.

CLMP-N and CLMP displayed a k_{cat} value of 0.56 sec^{-1}, and 0.58 sec^{-1}, demonstrating an increased V_{max} upon immobilization. Both of these values are higher than the native lipase k_{cat} of 0.42 sec^{-1}, and are in agreement with previously reported literature.[33] Kinetic trends were similar between days, but absolute values differed, such that significant differences may not exist when the microparticles are applied in industrial bioprocessing applications. The general increase in apparent turnover rate supports that immobilized lipase outperforms native lipase when situated at a hydrophobic interface.[44]

Table 1. Apparent kinetic parameters of native lipase, CLMP-N, and CLMP. Values represent 95% confidence intervals of n=4 determinations with superscripts indicating significant differences at p<0.05. Data are representative of experiments performed on two independent days.

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1}s^{-1})
CLMP-N	(20.65-21.48)[a]	(0.55-0.57)[a]	(2.6x10^4-2.7 x10^4)[a]
CLMP	(22.61-24.75)[b]	(0.57-0.60)[b]	(2.4 x10^4-2.5 x10^4)[b]
Native	(13.38-14.29)[c]	(0.40-0.43)[c]	(2.9 x10^4-3.0 x10^4)[c]

Taking into account the K_m and k_{cat} values, the catalytic efficiency, (k_{cat}/K_m), decreases upon immobilization and the addition of nanoparticles, compared to the native lipase, due to the observed increase in K_m values. These data show similar catalytic behaviors between CLMP-N and CLMP, suggesting the macrostructure of the microparticle may have a more significant impact on the apparent kinetic parameters than the presence of nanoparticles. Furthermore, these apparent kinetic parameters display overall advantageous properties of immobilized lipase at a hydrophobic interface.

1.3.3. pH Stability

After 24 hours at 30°C, CLMP-N retained 43%, 66%, and 76% activity at pH 3, 7, and 10, respectively. CLMP retained 35%, 61%, and 70% activity at pH 3, 7, and 10, respectively (Figure 5).
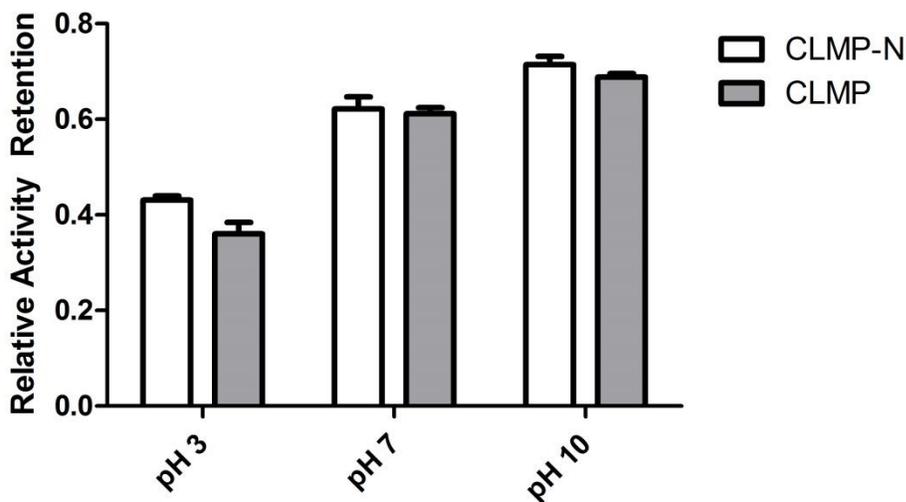
Figure 5. Relative activity retention of CLMP-N and CLMP after 24 hours exposure to pH 3, 7 and 10 buffers at 30 °C. Activity remaining after 24 hours was tested using resorufin butyrate (50 °C in 10 mM MES buffer, pH 7). Values represent average ± standard deviation of n=4 determinations. Data are representative of experiments performed on two independent days.

At neutral and basic pH values, no significantly different stabilization of lipase was observed for both CLMP-N and CLMP. These data suggest the main mechanism of stabilization is through conformational stability given by the polydicyclopentadiene core, similar to stabilization seen with traditional immobilization methods.[45,46] In addition, the core material could be influencing the microenvironment of the enzyme, providing protection from denaturation at basic pH values.

At pH 3, statistically significant stabilization of CLMP-N over CLMP was observed, supporting previous research demonstrating iron oxide nanoparticles coupled with lipase retain activity at acidic pH values.[47] Enhanced stability of CLMP-N may be provided by the presence of nanoparticles from multipoint covalent attachment stabilization imparted from the dopamine cap on the nanoparticle surface.[29,30] These data suggest the main form of stabilization throughout varying pH values comes from the core material. While significant differences were observed under these conditions, the proximity of the average retained activity values indicate that practical significant differences may not be observed in commercial applications. Nevertheless, these results demonstrate the potential of hierarchical systems to be tailored to specific applications for maximum stability.

1.3.4. Stability in Deep Eutectic Solvent (DES)

Both CLMP-N and CLMP were exposed to the deep eutectic solvent ChCl:U for 48 hours. ChCl:U was chosen as the solvent for the production of glucose esters because of the lack of excess hydroxyl groups. These hydroxyl groups, present in the deep eutectic solvent formed from choline chloride and glycerol, can interfere with the

esterification reaction, leading to the formation of secondary products.[37] Elevated

temperatures at 50 ℃ and 60 ℃ were used to decrease the viscosity of ChCl:U to

promote adequate mixing of the reactants and microparticles. Native lipase was not

included as a control, as previous research showed complete inactivation of native lipase

in less than two hours in buffer at 50 ℃ and 60 ℃.[33] Likewise, the commercially

available catalyst, Novozym 435 was unable to be included because of the inability to

accurately separate and recover the catalyst from ChCl:U for activity measurements with

resorufin butyrate. Previous stability testing of Novozym 435 required dry dilution in

sand to match protein concentration to the microparticles for accurate activity testing,

further complicating catalyst recovery.[33] As expected, both samples retained higher

activity at 50 ℃ than 60 ℃ (Figure 4). After 48 hours, CLMP-N retained 71% activity at

50 ℃, and 33% activity at 60 ℃, while CLMP retained 61% activity at 50 ℃, and 23%

activity at 60 ℃.
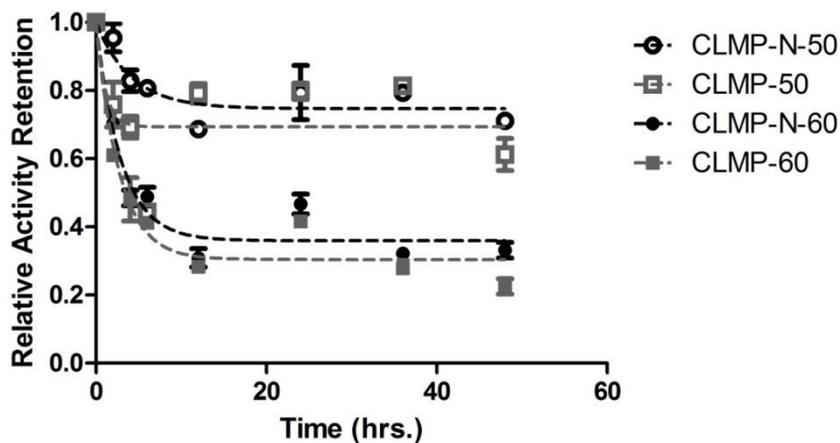
Figure 6. Relative activity retention of CLMP-N and CLMP after exposure to ChCl:U at
50 ℃ and 60 ℃. Residual activity was testing using resorufin butyrate as the substrate at
50 ℃ in 10 mM MES buffer, pH 7. Values represent average ± standard deviation of n=4
determinations. Data are representative of experiments performed on two independent
days.

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CLMP-N and CLMP displayed similar inactivation profiles: an initial drop in activity, followed by stabilization of the enzyme suggesting the stability lipase is being controlled by the core material. This finding supports previous research demonstrating immobilized lipases retain activity due to structural restrictions, which prevent aggregation.[45,46] As expected, a sharper decrease in activity is observed at 60 ºC than 50 ºC because of the more extreme environment. Retained activity was calculated based on the initial activity of the microparticles before exposure to ChCl:U and elevated temperature.

Approximately 70% activity retention after 24 hours by CLMP-N is in accordance with previously published data of the thermostability of CLMP-N in buffer.[33] However, CLMP-N activity observed in ChCl:U at 60 ºC is lower than that reported in buffer, suggesting increased interactions of ChCl:U, furthermore decreasing stability of the enzyme. However, the sustained activity exhibited by CLMP and CLMP-N over extended time further demonstrate feasibility of the use of ChCl:U for the production of glucose esters.

1.3.5. Influence of DES Exposure on Apparent Kinetic Parameters

The kinetic parameters of CLMP-N and CLMP after 12 hours exposure to ChCl:U at 50 and 60 °C were analyzed to determine the mechanism of lipase destabilization (Tables 2 and 3).

Table 2. Apparent kinetic parameters of CLMP-N and CLMP following exposure to 12 hours in ChCl:U at 50 °C. Values represent 95% confidence intervals of n=4 determinations with superscripts indicating significant differences at $p<0.05$. Data are representative of experiments performed on two independent days.

50 °C	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1}s^{-1})
CLMP-N	$(23.71 – 34.76)^a$	$(0.26 - 0.36)^a$	$(0.87x10^4 – 1.3x10^4)^a$
CLMP-N Initial	$(22.82 – 27.98)^{ab}$	$(0.40 -0.42)^b$	$(1.5x10^4 – 1.8x10^4)^b$
CLMP	$(24.96 – 33.44)^a$	$(0.33 - 0.36)^c$	$(1.1x10^4 – 1.3x10^4)^a$
CLMP Initial	$(23.17 – 24.08)^b$	$(0.45 -0.46)^d$	$(1.9x10^4 – 2.0x10^4)^c$

Table 3. Apparent kinetic parameters of CLMP-N and CLMP following exposure to 12 hours in ChCl:U at 60 °C. Values represent 95% confidence intervals of n=4 determinations with superscripts indicating significant differences at $p<0.05$. Data are representative of experiments performed on two independent days.

60 °C	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1}s^{-1})
CLMP-N	$(33.89 – 36.79)^a$	$(0.16 - 0.18)^a$	$(0.42x10^4 – 0.52x10^4)^a$
CLMP-N Initial	$(30.87 – 36.43)^a$	$(0.35 -0.38)^b$	$(1.0x10^4 – 1.1x10^4)^b$
CLMP	$(34.80 – 37.12)^a$	$(0.16 - 0.19)^a$	$(0.45x10^4 – 0.55x10^4)^a$
CLMP Initial	$(33.75 – 36.97)^a$	$(0.39 -0.42)^c$	$(1.1x10^4 – 1.2x10^4)^c$

At 50 °C and 60 °C, both CLMP-N and CLMP showed increased K_m values compared to the control, which had not been exposed to elevated temperatures or deep eutectic solvents. The increased K_m values of CLMP-N and CLMP were not significantly different from each other or the controls, suggesting limited restriction of the active site. Since the particles are recovered from the viscous ChCl:U with water, there is a potential for residual ChCl:U to be held on the surface from hydrogen bonding, resulting in higher deviations compared to the controls. The residual solvent coating the surface of the particle could restrict substrate interaction with the active site, explaining the increase in

K_m seen at 50 ºC for CLMP. However, this increase was not significantly different for CLMP-N at 50 ºC and both CLMP and CLMP-N at 60 ºC, suggesting limited impact of residual solvent.

Following exposure to both 50 ºC and 60 ºC in ChCl:U, both CLMP-N and CLMP displayed a significant decrease in the apparent turnover number, k_{cat}. Furthermore, both samples showed a significant decrease in catalytic efficiency (k_{cat}/K_m). The observed decrease in k_{cat} suggests conformational changes to the structure of lipase, which impact the rate of catalysis. The insignificant changes in K_m observed for CLMP-N at 50 ºC, and CLMP-N and CLMP at 60 ºC suggest active site binding is not impacted by these conformational changes. As expected, higher decreases in kinetic parameters were observed at 60 ºC than 50 ºC.

At each temperature, no practical differences between CLMP-N and CLMP were displayed, suggesting enzyme stabilization in ChCl:U is imparted from the macrostructure.   These data show the entrapment of the enzyme structure in the polydicyclopentadiene impacts enzyme stability greater than nanoparticles, agreeing with previous research showing entrapment of lipase in a hydrophobic material improves thermostability.[45]

1.3.6. Glucose Ester Synthesis in DES

CLMP, CLMP-N and Novozym 435 (N435) were exposed to direct esterification reaction conditions for 12 hours at 50 ºC. Temperature selection was based on stability testing, where CLMP and CLMP-N retain over 60% activity at 50 ºC in ChCl:U throughout 12 hours of exposure.   Novozym 435 was selected as a commercially available form of immobilized lipase. Initial glucose, lauric acid, and Novozym 435

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concentrations were selected based on prior reports of lipase-catalyzed direct esterification within ionic liquids.[41,48,49] From empirical calculations, the amount of protein present in Novozym 435 was estimated to be 2500 µg protein/50 mg solid support, while CLMP and CLMP-N estimated to be 50 µg protein per reaction vial. To account for the large difference in protein content, reported values were normalized to represent percent of glucose converted per µg protein. After 12 hours, Novozym 435 converted 0.002±0.001% glucose per µg protein (Figure 7a), and CLMP and CLMP-N converted 0.23±0.06%, and 0.14±0.07% glucose per µg protein, respectively. Previous research has shown correlation between the DNS method for reducing sugar quantification and HPLC quantification.[48]
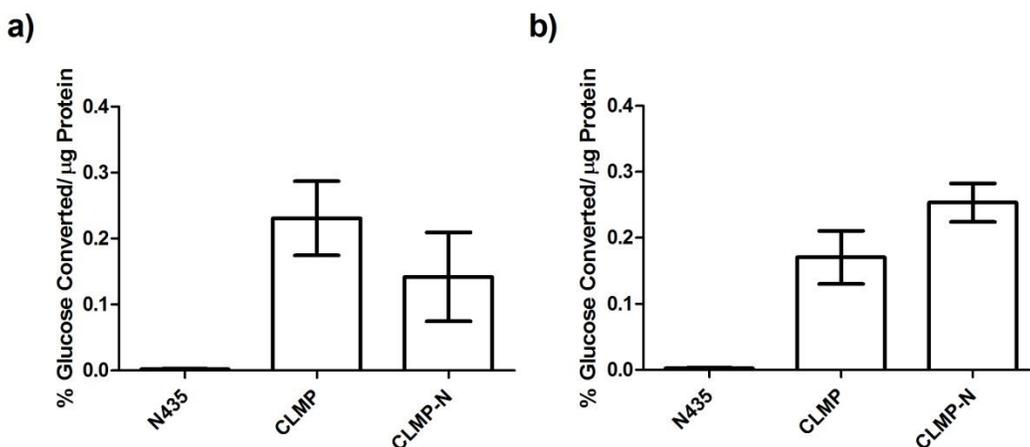
Figure 7. Percent conversion of glucose per µg protein in ChCl:U at 50 ºC after 12 hours exposure to Novozym 435, CLMP, and CLMP-N for a) direct esterification and b) transesterification. Values represent average ± standard deviation of n=8 determinations. Data are representative of experiments performed on two independent days.

Per unit protein, CLMP is approximately 114 times more active than the industry standard, and CLMP-N is approximately 70 times more active. After exposure to ChCl:U, CLMP retained a higher turnover rate (k_{cat}) than CLMP-N, possibly explaining

the higher activity exhibited by CLMP over CLMP-N when applied to direct esterification. Previous reports of kinetic analysis using a synthetic substrate demonstrated that CLMP-N is more efficient than Novozym 435, further supporting the findings.[33] The increased surface area of CLMP/CLMP-N compared to Novozym 435 may reduce mass-transfer limitations and steric hindrance, thus increasing conversion to product. Since Novozym 435 could not be tested for stability in ChCl:U because of handling considerations with the synthetic substrate, Novozym 435 may experience steric hindrance from the high amount of protein loaded onto the support. The viscosity of ChCl:U increases upon the addition of reactants, which may impart mass transfer limitations, inhibit the reaction, and increase deviation. Direct esterification is governed by the buildup of the secondary product, water.[50] Thus, molecular sieves were added to absorb water prior to and throughout the reaction, however small amounts of water may still inhibit the process. As a result, transesterification was studied to circumvent the issue of aqueous inhibition.

Transesterification is considered a more favorable pathway for lipase because the secondary product, ethenol, is rapidly tautomerized to acetaldehyde, driving the reaction towards completion. Following 12 hours exposure at 50 ºC, Novozym 435 converted 0.002±0.001% glucose per µg protein, while CLMP and CLMP-N converted 0.17±0.04% and 0.25±0.03% glucose per µg protein, respectively (Figure 7b). As observed with direct esterification, CLMP and CLMP-N perform significantly better than Novozym 435, per unit protein. Novozym 435 activity was similar for direct esterification, suggesting inactivation of the enzyme, or mass transfer limitations, due to the viscosity of the deep eutectic solvent. CLMP-N showed an increase in activity

compared to direct esterification, which was expected due to the reaction mechanism. However, CLMP activity for transesterification was not significantly different from the activity seen during direct esterification. The difference in activity during transesterification between CLMP and CLMP-N may imply that the presence of nanoparticles does influence substrate affinity for certain substrates. Apparent kinetic parameters determined with a synthetic substrate, are specific to that given substrate, and can change with the substrate; hence differences were observed between direct esterification and transesterification. Because of the inherent error associated with the viscous deep eutectic solvent, differences in percent conversion may also be due to insufficient mixing or aggregation of the microparticles. Nonetheless, CLMP and CLMP-N demonstrate significant advantages over Novozym 435 for both direct esterification and transesterification, per unit protein.

1.4. Conclusions

Hierarchical structures, incorporating both macrostructures and nanostructures, can provide enhanced stability to immobilized enzyme systems through stabilizing the structure and microenvironment of the enzyme. Apparent kinetic parameters reveal similarities between CLMP-N and CLMP, the macrostructure dictates kinetic parameters for this hierarchical system. Stability testing in varying pH values demonstrated that nanoparticles may influence the microenvironment of the enzyme, providing additional stability for this system. Stabilization of lipase in deep eutectic solvents, elevated temperatures and alkaline conditions is likely due to protein structure and microenvironment stabilization from the macrostructure, polydicyclopentadiene. Lipase

microparticles exhibited stabilized activity for 48 hours of time in ChCl:U, and performed better than the commercially available immobilized lipase when applied to the synthesis of glucose esters. For direct esterification, the macrostructure dictated lipase activity in ChCl:U; however, for transesterification, the presence of nanoparticles may influence activity towards vinyl esters. Further optimization of reaction conditions has the potential to increase total product yield and activity. A greater understanding of hierarchical systems can lead to tailor-made biocatalyst systems with enhanced stability and practical application in the food and bioprocessing industries.

CHAPTER 2

RECOMMENDATIONS FOR FUTURE RESEARCH

Hierarchical systems show potential for enhanced enzyme activity and stability in extreme environments. The aforementioned research showed the application of hierarchical systems for the production of value-added agricultural products in deep eutectic solvents. However, the feasibility of commercial application of the deep eutectic solvent, ChCl:U, is low due to the high viscosity of the solvent. Additionally, for the production of sugar esters using glucose and lauric acid/vinyl laurate, a different solvent should be explored, as the product is difficult to extract from the reaction mixture. To continue optimization of the production of sugar esters in ChCl:U, I recommend the following:

- Increase the temperature of the reaction to 60 ℃ to further reduce viscosity.

- Explore other sugars (e.g. fructose, maltose) and both lower and higher chain lengths of fatty acids- to find a product less soluble in ChCl:U.

- Use a method other than rotation for mixing- possibly magnetic stirring in a reaction block.

It is recommended to explore other solvents than ChCl:U for the production of sugar esters, especially those with lower viscosities. Additionally, because of the inherent difficulties in the recovery of a surfactant product, other reactions should be explored using hierarchical systems. This type of system has the ability to be used for various applications such as biodiesel production and in biosensors. This research presents methods for understanding how hierarchical systems can be tailored for specific processing applications.

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