High-Activity Enzyme-Polyurethane Coatings

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Abstract: The synthesis of water-borne polyurethane coatings in the presence of diisopropylfluorophosphatase (DFPase, E.C. 3.8.2.1) enabled the irreversible attachment of the enzyme to the polymeric matrix. The distribution of immobilized DFPase as well as activity retention are homogeneous within the coating. The resulting enzyme-containing coating (ECC) film hydrolyzes diisopropylfluorophosphate (DFP) in buffered media at high rates, retaining approximately 39% intrinsic activity. Decreasing ECC hydrophilicity, via the use of a less hydrophilic polyisocyanate during polymerization, significantly enhanced the intrinsic activity of the ECC. DFPase-ECC has biphasic deactivation kinetics, where the initial rapid deactivation of DFPase-ECC leads to the formation of a hyperstable and active form of enzyme.
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INTRODUCTION

Immobilization has been widely employed to enable and enlarge the application of enzymes as catalysts in industrial processes. The insertion of biological molecules in coatings and thin films would drive a large range of applications. For example, potentiometric biosensors often involve the covalent attachment of enzyme onto an inner film adjacent to the sensing surface of the electrode and the subsequent protection of the enzyme layer with an outer film (Jung et al., 1996; Marzouk et al., 1997). Another immobilization method for the fabrication of amperometric biosensors relies on the entrapment of enzyme in a gel layer, which is further coated by an external protective film (Mădăraş et al., 1995). The lifetime and use of such systems are often limited by the diffusion of enzyme through the external membrane (Peteu et al., 1996). To overcome this main disadvantage, the enzyme has to be directly and covalently immobilized into the coating. The covalent incorporation of biocatalyst into coatings would also be beneficial for other bioprocesses, such as biocatalytic separation and filtration (Flickinger et al., 1998, 1999), microchips (Chován et al., 2002; Kim et al., 2001a), and antifouling (Novick et al., 2002).

Direct covalent immobilization of highly active enzymes into coatings and films has remained an elusive goal, with some of the most successful approaches (Kim et al., 2001b) exhibiting only up to 0.5% activity. Water-borne polyurethane coatings result from the polymerization of aqueous polyester-based polyol dispersions and water dispersible aliphatic polyisocyanates. As the film is cured at room temperature, water evaporates and cross-linking occurs through the condensation between hydroxyl groups and isocyanate functionalities. Two-component water-borne polyurethanes are increasingly used in industrial applications and they exhibit properties similar to those of solvent-borne polyurethane coatings (Boudreaux et al., 1999; Feng et al., 1999; Melchiors et al., 2000). Water-borne polyurethane coating represents a potentially ideal polymeric matrix for multipoint and covalent immobilization of enzymes, and given our depth of understanding of monolith polyurethane-enzyme composites we have begun to explore whether an enzyme can be directly added to the aqueous phase of a two-component system prior to polymerization. The immobilization process relies on the ability of amines at the enzyme surface to react with isocyanate functionalities at a faster rate than hydroxyl groups on the polyol.

In the study reported herein, we investigate the immobilization of diisopropylfluorophosphatase (DFPase,

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EC 3.8.2.1) into water-borne polyurethane coatings. Native DFPase catalyzes the hydrolysis of toxic organophosphorus nerve agents such as soman and DFP (Hartleib et al., 2001a,b; Scharff et al., 2001a,b). DFPase was previously copolymerized into monolithic polyurethane foams with a 67% activity retention (Drevon et al., 2000) and an enhanced thermostability (Drevon et al., 2001). Since alterations in enzyme-containing coating (ECC) hydrophilicity could influence activity retention and stability, we performed the immobilization process using polyisocyanates with various hydrophilicities. The degree to which the enzyme was irreversibly attached to the support was determined. The enzyme distribution within the coating was observed by means of gold-labeling. The influence of mass transfer on the activity of enzyme-polymers was examined using a diffusion cell apparatus. The enhancement of DFPase thermostability via immobilization was also investigated.

MATERIALS AND METHODS

BAYHYDUR polyisocyanates XP-7063, XP-7007, XP-7148, BAYHYDROL polyol XP-7093, and Desmodur N3400 as well as thermoplastic polyolefin (TPO) panels, used in the synthesis and curing of protein-containing coatings were kindly provided by Bayer Corp. (Pittsburgh, PA). The surfactant BYK-345 was obtained from BYK-Chemie (Wallingford, CT). Diisopropylfluorophosphate (DFP), Bradford reagent, bovine serum albumin (BSA), Bis-Tris Propane, Tris(hydroxymethyl) aminomethane-HCl (Tris-HCl), $CaCl_2$, NaCl, K_2CO_3, and isopropanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DFPase was purchased from BioCatalytics (Pasadena, CA). Polybed 812 embedding resin was obtained from Polysciences (Warrington, PA).

ECC Synthesis

ECCs were prepared using buffered aqueous mixtures (10 m*M* Bis-Tris-Propane buffer, pH 7.5, 5 m*M* $CaCl_2$). Water-borne two-component polyurethanes were synthesized using water-dispersible aliphatic polyisocyanates based on hexamethylene diisocyanate (HDI) BAYHYDUR and the polyol dispersion coreactants BAYHYDROL. During ECC synthesis, a ratio between isocyanate and hydroxyl functionalities of 2 was used. Typically, BAYHYDROL XP-7093 (2.5 g) (water content of 70 w%), BYK-345 surfactant (0.01 g) and buffered medium (1.2 g) were poured into a cylindrical vessel, and followed by the addition of enzyme (0.02–9 mg). The aqueous solution was further stirred mechanically (300 rpm) for 1 min. The amounts of BAYHYDUR XP-7063, XP-7007, XP-7148 required for ECC synthesis were calculated knowing the polyisocyanate equivalent molecular weights. When using XP-7007, the polyisocyanate (1 g) was added to the aqueous solution and the biphasic mixture was agitated for 20 sec with a custom-designed head attached to a 2500 rpm hand-held drill. After mixing, a white emulsion with a 63 w% water content was obtained and applied (0.45 g) on thermoplastic polyolefin (TPO) panels previously cleaned with isopropanol and dried under ambient conditions. The ECC was then allowed to cure for 12 h under ambient conditions and weighed again (0.24 g).

Bis-Tris-Propane contains hydroxyl groups and secondary amines, which might react with the isocyanates during the coating synthesis. The amount of buffer salt added to the reaction mixture was negligible compared to the reactive functionalities of the polyisocyanate and polyol dispersion and, hence, did not affect the properties of the resulting two-component water-borne polyurethanes.

Protein Concentration Determination

Protein concentrations were evaluated using the Bradford reagent, as described previously (Drevon et al., 2000).

Synthesis of Enzyme/Gold Conjugates

Gold colloids with diameters ranging from 25–30 nm were prepared as previously described (Lococq et al., 1993) and conjugated to DFPase in aqueous medium (Albrecht et al., 1993). During conjugation the pH was adjusted slightly above the enzyme isoelectric point (pI 5.8) with K_2CO_3. The pH was measured with litmus paper. Typically, an enzyme weight of 0.12 g was needed to stabilize 30 ml of gold colloid solution (gold concentration: 0.01%). After addition of DFPase, the enzyme-gold solution was gently agitated and BSA solution (10% [w/v]) was added to a final concentration of 0.1% (w/v). BSA blocked areas of the colloidal surface that were not coated with the enzyme. The resulting solution was centrifuged for 1 h at 100,000 rpm and the enzyme-gold conjugate was recovered in the precipitate, which was resolubilized in buffered medium (10 m*M* Tris-HCl, pH 7.5). Centrifugation lead, to a certain extent, to the formation of gold clusters. The largest clusters were found in dense areas of the precipitate, which were discarded. Smaller clusters were still present among the colloidal gold conjugates. Coatings were further prepared with BAYHYDUR XP-7007 as described above using two different concentrations of colloidal gold conjugated to enzyme (0.001 $mg_{gold}/g_{coating}$ and 0.012 $mg_{gold}/g_{coating}$).

Localization of Gold-DFPase Conjugate in Coating

To embed the films for transmission electron microscopy (TEM), small strips were washed several times in

100% ethanol, then incubated in several 1-h changes of Polybed 812 embedding resin. Films were cut into 1×2 mm strips, placed in embedding molds, and embedded in Polybed 812. Blocks were cured overnight at 37°C, then cured for 2 days at 65°C. Ultrathin cross sections (60 nm) of the films were obtained on a Riechart Ultracut E microtome. Sections were viewed on a JEOL JEM 1210 or 100CX TEM at 80 KV.

Activity of ECCs Using a Fluoride Ion Electrode

ECC was assayed using pieces of peeled DFPase-film ranging in weight from 0.009–0.012 g. Typically, the pieces were placed in 10 ml of 3 m*M* DFP buffered solution (5 m*M* $CaCl_2$ and 10 m*M* Bis-Tris-Propane, pH 7.5) and agitated by magnetic stirring. As DFPase acts by binding and hydrolyzing DFP (see below), the activity was measured by following fluoride release with a fluoride ion electrode at room temperature (Drevon et al., 2000). Fluoride bulk solution concentration was measured every 20 sec for 5 min.

The enzyme concentration in the coatings were varied between 0–2 $mg/g_{coating}$. The ECCs with higher enzyme concentrations were too active for the initial velocities to be determined.

Determination of Kinetic Constants Using a Fluoride Ion Electrode

The kinetic constants were determined by means of a fluoride sensor as described in the previous section. The substrate concentrations varied from 0–20 m*M*. The data were fit to the Michaelis-Menten equation using a nonlinear regression (Sigma Plot Version 2).

Diffusion Cell Experiments

The diffusion apparatus is composed of a donor and a receptor compartment, each of them equipped with a water jacket. The diffusion system was previously described in detail (Andreopoulos et al., 1998). The ECC was mounted between the two compartments and the experiments were conducted at room temperature (22°C).

Determination of Susbtrate Effective Diffusion Coefficient, D_{eff}

The substrate effective diffusion coefficient, D_{eff} (m^2/min), was estimated by following the procedure developed by Page et al. (1981). Urease was immobilized into the coating (3.6 $mg/g_{coating}$) to mimic the presence of DFPase. Initially, a 3-ml volume of buffered medium (5 m*M* $CaCl_2$, 10 m*M* Bis-Tris-Propane, pH 7.5) supplemented with DFP (4 m*M*) was placed in the donor cell, while the receptor cell was filled with buffered medium (3 ml). Each cell was well mixed by magnetic stirring. After a fixed period of time (5–300 min) the contents were removed and diluted four times with buffer medium (5 m*M* $CaCl_2$, 10 m*M* Bis-Tris-Propane, pH 7.5). The DFP concentration of each sample was then determined by an activity assay with soluble DFPase. D_{eff} was calculated at quasi-steady state (Page et al., 1981):

$$[DFP]_R = \frac{D_{\text{eff}} A [DFP]_D}{V_{\text{cell}} \delta'} (t - t_0) \tag{1}$$

$[DFP]_D$ and $[DFP]_R$ are the DFP concentrations in the donor and receptor cell, respectively (mol/m^3). V_{cell} (3.10^{-6} m^3) and A ($6.36.10^{-5}$ m^2) are the cell volume and diffusion cross-section area, respectively. Assuming that the swelling of polyurethane film occurs predominantly in thickness, the thickness of wetted ECC, δ', was estimated as follows:

$$\delta' = \frac{1}{1 - \varepsilon} \delta \tag{2}$$

The dry coating thickness, δ (10 μm), was determined using scanning electron microscopy. ε (0.7) is the fraction of the total volume occupied by the liquid phase in the wetted coating.

Activity Measurements

The cells were filled with buffer (5 m*M* $CaCl_2$, 10 m*M* Bis-Tris-Propane, pH 7.5). The donor cell was initially supplemented with DFP (4 m*M*). The initial DFP concentration in receptor cell was either 0 or 4 m*M*. The experiments were conducted using a fixed DFPase-ECC concentration (3.6 $mg/g_{coating}$), for which the substrate complete degradation occurred on a reasonable time scale. Each cell was well mixed by magnetic stirring. After a fixed period of time (5–120 min), the contents were removed and diluted 4 times with buffer (5 m*M* $CaCl_2$, 10 m*M* Bis-Tris-Propane, pH 7.5). The DFP concentration of each sample was then determined by an activity assay with soluble DFPase.

Figure 1 is a schematic of the DFP concentration profile in the case of simultaneous diffusion and enzymatic reaction in the DFPase-containing coating when the receptor cell does not contain DFP at $t = 0$ sec. If the diffusional resistance of boundary layer is neglected, the concentration profiles of DFP in the DFPase-ECC at unsteady state are given by Eq. (3) (Bird et al., 1966; Gray, 1975; Van Stroe-Biezen et al., 1996):

$$\frac{d[DFP]_{lc}}{dt} = \frac{D_{\text{eff}}}{\varepsilon} \frac{d^2[DFP]_{lc}}{d^2x} - \frac{k_{\text{cat,int}}[DFPase]_{lc}[DFP]_{lc}}{K_{M,\text{int}} + [DFP]_{lc}} \tag{3}$$

$[DFP]_{\text{lc}}$ (mol/m^3) is the DFP concentration in the liquid phase in the coating. $k_{\text{cat,int}}$ (s^{-1}) and $K_{M,\text{int}}$ (mol/m^3) are the intrinsic kinetic constants for the ECC.

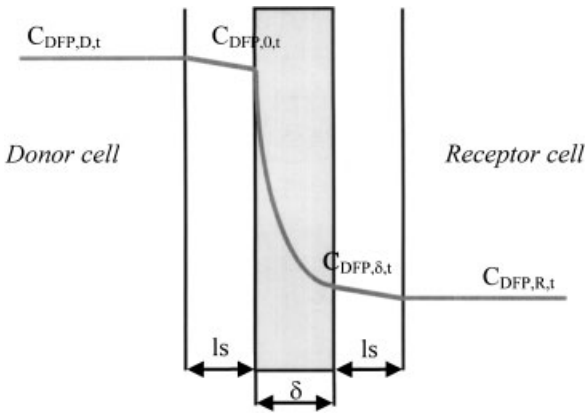

Figure 1. Schematic of the DFP concentration profile in the case of simultaneous diffusion and enzymatic reaction in the DFPase-containing polyurethane coating. 1s, δ′ are the stagnant solution layer and the wetted coating thickness, respectively. $[DFP]_{D,t}$, $[DFP]_{R,t}$ are the bulk DFP concentrations at a time t in the donor and receptor cell, respectively. $[DFP]_{0,t}$, $[DFP]_{\delta,t}$ are the DFP concentration in the liquid phase of coating at the surfaces and at a time t.

The initial conditions are as follows:

$$x = 0 \text{ and } t = 0, \quad [DFP]_{lc} = 4 \tag{4}$$

$$x(x \neq 0) \text{ and } t = 0, \quad [DFP]_{lc} = 0 \tag{5}$$

At the interface between the ECC and the donor cell we have:

$$\frac{d[DFP]_0}{dt} = \frac{AD_{\text{eff}}}{V_{\text{cell}}} \frac{d[DFP]}{dx}\bigg|_0 - (V_{\text{Surface}} + V_{\text{Release}}) \tag{6}$$

where $[DFP]_0$ represents the DFP concentration in the liquid phase at the surface of the ECC ($x = 0$). (mol/[m^3.s]) represents the rate of DFP hydrolysis at the coating surface ($x = 0$) (Eq. [7]), and (mol/[m^3.s]) the rate of reaction catalyzed by the enzyme not covalently immobilized during the ECC synthesis and released in the donor cell (Eq. [8]):

$$V_{\text{Surface}} = \frac{k_{\text{cat,int}}[DFPase]_{\text{Surface}}[DFP]_0}{K_{M,\text{int}} + [DFP]_0} \tag{7}$$

where $[DFPase]_{\text{Surface}}$ (mol/m^3) is the number of moles of enzyme at the coating surface per unit volume of donor cell:

$$V_{\text{Release}} = \frac{k_{\text{cat,native}}[DFPase]_{\text{Release}}[DFP]_0}{K_{M,\text{native}} + [DFP]_0} \tag{8}$$

where $[DFPase]_{\text{Release}}$ (mol/m^3) is calculated with respect to the donor cell volume. $k_{\text{cat,native}}$ and $K_{M,\text{native}}$ are given in Table I (Experiment 1[a*]).

Given the experimental DFP concentration profiles in donor and receptor cells, Eq. (2) was solved numerically using Eqs. (4–8) with Athena Visual v. 7.1.1. The intrinsic kinetic constants of the ECC, $K_{M,\text{int}}$ and $k_{\text{cat,int}}$, were then calculated.

Enzyme Modification With Desmodur N3400

DFPase-containing solution (1 ml) (50 m*M* MOPS, 5 m*M* $CaCl_2$, pH 7.5) was added to Desmodur N3400 (1 g), which is composed of the dimer and trimer of HDI. The biphasic mixture was stirred at room temperature. The activity of modified enzyme was determined by means of a fluoride sensor, as described previously .

Since the degree of DFPase modification could not be determined directly, the reaction of Desmodur N3400 and enzyme Lysine residues was mimicked using Bradykinin potentiator B, a low molecular weight peptide (1182.4 Da) containing one Lysine residue. The extent of

Table I. Kinetic parameters for DFPase-containing coatings and soluble DFPase.

Experiment	K_M (m*M*)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} \cdot mM^{-1}$)
1; [a]*intrinsic native DFPase	0.79 ± 0.02	232 ± 2	293 ± 3
1; [b]*apparent ECC	1.3 ± 0.2	43 ± 3	33 ± 7
1; [b#]apparent ECC	1.3 ± 0.2	70 ± 6	54 ± 13
2; [b]**intrinsic ECC	0.96 ± 0.01	102 ± 1	106 ± 2

The errors on specific constants were calculated as follows:

$$\Delta\left(\frac{k_{\text{cat}}}{K_M}\right) = \left(\frac{k_{\text{cat}}}{K_M}\right) \cdot \left[\frac{\Delta k_{\text{cat}}}{k_{\text{cat}}} + \frac{\Delta K_M}{K_M}\right]$$

[a]Native DFPase (Drevon et al., 2000).

[b]Polyurethane coatings.

*The kinetic parameters were evaluated at room temperature in buffered media (10 m*M* bis-tris-propane, 5 m*M* $CaCl_2$, pH 7.5) using substrate concentrations varying from 0 to 20 m*M* and fluoride ion electrode, by applying the Michaelis-Menten equation as a model and using a nonlinear regression (SigmaPlot V. 2).

**The kinetic parameters were evaluated at room temperature in buffered media (10 m*M* bis-tris-propane, 5 m*M* $CaCl_2$, pH 7.5) using the diffusion cell apparatus.

[#]DFPase was modified with Desmodur N3400 prior to immobilization into polyurethane coatings.

Lysine modification was determined using MALDI-TOF for various reaction times (15 min to 17 h).

MALDI-MS analyses were performed with a Perseptive Biosystems Voyager elite MALDI-TOF. The acceleration voltage was set to 20 kV in a linear mode. The PEGylated enzyme solution (1–2 mg/ml) was mixed with an equal volume of the matrix solution (0.5 ml water, 0.5 ml acetonitrile, 2 μl TFA, and 8 mg α-cyano-4-hydroxycinnamic acid) and 2 μl of the final solution was spotted on the plate target. Spectra were recorded after evaporation of the solvent mixture and were calibrated externally with FMRP and ACTH.

DFPase modified with Desmodur N3400 was further immobilized into polyurethane coatings as described previously.

ECC Thermostability

Native and immobilized DFPase were added to buffer (10 m*M* BTP, 5 m*M* $CaCl_2$, pH 7.5) incubated at 65°C and assayed at room temperature in buffered media (10 mM BTP, 5 m*M* $CaCl_2$, pH 7.5) as described above.

The thermostability of dry ECCs was determined at room temperature. After fixed periods of storage under ambient conditions, the ECC samples were assayed for activity at room temperature in buffered media (10 m*M* BTP, 5 m*M* $CaCl_2$, pH 7.5) as described above.

RESULTS AND DISCUSSION

Reversibility of DFPase Attachment to ECCs

The extent to which DFPase is irreversibly attached to the polymer was determined using the Bradford reagent. DFPase-containing polyurethane coatings were peeled from panels, cut into small pieces, and extensively rinsed with distilled water. Less than 4% (w/w) of the protein loaded to the ECC was detected in the rinsates, indicating that the immobilization efficiency approached 100%.

Enzyme Distribution in ECCs

When enzymes are incorporated into films, a key issue is whether the enzyme is equally distributed in the film. Gold labeling has been used to localize immobilized enzyme in polyurethane monolith foams (Hu et al., 1993). Therefore, we decided to localize DFPase in ECCs via conjugation to colloidal gold particles. Figure 2A,B are micrographs of gold/DFPase conjugate-containing coatings obtained by dark-field microscopy (0.001 $mg_{gold}/g_{coating}$) and inverse image light microscopy (0.012 $mg_{gold}/g_{coating}$), respectively. As the concentration of immobilized colloidal gold/enzyme conjugate is increased by 12-fold it becomes apparent that the immobilized gold/enzyme complexes are uniformly distributed within the coating. TEMs of the cross section of gold/enzyme-containing coating (0.012 $mg_{gold}/g_{coating}$) are given in Figure 2C (originally 2,500-fold enlargement) and 2D (10,000-fold magnification). Similar to light microscopy, TEM shows that the gold/enzyme particles and clusters are randomly distributed at the microscale level. This implies that the synthesis of gold/DFPase conjugate-containing coating leads to the homogeneous immobilization of gold/DFPase complexes in the polymeric matrix. By extrapolation one can predict that the DFPase local concentration in a film should not be location-dependent.

Activity of ECCs

ECCs were prepared using the polyisocyanates XP-7007, XP-7148, and XP-7063. Figure 3 shows the activity of each ECC as a function of initial DFPase loading. The activity is directly proportional to the enzyme concentration, which implies that there is no significant mass transfer limitation. Since Figure 2 indicates that the films are nonporous, this result implies (as we will discuss in detail below) that only enzyme in a thin external layer of the film is accessible to substrate.

The hydrophilicity of polyisocyanate decreases in the order XP-7148 > XP-7063 > XP-7007. Interestingly, the apparent activity retention of ECCs increases as the hydrophilicity of polyisocyanate decreases (Fig. 3). Studies of enzyme activity in dehydrated organic solvents demonstrate that enzymes prefer hydrophobic environments. It may not be coincidental that less hydrophilic polyisocyanates are superior ECC materials.

The use of polyisocyanate XP-7007 generates ECCs with the highest levels of apparent activity retention and thus subsequent experiments were performed with XP 7007-containing-ECCs.

The apparent kinetic characteristics calculated by assuming all the loaded enzyme is available (Table I, Experiment 1^{b*}) lead to an observable activity retention (11%) rather than intrinsic retention.

Effective Diffusivity of DFP in ECC, D_{eff}

Clearly, to understand activity retention in ECCs we must assess the diffusivity of the substrate in the film. Using Eq. (1), D_{eff} was found to be $(5 \pm 1) \times 10^{-10}$ m^2/min (Fig. 4). D_{eff} is two to three orders of magnitude lower than the diffusion coefficients of gases into liquids or organic solutes into hydrogels (Reid et al., 1987; Van Stroe-Biezen et al., 1993). Similarly, Buenfeld et al. (1998) observed high resistance of two-component water-borne polyurethane coatings to diffusion of chloride ions. The accessibility of enzyme located within the coating to substrate is clearly limited by the low coating permeability. Once again, this result indicates that the degree of penetration of DFP into coating should be taken into account in order to determine the activity retention of ECCs.

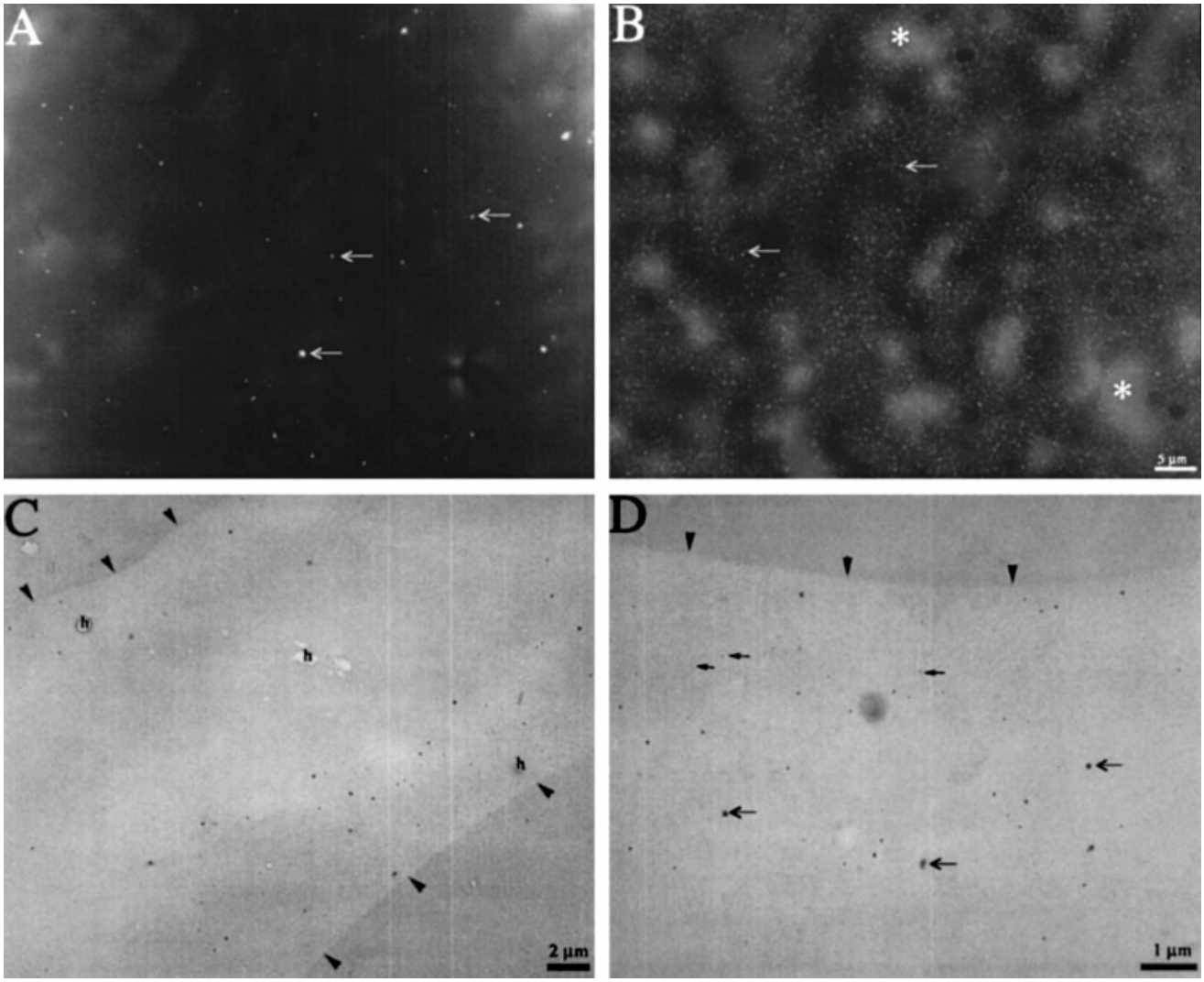

Figure 2. Enzyme distribution in polyurethane coating. Gold/DFPase-containing coatings were analyzed using dark-field microscopy (**A**; 0.0007 $mg_{gold}/g_{coating}$) and inverse (negative) images taken using light microscopy (**B**; 0.0116 $mg_{gold}/g_{coating}$). Negative images were used in this case because the thickness of the coating and the high concentration of gold particles made it difficult to obtain focused images. Cross sections of the coatings were obtained using TEM (**C,D**). The arrows with filled heads show some of the gold/enzyme particles, while the arrows with emptied heads show some of the gold/enzyme conjugate clusters. The arrowheads indicate the extremities of coating samples within the embedded resin. The stars designate some unfocused areas as a result of high gold particle concentration and uneven surface. Bubbles in the coating are indicated by the letter h. Scale bar in **B** represents **A** and **B**. Scale bars in **C** and **D** indicate sizes in those panels.

Figure 5 shows the profile for DFP concentration in donor and receptor cell over time when using a DFPase-ECC (3.6 $mg/g_{coating}$) and an initial concentration of 4 m*M* DFP in both cells. The profiles for the decrease in DFP concentration in donor and receptor cells follow similar trends. Assuming immobilized DFPase is homogeneously distributed in the coating (as implied in Fig. 2), the enzymatic activity retention is therefore almost the same on both sides of the coating. During curing, the ECC upper and lower surfaces are in contact with the TPO panel and exposed to air, respectively. As given by the little difference in activity retention of the ECCs external surfaces, the air interface and the polymeric/hydrophobic environment do not influence ECC activity retention.

DFP concentration profiles in the donor and receptor cells were also measured for a DFPase-ECC (3.6 $mg/g_{coating}$) with no DFP in the receptor cell (Fig. 6). Eq. (3) describes well the experimental results (Fig. 6a). The estimated intrinsic Michaelis constant of immobilized DFPase, $K_{M,int}$ (Table I, Experiment 2^{b**}), is similar to that obtained without the diffusion apparatus (Table I, Experiment 1^{b*}). Interestingly, by taking into account the coating resistance to substrate diffusion, $k_{cat,int}$ (Table I, Experiment 2^{b**}) was found to be 2.4 times higher than the apparent $k_{cat,app}$ measured without the diffusion apparatus (Table I, Experiment 1^{b*}). As shown by the simulated substrate profiles within the coating at different experimental times (Fig. 6b), the substrate penetrates a third of the coating over the time

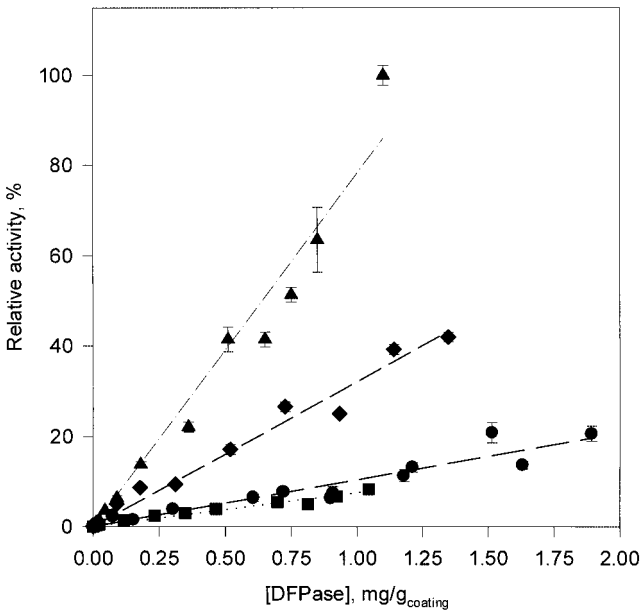

Figure 3. Effect of DFPase concentration on DFPase-containing coating efficiency. Coatings were synthesized with polyol XP-7093 and polyisocyanates XP-7007 (closed diamond), XP-7063 (closed circles), and XP-7148 (closed squares) in buffered solutions (10 m*M* bis-tris-propane, 5 m*M* $CaCl_2$) at pH 7.5. The closed triangles correspond to the apparent activity of coatings synthesized starting from DFPase modified with Desmodur N3400, polyol XP-7093, and polyisocyanate XP-7007. The activity of the bioplastic is reported at a 3 m*M* DFP concentration.

course of the experiment. Clearly, the estimation of apparent kinetic parameters involves solely the degradation of DFP in a layer of immobilized enzyme at the coating surface. Consequently, the apparent enzymatic

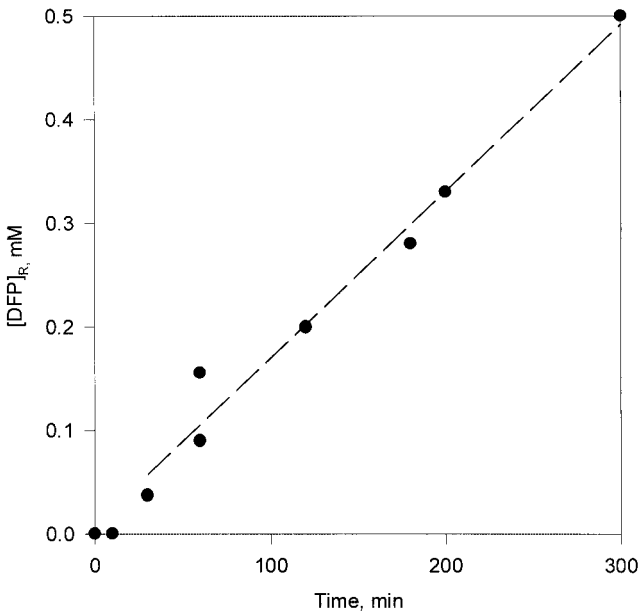

Figure 4. Effective diffusion of DFP through coatings. Coatings were synthesized using the polyol XP-7093 and polyisocyanate XP-7007 and the experiment was conducted in buffered medium (10 m*M* bis-tris-propane, 5 m*M* $CaCl_2$) at pH 7.5 by means of a cell diffusion apparatus.

efficiency of DFPase-ECCs is based on the activity retention of this external layer of immobilized DFPase. As given by the intrinsic kinetic constants of DFPase-ECC, the intrinsic activity retention within this layer is 38%. The ratio of apparent to intrinsic k_{cat}, gives the proportion of immobilized DFPase in ECCs reachable by the substrate during activity measurements without the diffusion apparatus.

Desmodur N3400-Modified ECCs

The vigorous mixing of Bradykinin potentiator B-containing aqueous solution with Desmodur N3400 ensured the chemical modification of the peptide Lysine residue with the dimer of HDI, as observed using MALDI-TOF. A reaction yield fluctuating between 70–90% was reached for a 15-min reaction time and was not increased by further mixing of the peptide solution with the Desmodur N3400 phase.

Polyisocyanate Desmodur N3400 is based on the uretdione of HDI, which is known to migrate from the bulk to the polymer/air interface during coating curing. By modifying *DFPase* with Desmodur N3400 prior to its immobilization into coatings, we expected the

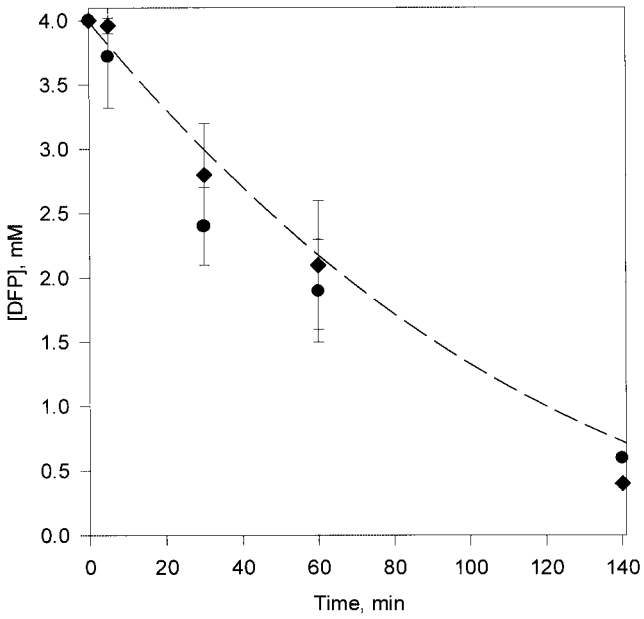

Figure 5. Profiles for DFP consumption in diffusion cells. Coatings were synthesized using polyol XP-7093 and polyisocyanate XP-7007 and a DFPase loading of 3.6 mg/g$_{coating}$. The experiments were conducted in buffered medium (10 mM bis-tris-propane, 5 m*M* $CaCl_2$) at pH 7.5 using an initial DFP concentration of 4 m*M* in both donor and receptor cells. The DFP concentrations in donor (closed diamonds) and receptor (closed circles) cells were determined over time. The theoretical DFP profiles in the donor and receptor cells are identical due to symmetry. The experimental concentration curves in donor and receptor cells are, thus, described by the same simulated profile (dashed line) using Eqs. (2–7) and Athena Visual 7.1.1.

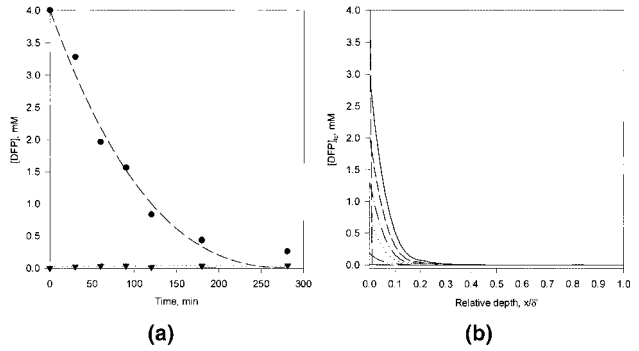

Figure 6. Profiles for DFP consumption in diffusion cells. Coatings were synthesized using polyol XP-7093 and polyisocyanate XP-7007 and a DFPase loading of 3.6 mg/$g_{coating}$. The experiments were conducted in buffered medium (10 m*M* bis-tris-propane, 5 m*M* $CaCl_2$) at pH 7.5 starting with DFP (4 m*M*) in the donor cell and no DFP in the receptor cell. **a:** The DFP concentrations in donor (closed circles) and receptor (closed triangles) cells were measured over time. The simulated DFP concentration profiles in donor (dashed line) and receptor (dotted line) cells were determined using Eqs. (2–7) and Athena Visual 7.1.1. **b:** The substrate concentration profile in the ECCs was calculated at 0 (medium dashed line), 30 (solid line), 60 (small dashed line), 90 (dashed-dotted line), 120 (dotted line), 180 (dashed-dotted-dotted line), and 280 min (long dash line).

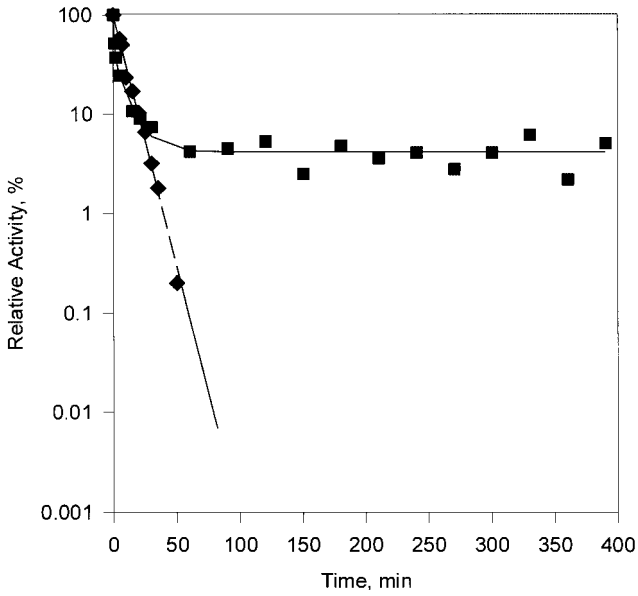

Figure 7. Thermoinactivation of DFPase-containing coating at 65°C. Deactivation of immobilized DFPase (closed squares) and native DFPase (closed diamonds) were conducted in buffered solution (10 m*M* BTP, 5 m*M* $Cacl_2$, pH 7.5). The remaining enzymatic activity was measured over time at room temperature in buffered media (10 m*M* BTP, 5 m*M* $CaCl_2$, pH 7.5) using DFP (3 m*M*) as a substrate. The biphasic behavior was described with a four-parameter model and the kinetic constants α_1 (0.34 ± 0.03), α_2 (0.10 ± 0.01), k_1 (1.3 ± 0.1) and k_2 (0.042 ± 0.003) were determined using the algorithm of Marquardt-Levenberg (SigmaPlot v. 2.0).

immobilized enzyme to be mainly concentrated within an external layer at the coating surface. Consequently, immobilized *DFPase* would be well accessible to substrate, leading to an increased apparent activity retention. Given the fast favorable reaction between isocyanates of the dimer of HDI and the Lysine residue of Bradykinin potentiator B, DFPase was reacted with Desmodur N3400 for 15 min. No loss of enzymatic activity was observed. As shown in Figure 3 and Table I (Experiment 1b$^{\#*}$), pretreatment of DFPase with Desmodur N3400 produced a 64% increase in apparent efficiency of ECCs.

Thermostability of ECCs

As explained in the previous section, not all the immobilized enzyme is seen by the substrate during activity measurement. Since the inaccessible enzyme does not interfere with rate determinations the thermal stability of the film can be determined without special consideration of diffusion resistances.

Unlike native *DFPase*, immobilized DFPase has a biphasic thermoinactivation profile at 65°C (Fig. 7). An elevated temperature of 65°C was used to inactivate the enzyme in order to perform experiments on a reasonable time scale. For this range of incubation periods, the two-component polyurethane coatings did not dissolve significantly into the aqueous phase. Initially, the ECC follows a deactivation trend similar to that for native enzyme. This initial rapid deactivation leads, however, to the formation of a stable and active form of immobilized enzyme with a 6–7% residual activity. No significant change in the activity of the highly stable form of the DFPase-ECC was observed over 350 min. The biphasic deactivation kinetics of the ECC can be modeled by a four-parameter model (Henley et al., 1985), which assumes the following scheme:

$$E \xrightarrow{k_1} \overset{\alpha_1}{E_1} \xrightarrow{k_2} \overset{\alpha_2}{E_2} \tag{9}$$

E, E_1, and E_2 correspond to the initial, intermediate, and final state of enzyme. α_1 and α_2 are the residual activities of E_1 and E_2, respectively, while k_1 and k_2 represent first-order deactivation rates. The analytical solution for the enzymatic activity, a, is given by Eq. (10):

$$a = \left(1 + \left(\frac{\alpha_1 k_1 - \alpha_2 k_2}{k_2 - k_1}\right)\right)\exp(-k_1 t) + \left(\frac{k_1(\alpha_2 - \alpha_1)}{k_2 - k_1}\right)\exp(-k_2 t) + \alpha_2 \tag{10}$$

Where t represents the time of deactivation. The fit of the data to Eq. (10) are given in Figure 7.

Another kinetic model assuming the existence of two different forms of DFPase in ECCs with different deactivation pathways and requiring only four physical parameters did not adequately describe the experimental data. Further, more complex mechanisms were not considered as they involved five or more parameters (Henley et al., 1985).

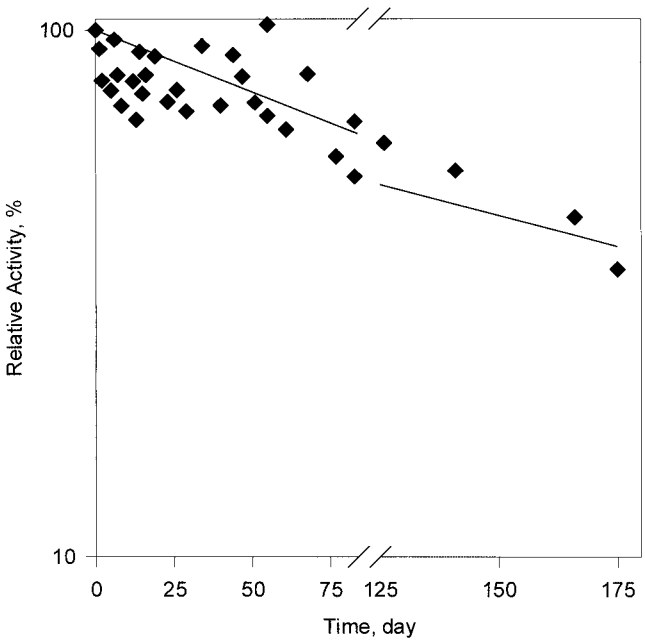

Figure 8. Thermoinactivation of DFPase-containing coating at room temperature. The remaining enzymatic activity was measured over time at room temperature in buffered media (10 m*M* BTP, 5 m*M* $CaCl_2$, pH 7.5) using DFP (3 m*M*) as a substrate.

The immobilization of DFPase in polyurethane foam and PEGylation also induced a transition from first-order to biphasic inactivation kinetics (Drevon et al., in press). We believe that thermoinactivation of the DFPase-ECC results from structural changes similar to those described previously for the thermoinactivation of DFPase-containing polyurethane foam monoliths.

DFPase-ECCs exhibit a higher stability at room temperature than at 65°C. Indeed, DFPase-ECCs lose only 40% activity after 100 days of storage at room temperature (Fig. 8). Given the high stability of ECCs maintained dry under ambient conditions, the resulting catalyst should be an effective decontaminant for a variety of applications.

CONCLUSION

Covalent incorporation of DFPase into water-borne polyurethane coatings was performed in a single step protein-polymer synthesis using polyol and polyisocyanates. The use of polyisocyanate XP-7007 and enzyme modified with Desmodur N3400 during the immobilization process leads to the highest intrinsic catalytic efficiency. At high temperature, DFPase-ECCs lose 93% of their activity quickly, but then become hyperstable. We expect the approach we describe to be generic and we are currently exploring a wide range of applications of ECC materials. We are also investigating methods to increase the apparent efficiency of ECCs.

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