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ABSTRACT

Confocal laser scanning microscopy (CLSM) in combination with a fluorescently labeling enzyme dye, LavaPurple™, was demonstrated as a technique for the visualization of Thermomyces (Humicola) lanuginosa lipase (LIPHLL) and Candida antarctica lipase A (LIPCA) within a transparent latex coating. Addition of Teric surfactants (1.8 %wt/wt addition of C16 non-ionic Teric 475 or 2.0 %wt/wt addition of C10 non-ionic Teric 460) significantly increased the accumulation of both LIPHLL and LIPCA to the surface of a latex coating. An $\alpha$-naphthyl acetate substrate assay was used to quantify the accumulated lipase. The results derived from the acetate assay correlated with the enzyme accumulation (at the surface) observed in the CLSM images of the latex coating. This correlation demonstrated that the increased enzyme accumulation within the top 2 $\mu$m of the latex film was responsible for the increase in surface enzymatic activity. The combination of CLSM imagery and quantifiable image analysis provided a valuable tool for the optimization of surfactant concentrations for maximizing the activity of an enzyme (and potentially other additives) within a latex coating.

Keywords: confocal laser scanning microscopy; lipase; catalytic coating; surface enzyme activity; surfactant; latex
1. INTRODUCTION

Environmental sustainability requires the design and engineering of biointerfaces between synthetic surfaces and natural environments. Biological-synthetic composites are topical at present and represent a means of improving the interface between synthesized surfaces and the natural environment.[1] Enzymatic immobilization in commercial coatings is one such biological-synthetic composite, combining synthetic coating engineering with the utility of nature's catalysts, enzymes.[2-7] Direct incorporation of enzymes into solutions, prior to coating, represents an economical, industrially adaptable and scalable method of producing enzyme catalytic coatings.[8]

Rapid evaporation of water and solvent from the surface of a freshly applied coating can cause a skin to form before the film has completely dried. This effect may reduce or prevent migration of enzymes to the surface from the bulk of the film before drying. Concentrating active enzyme to the air-film surface interface (referred to hereafter as the surface) of the coating provides greater activity by increasing catalytic sites per unit area of surface and can decrease the amount of enzyme needed to produce an equivalent catalytic affect at the surface.

Surfactants can be used as surface concentrating agents in commercial latex coatings, and are known to closely associate with lipases.[9] Commercially available surfactants commonly used in paint manufacturing can combine with enzymes without compromising the activity of the biocatalyst, for example, ionic surfactants such as SDS have been demonstrated to form a complex solution with pancreatic lipase.[10] Ethoxylated nonyl phenol products marketed under the proprietary name “Teric” (e.g., Teric 475, Teric 460 - non-ionic surfactants) also interact with enzymes. Previous studies of Teric 475 surfactant affected the surface activity of
lipase-surfactant complexes.[11, 12] The surfactant-enzyme combination could result in enzymes concentrating to the surface of a coating.

Confocal laser scanning microscopy (CLSM) in combination with a fluorescent labeling enzyme dye can be used as a visualization tool to confirm a surfactant mediated accumulation of enzymes at the surface of a catalytic coating. While CLSM has been used to visualize surface-bound enzymes in films[13] and in the identification of reactive amino groups of chitosan films[14], polyurethane coatings containing lysozyme represent the only example of enzymes imaged by CLSM.[3] Image analysis and quantification of the CLSM acquired images measure accumulated enzyme within a coating and thus enable the screening of additives that concentrate enzymes to the surface.

Here, we report the use of CLSM in combination with LavaPurple™ labelled Thermomyces (Humicola) lanuginosa lipase (LIP_HLL) and Candida antarctica lipase A (LIP_CA) to visualize the depth distribution of enzymes in a transparent latex coating. Commercially available surfactants, Teric 475, 460 and SDS were added to these coatings to produce accumulated lipase regions at the surface of the resulting catalytic latex films. Statistical analysis (ANOVA followed by Bonferroni posthoc testing) and quantification of the CLSM images using software (ImageJ) confirmed that concentrating the enzyme in the upper 2 µm of the latex coating is critical to significantly improving activity of the catalytic latex film. The combination of CLSM imagery and quantifiable image analysis can provide a valuable tool for the optimization of surfactant concentrations for maximizing the activity of an enzyme containing latex film.
2. Material and Methods

Lipase enzymes and fluorescent labeling

*Thermomyces (Humicola) lanuginosa* lipase (LIP<sub>HL</sub>) and *Candida antarctica* lipase A (LIP<sub>CA</sub>) lipases were supplied by Novozymes Inc. (Denmark) and used after filtration through Durapore sterilizing-grade 0.22 µm hydrophilic Polyvinylidene difluoride (PVDF) low protein-binding membranes (Millipore Inc.). LavaPurple™.[15-17] Total Protein Stain (Fluorotechnics, Sydney) was used to stain lipases for confocal microscopy. Ten µL of lipase (50 µg) and 5 µL of LavaPurple™ dye (1:200 dilution) were mixed at room temperature for 1 hour. The Novozymes published activity for both lipases is 50 Kilo Lipase Units per gram (KLU/g). α-naphthyl acetate and FAST Blue Dye BB Salt obtained from Sigma Aldrich (USA), were used as an esterase-proxy for lipase activity. [18]

Surfactants

Concentrations of Teric 475 (a C<sub>16</sub> ethoxylated non-ionic surfactant) and Teric 460 (a C<sub>10</sub> ethoxylated non-ionic surfactant) ranging from 0.5 % - 3.0 %wt/wt and different concentrations of surfactant Sodium Dodecyl Sulphate (SDS) ranging from 0.2 - 1.0 %wt/wt were examined for their effect on the elevation of lipase concentration at the surface of the latexes.

Preparation of latex films

Model latex films consisted of mix of styrene-acrylic latex (DuluxGroup, Melbourne, Australia) and 2,2,4-trimethyl-1,3-pentanediol monoisobutyrate (2%) (Texanol Ester Alcohol - Eastman) used as a coalescing, non-volatile solvent to stabilize the film. To ensure a transparent film for CLSM analysis, the pigment particles typical in a standard paint
formulation were substituted with 3 μm dia. clear solid beads at 20 %wt/wt. The LavaPurple™ labeled lipase was mixed in the latex formulation at a concentration of 15 μL per mL of latex. Wet latex films of 80 μm thickness were created by spreading 0.5 mL of latex mixture on thin transparent Melinex® polymer sheets using a Multicator 411 (Erichsen, Germany). These were dried at 25 ºC for 48 hours giving a dry film thickness of ~ 40 μm. The latex had a pH of 7-7.5 and the addition of Texanol resulted in a pH of 6.5 in the final emulsion.

**Confocal Laser Scanning Microscopy**

Twenty μL of MilliQ water was placed on the dried latex film, covered with a cover slip and the film was placed inverted on the viewing stage of an Olympus FV1000 Confocal Inverted Microscope. The transparent film was viewed using the 40x water immersion lens and at an excitation wavelength of 488 nm. The LavaPurple™-lipase complexes fluoresced red. The fluorescent region was viewed in the Z (depth) direction. The cross sectional projections of the lipase-incorporated films were integrated by stacking the X-Y plane intensities along their Z-axis. Laser intensity and detector settings were optimized and kept constant throughout all experiments.

**CLSM image and statistical analysis**

The cross-sectional (Z-plane) images of all lipase-incorporated films were quantified with ImageJ® to determine the percent coverage of lipase in the top 8 μm of the transparent films. Each 1 μm slice from the surface to 8 μm depth was measured for the area coverage by the LavaPurple™ labeled lipase molecules. One-way and two-way ANOVA with Bonferroni post-hoc testing was performed using PRISM (Mac v5.0b).
**Enzymatic activity of films**

Surface activity of the embedded lipases on paint films was determined by a surface esterase assay using α-naphthyl acetate as a substrate. Paint-enzyme mixes were cast as films on Melinex® polyester film as described above. After drying, circular pieces (44 mm in diameter) were cut and placed inside each well of a 12 well culture plate. These dry films were treated with 0.23 mL of naphthyl acetate (1 mM) dissolved in acetone:water (ratio 1:1, pH 7.0) and distributed evenly across the surface. The plates were shaken under constant agitation (5 min, 50 rpms). Afterwards, the reaction was stopped by adding 20 µL of stop reagent (0.2% FAST Blue dye BB in 5% SDS). The color was developed for 10 min at room temperature and absorbance was measured at 600 nm.

3. RESULTS & DISCUSSION

3.1 Confocal imagery of catalytic latex films

Confocal laser scanning microscopy (CLSM) in combination with the fluorescently labeling enzyme dye, LavaPurple™, enabled visualization of enzyme accumulation, with respect to depth within a film, of *Thermomyces (Humicola) lanuginosa* lipase (LIP_HLL) and *Candida antarctica* lipase A (LIP_Ca) to 20 µm below the surface of a transparent latex coating. Addition of LIP_HLL or LIP_Ca to the latex mixture (without surfactants) resulted in the homogenous distribution of lipases throughout the depth of the coating (Figures 1, 2, 3). Due to the surface concentrating effect of surfactants, it was predicted that the movement of surfactants in a drying latex film could interact with the added lipases and form a complex thus moving the lipases to the surface. Hence, surfactants Teric 475, 460 and SDS were added to the lipase containing latex coatings. This addition produced an accumulation of lipase toward the latex surface (Figures 1, 2, 3 respectively). This observation is consistent with
previous studies indicating increased enzymatic activity in surfactant containing emulsions.[4, 19] For example, surfactants such as Triton X-100 and Tween-80, enhanced activity and stability in solutions of enzymes.[4, 20]

In this study, the maximum amount of surface accumulated lipase was observed with the addition of Teric 475 (1.8wt/wt%, Figure 1), Teric 460 (2.0wt/wt%, Figure 2) and SDS (0.2wt/wt%, Figure 3). Teric 460 appeared to be less effective in concentrating the lipase to the surface of the latex coating. This may be due to the lower molecular weight of Teric 460 in relation to Teric 475 (C10 vs C16). At concentrations of surfactant beyond that needed to maximize enzyme surface accumulation, a more distributed fluorescent lipase band using Teric 475 (3.0%) or SDS (0.5%) was observed (Figures 1 and 3 respectively). An excess of surfactant can reduce activity of immobilized lipases. Wannerberger et al.[21] noted a reduction in lipase activity at high surfactant concentrations. At high surfactant concentrations, enzyme-surfactant interactions are estimated to be weak due to the formation of micellar aggregates. If surfactants are present above their respective critical micelle concentrations, they have higher surface activity and are present in greater concentrations over proteins at interfaces.[22, 23] The observed concentration versus homogenous distribution of the lipase band in our CLSM images suggested an optimal amount of surfactant required to maximize the accumulation of lipase to the surface of the latex films that should correspond to a maximum level of lipase activity at the surface. A surface enzymatic assay was used to test this suggestion.

### 3.2 Enzymatic assay of catalytic latex films

An α-naphthyl acetate assay was applied to the surface of the lipase containing latex films to determine surface activity of the lipases. The absorbance of the α-naphthyl acetate was measured for each of the surfactant percentage additions and normalized against the zero
surfactant addition control for both LIP_{HLL} and LIP_{CA} containing latex coatings. The effect on enzymatic activity for all three surfactants, Teric 475 (Figure 4), Teric 460 (Figure 5) and SDS (Figure 6) was measured. The surfactants increased enzyme activity by as much as 100% over the control with Teric 475 at 1.8 and 2.5 wt/wt% facilitating the greatest enzyme activity for both LIP_{HLL} and LIP_{CA} latex films. This corresponded well with the fluorescently labeled enzyme band at the surface in Figure 1 at 1.8 and 2.5 wt/wt% (Teric 475) and confirmed the utility of CLSM imaging of latex films as a screening tool for determining the optimal accumulation and corresponding activity of enzyme at the latex film-air interface. This correlation was also observed for Teric 460 additions (Figures 2 and 5) with a significantly greater activity shown for 2.0 wt/wt% added Teric 460. The LIP_{HLL} and LIP_{CA} accumulated to the film surface as predicted with the addition of 0.2% SDS, comparable to 1.8 and 2.0 wt/wt% of Terics 475 and 460 respectively. Despite the observed contraction of the enzyme band to the surface in the latex film with added SDS, there was only a marginal, non-significant (30%) increase in α-naphthyl acetate activity (Bonferroni post-hoc tests). Furthermore, increased amounts of added SDS did not produce a significantly increased enzyme activity at the surface. SDS surfactant is known to bind readily to and alter the activity of proteins[10, 24]. For example lipases from the fungus *Metarhizium anisopliae* (spore surface), and a *Rhizopus sp.* showed both increases and decreases in enzymatic activity in the presence of SDS.[25, 26]. Martinelle and co. workers [27] observed the level of enzymatic activity change of LIP_{CA}, in the presence of SDS, was found to be insignificant but in contrast, measured an increase in enzymatic activity for LIP_{HLL} in the presence of SDS. This is supportive of the findings we observed of SDS in our study and although we measured LIP_{HLL} exhibiting a trend towards greater activity in the presence of SDS (Figure 6), this was not determined to be significant.

### 3.3 Quantification of confocal images of catalytic latex films
To further analyze the CLSM data, we applied image (ImageJ) and statistical analysis (ANOVA followed by Bonferroni post hoc testing) to the CLSM images. Using 1 µm slices starting from the surface interface of the CLSM images, the percent coverage of fluorescence in each slice was determined down to a depth of 8 µm below the surface. The percent coverage was plotted versus the depth of slice for LIP_{HLL} with added T475 (Figure 7a), LIP_{CA} with added T475 (Figure 7b), LIP_{HLL} with added T460 (Figure 8a) and LIP_{CA} with added T460 (Figure 8b). T475 and T460 at 1.8 and 2.0 wt/wt% respectively had a significant increase in enzyme density in the top 2 µm for all four lipase and Teric surfactant combinations (Supporting information Tables 1, 2, 3 and 4). This observation, across two lipases and two surfactants, highlighted the importance of concentrating enzyme to within 2 µm of the surface for maximum enzymatic activity. In contrast, significantly less enzyme was measured in the bulk of the coating (4 - 8 µm) confirming that the majority of the enzyme had accumulated to the top of the latex thus providing the maximum surface enzyme activity. Due to no significant increase in enzyme activity at the surface of the lipase containing latex with added SDS, the image analysis was not carried out on these CLSM images.

**Acknowledgements**

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REFERENCES


List of figure captions

**Figure 1.** CLSM (X-Z) sections of latex films containing LavaPurple™-labelled lipase LIP_{HLL} or LIP_{CA} in 20 µm of latex with Teric 475 at 0.0, 1.0, 1.8, 2.5 and 3.0 %wt/wt. Images at 40x magnifications

**Figure 2.** CLSM (X-Z) sections of latex films containing LavaPurple™-labelled lipase LIP_{HLL} or LIP_{CA} in 20 µm of latex with Teric 460 at 0.0, 1.0, 2.0 %wt/wt. Images at 40x magnifications

**Figure 3.** CLSM (X-Z) sections of latex films containing LavaPurple™-labelled lipase LIP_{HLL} or LIP_{CA} in 20 µm of latex with SDS at 0.0, 0.2, 0.5 and 1.0 %wt/wt. Images at 40x magnifications

**Figure 4.** α-naphthyl acetate enzyme activity of LIP_{HLL} and LIP_{CA} containing latex coating with increasing wt% of additional Teric 475 (proportional to 0% added T475). n = 3. Letters denote groups of significance, p < 0.05

**Figure 5.** α-naphthyl acetate enzyme activity of LIP_{HLL} and LIP_{CA} containing latex coating with increasing wt% of additional Teric 460 (proportional to 0% added T460). n = 3. Letters denote groups of significance, p < 0.05

**Figure 6.** α-naphthyl acetate enzyme activity of LIP_{HLL} and LIP_{CA} containing latex coating with increasing wt% of additional SDS (proportional to 0% added SDS). n = 3

**Figure 7.** ImageJ software quantification of CLSM images of additional Teric 475 wt% to a) LIP_{HLL} and b) LIP_{CA} latex coatings. One µm slices from surface of the coating to an 8 µm depth were measured.

**Figure 8.** ImageJ software quantification of CLSM images of additional Teric 460 wt% to a) LIP_{HLL} and b) LIP_{CA} latex coatings. One µm slices from surface of the coating to an 8 µm depth were measured.
Figure 3
Figure 4
Proportional change relative to 0% added Teric 460

Lipases

Figure 5
Figure 6
Figure 7a
Figure 8a
Figure 8b
Supporting information Table 1. and 2. Statistical analysis of ImageJ software quantification of CLSM images of additional Teric 475 wt% to a) LIP_{HLL} and b) LIP_{CA} latex coatings. One µm slices from surface of the coating to an 8 µm depth were measured.

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[a] Level of significance  [b] *p<0.05, **p<0.01, ***p<0.001
Supporting information Table 3. and 4. Statistical analysis of ImageJ software quantification of CLSM images of additional Teric 460 wt% to a) LIPHLL and b) LIPCA latex coatings. One µm slices from surface of the coating to an 8 µm depth were measured.

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[a] Level of significance [b] *p<0.05, **p<0.01, ***p<0.001