Combinatorial Discovery of Reusable Noncovalent Supports for Enzyme Immobilization and Nonaqueous Catalysis

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A simple and effective method is described for the preparation of enzyme-containing materials that possess excellent catalytic activity, mechanical strength, and reusability. Uniform spherical beads were produced via the colyophilization of α-chymotrypsin with the support materials, leaving the active enzyme entrapped within the porous "ice-templated" support matrix. The composites were assayed for catalytic activity by monitoring a nonaqueous transesterification reaction. The mechanical strength for each composite was measured using a compression assay. Initial screens identified a set of six support materials that contributed favorably to either the enzyme activity or to the mechanical strength of the composite. A design of experiments (DoE) methodology was employed to screen 80 combinations of these six "base" materials. A model representing this formulation space was constructed which could be used to predict both the catalytic activity and mechanical strength with reasonable accuracy for any combination of the six base component materials. This model was used to predict optimized materials with an enzyme activity that was 50 times greater than that of the free enzyme. The model was also used to set a minimum acceptable mechanical stability for these composites, and the resulting materials were shown to be reusable for at least ten reaction cycles.

Introduction

The use of enzymes in nonaqueous media for synthetic, analytical, and biomedical applications has increased significantly in recent years.[1−5] In particular, the use of enzymatic catalysis in organic synthetic transformations is of growing importance.[5,6] There are, however, a number of problems associated with the long-term stability and reusability of enzymatic catalysts, and this has restricted the industrial use of these systems. Enzyme agglomeration is a common problem that is encountered when powdered preparations of lyophilized enzyme are employed in organic solvents.[7,8] This is caused by the repulsive hydrophobic interactions with the nonpolar solvent, combined with the hydrophilic attraction between the enzyme particles.[9] Agglomeration reduces the catalytic rate by blocking the active site through protein−protein contacts.[10] If water is present in the reaction media, the enzyme may also be deposited as an intractable, sticky mass which makes cleaning difficult and prevents reuse of the enzyme.[7,10] It is well-known that these problems can be overcome by immobilization of the enzyme on a suitable support material,[7,11,12] either by covalent or noncovalent attachment. Immobilized enzyme preparations often exhibit higher catalytic activities than lyophilized powders: this is in part caused by an increase in the number of active sites that are available when the enzyme is spread over a larger surface area and efficient mass transfer is promoted.[13,14] Immobilization on a support material can impart increased conformational stability to the enzyme[9,15] and enhance both the catalytic activity and its resistance to environmental extremes of temperature, polarity, and pH.[10,16] Enzyme immobilization can also facilitate the use of continuous-flow reactors which are usually impractical with lyophilized powders.[17,18]

For noncovalent immobilization on supports, the nature and morphology of the support material greatly influences the quantity of enzyme that can be immobilized, the enzyme accessibility, and the resultant enzyme activity.[12,15,19] The accessible surface area and the nature of the porosity in the material are particularly important in this respect, although the chemical nature of the support is also known to affect enzyme performance.[15,20] The interaction of the support material with water is also important. In nonaqueous media, water can partition between the enzyme, the solvent, and the support material; as such, the capacity of the support to absorb water may also influence the course of the reaction.[12] From a recycling perspective, the mechanical stability of the support is important. For example, weak, brittle supports may be fragmented into "fines" upon stirring which may lead to difficulties in separation and loss of enzyme between reactions. Similarly, continuous-flow reactors will require supports with suitable mechanical properties.

The de novo design of optimal enzyme supports presents a significant challenge because a number of the factors that affect activity and reusability are difficult to predict. Moreover, it is obvious that many design parameters will need to be optimized carefully to generate practically useful

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support materials; for example, the "ideal" support may be highly porous, but materials with maximized porosity may exhibit very poor mechanical properties. Similarly, microporous materials may exhibit the highest possible surface areas, but it may not be possible to load the enzyme into such small pores nor will the entrapped active sites necessarily be accessible to the reagents. In this study, we have tackled these problems by using a novel combinatorial approach coupled with the use of design of experiments (DoE). A library of 80 supported chymotrypsin materials was produced in the form of spherical beads using a set of six organic and inorganic materials as "building blocks". This library was characterized with respect to both mechanical strength and catalytic activity, and a response surface model was constructed to relate these properties to the composition of the support. This model was then used to predict "optimized" materials which were shown to be readily recycled and much more active than the lyophilized enzyme.

Experimental Section

Materials. Poly(styrene sodium sulfonate) (PSS, 100,000 g mol^{-1}), poly(vinyl alcohol) (PVA, 9000−10 000 g mol^{-1}), dextran (DEX, 100 000 g mol^{-1}, Fluka), Ludox HS30 silica nanoparticles (HS30, 30 wt % aqueous colloidal silica suspension), poly(ethylene glycol) (PEG, 8000 g mol^{-1}), sodium dodecyl sulfate (SDS, P & R Laboratory Supplies), *N*-acetyl-L-tyrosine ethyl ester (ATEE, Sigma grade), propan-1-ol (99+ %), and α-chymotrypsin (α-CT, type II from bovine pancreas) were obtained from the Aldrich, unless otherwise stated. All solvents were obtained from VWR and were of HPLC grade.

Design of Experiments (DoE). An experimental design was generated using Umetrics MODDE 6.0 software. A cubic simplex centroid formulation design was adopted,[21] which led to a total of 80 formulations (77 plus 3 center points), incorporating between one and six of the set of matrix building blocks (SDS, DEX, PSS, PEG, HS30, and PVA, see Supporting Information for full dataset and further details; Tables S1 and S2, Figures S1−S6).

Preparation of Composite Bead Library. A library of 80 solutions of the various chemical components of the support materials was prepared by dissolution of 570 mg total mass in 2.7 mL water; that is, each solution contained 570 mg of the solid-support precursors in the ratios prescribed by the experimental design. To each of these 80 solutions was added 300 μL of α-CT in 20 mM phosphate buffer (pH 7.8, 100 mg mL^{-1} α-CT). For consistency in bead preparation, the total mass of dissolved solid (enzyme plus support material) in each solution was kept constant at 20 wt %. The loading of α-CT for each material was fixed at 5% w/w based on the total mass of the support. It is often very difficult to make direct comparisons between the results obtained from different research groups for catalytically active enzyme preparation because of variations in reaction conditions and protocols used for assaying the reactions. Hence, a solution of α-CT (20 wt % in 20 mM KH_2PO_4 pH 7.8 buffer solution) was lyophilized and used as a control for catalytic activity. The composite beads were prepared by continuously injecting droplets of these solutions directly into liquid nitrogen. The sedimented frozen droplets were then collected, and the water was removed by freeze drying for 2 days under vacuum (Lyolab 3000, Heto). The dry material was then recovered as uniform spherical beads with a diameter of approximately 2 mm. A 16-channel peristaltic pump (205u Watson Marlow) was used to inject the solutions into multiple separate liquid nitrogen dewars, thus allowing us to prepare several samples in parallel and thereby to increase sample throughput significantly.

Physical Characterization. The mechanical strength of the beads was determined using a Lloyd instruments LR30K series tensile testing machine. The instrument was used in compression mode with a 100 Newton probe. The energy required to deform each bead by 1 mm was calculated (units of N mm) by measuring the area under a force/distance plot. The strength of the sample in the library was averaged from three concordant results. The average strength values for all 80 composites are presented in Table S1.

The surface area of the composite beads was characterized using a Micrometrics ASAP 2010 and by application of the BET (Brunauer Emmett Teller) method. Pore volumes were measured by mercury intrusion porosimetry with a Micrometrics AutoPore IV. Absolute densities were obtained by helium pycnometry (Micrometrics AccuPyc 1330). Scanning electron microscope (SEM) images were captured using a Jeol 840 instrument. Samples were sputter-coated with approximately 2 nm of gold (Polaron E5000 coating unit) prior to analysis and mounted on 12 mm carbon coated aluminum stubs (Agar Scientific).

Enzyme Activity Assay. The catalytic activity of the immobilized enzyme composite beads was assayed by monitoring the transesterification reaction between ATEE and propan-1-ol using HPLC. All enzyme-catalyzed reactions were pre-equilibrated over a K_2CO_3 saturated salt solution to a water activity of 0.43 and carried out in triplicate. The reactions were carried out in 3 mL of hexane containing a known mass of the enzyme preparation. The reaction was initiated by the addition of ATEE (0.028 mmol) dissolved in dry propan-1-ol (1.5 mmol). All reactions were carried out at a constant temperature of 22 °C with orbital shaking at 100 rpm; 50 μL aliquots were removed from the reaction mixture at regular time intervals and passed through a 0.22 μm syringe filter to remove any enzyme particles, thereby terminating the reaction. The determination of the relative proportions of substrate (ATEE) and product (*N*-acetyl-L-tyrosine propyl ester, ATPE) was carried out by reverse-phase HPLC. The instrument employed for this analysis consisted of a Spectra-Physics SP8800 pump, Kontron 465 autosampler and Applied Biosystems 757 UV absorbance detector (245 nm) processed with Datalys Azur, version 3.0, software. The eluent consisted of a 70:30 water/acetonitrile mixture adjusted to pH 2 with orthophosphoric acid. The flow rate was set to 1 mL min^{-1}. An injection volume of 10 μL was passed through a 250 × 4.6 mm i.d. Hichrom column packed with Waters Spherisorb ODS2. The measured activity values for all 80 composites are presented in Table S1.

Enzyme Reuse Studies. A number of the optimized enzyme composites were assayed for their reusability by assessing catalytic activity. Samples of five whole beads in

hexane were hydrated to a water activity of 0.43. After determination of the initial catalytic activity, the reaction medium was decanted from the vessel, and the beads were washed with 3 × 3 mL portions of dry hexane. The transesterification assay was then repeated using fresh hexane with a water activity of 0.43. This process of washing and rehydration was repeated for each subsequent use of the preparations. Each of the repeat reactions was carried out in triplicate at 22 °C.

Results and Discussion

Selection of Support Building Blocks. The colyophilization technique is commonly used to prepare immobilized enzymes for application in organic media.[7,22] We have adapted this technique to prepare enzyme-containing beads by freezing aqueous droplets and then removing the water from the frozen droplet under vacuum. Although many attempts have been made to establish parameters such as porosity, hydrophobicity, aquaphilicity, and chemical functionality as criteria for selecting support materials, the combined influence of these parameters on enzyme activity is still not fully understood, and little research has been done on the mechanical strength of such materials. The addition of additives such as polyols and saccharides[23] and PEG[24] during the lyophilization process is also known to increase enzyme activity. Rather than rely on data for existing materials, we conducted an initial screening study prior to the construction of the catalyst library. This was to identify potential support materials that gave rise to high enzyme activities, increased mechanical strength, or in the ideal case, both. In this screening process, we generated a number of two-component systems, that is, α-CT supported on a single support material, and evaluated the catalytic activity and mechanical strength of the resulting composites. A broad range of water-soluble support materials were examined including inorganic salts, low molecular weight organic compounds, polyelectrolytes, polysaccharides, inorganic nanoparticles, and more traditional polymeric supports such as poly(ethylene glycol) and poly(vinyl alcohol). We chose materials that would be insoluble and nondispersible in organic reaction solvents such as hexane to allow their subsequent use as heterogeneous catalyst supports in such media. From this initial study, six compounds were selected as building blocks for the composites: poly(ethylene gylcol) (PEG), poly(vinyl alcohol) (PVA), Dextran (DEX), poly(styrene sodium sulfonate) (PSS), Ludox silica nanoparticles (HS30, 15 nm diameter), and sodium dodecyl sulfate (SDS). HS30 and PEG were chosen because they produced the most catalytically active immobilized enzyme preparations, albeit with low mechanical stability when prepared via this "co-freeze-drying" route. DEX and PSS were chosen because these materials gave rise to the most mechanically stable composites (although with relatively low enzyme activities). PVA represented a reasonable compromise since it showed good mechanical strength and promising catalytic activity. SDS was chosenbecause it is known to stabilize emulsions, offering the future possibility of introducing porosity into these materials. Although it is known to denature proteins it has also successfully been used to solubilize enzymes via ion-pairing.[25] Additionally, initial experiments suggested that SDS/PEG support mixtures might exhibit a positive synergy, even though both the mechanical strength and enzyme activity of composites prepared from SDS alone were very poor.

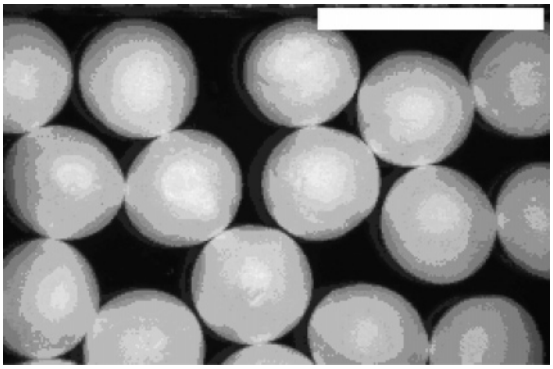

Figure 1. Optical image of composite beads (scale bar = 5 mm). The beads produced by this injection freezing method are typically very monodisperse (<5% standard deviation in bead diameter).

Negative control experiments were conducted on each of these structural building blocks where beads were formed from 20 wt % solutions of the six materials with no enzyme present and also from solutions that contained a small quantity of buffered enzyme (19% w/v structural material and 1% w/v α-CT). Analysis confirmed that the beads formed in the absence of α-CT did not catalyze the transesterification reaction. It was also shown that the inclusion of α-CT in the dried composite at this 5% w/w level did not significantly affect the mechanical strength of the beads. The catalytic activity of particles ground to a fine powder exhibited only a very small increase in catalytic activity compared to the differences in activity between beads composed of different materials. Hence, the differences in activities observed are caused by differences in mass transfer between different beads.

The composite bead library was produced using a parallel formulation approach (see Experimental Section) that allowed all 80 materials to be produced in the form of highly monodisperse spherical beads (Figure 1). The large-beaded format is convenient in terms of use and separation providing that the mechanical stability of the material is adequate. In general, the mechanical strength of the composites was found to vary markedly and materials were produced which ranged from very weak (i.e., hard to handle without fragmentation) to very strong (i.e., tough and hard to fragment without the application of significant mechanical pressure). The beads were found to be macroporous by SEM imaging (Figures 2, S7, anD S8). This porosity results from the templating of the ice crystallites which are formed during rapid freezing and then removed by sublimation during freeze-drying to produce permanent pores.[26]

Experimental Design. MODDE 6.0 software (Umetrics) was used to design a series of 80 composite materials with the aim of finding optimum supports that combine both high enzyme activity with good mechanical properties. This DoE approach was employed for three main reasons. First, it allows the construction of a manageable and representative subset of the many thousands of possible combinations of

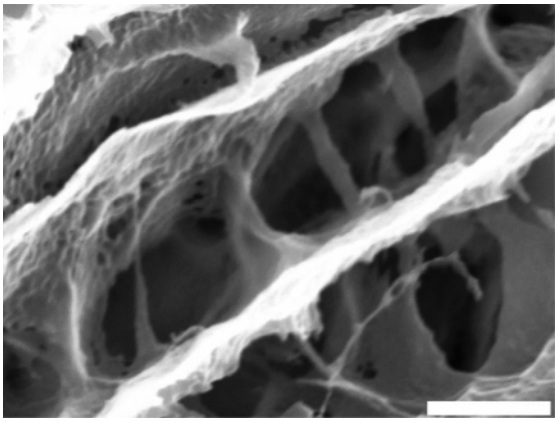

Figure 2. High-magnification SEM image of chymotrypsin−PVA composite bead interior showing the ice-templated macropore structure (scale bar = 3 μm).

the six structural building blocks. Second, the approach facilitates the search for positive synergies, that is, combinations of support materials that give rise to composites that are stronger or more catalytically active than any of the structures formed from the individual building blocks. Third, response surface modeling can be used to predict optimal combinations of materials for particular applications, with the possibility of applying specific weightings to the objectives in favor of either mechanical strength or enzyme activity.

The library of 80 enzyme-containing composites was prepared and characterized with respect to both the catalytic activity and the mechanical strength of the beads. A single mathematical model was then constructed to relate both of these parameters to the composition of the materials by applying a partial least-squares (PLS) fit. The predictive power of the model can be evaluated from the plots shown in Figure 3. These plots indicate that the model fits the data moderately well (r^2(strength) = 0.959; r^2(activity) = 0.824), although it is clear that the catalytic activity data (Figure 3a) show much more scatter than the mechanical strength data (Figure 3b). We believe that this is the result of systematic variability in the measurement of the catalytic activity, and indeed this variability is reflected in the typical standard deviation for these measurements (10−15%). Both fits, however, are sufficiently good to allow some general predictions and optimizations to be made with reasonable accuracy in terms of both enzyme activity and mechanical strength. A double log plot of *N* probability against standardized residuals (see Supporting Information, Figures S2 and S4) showed a distribution of residuals for both responses (activity and strength) that was close to the regression line and that contained no obvious unique outlying data points, despite the scatter in the data; as such, we chose not to exclude any data points from the model. Coefficient plots for the fitted model (Figures S1 and S3) confirmed what we expected from our initial screening studies: both DEX and PSS are correlated with strength rather than activity, HS30 and PEG are correlated with activity rather than strength, and PVA is positively correlated (to some extent) with both responses. These plots further confirmed our practical experience in that materials generated with high loadings of PEG, for example, tended to be mechanically weak. This is

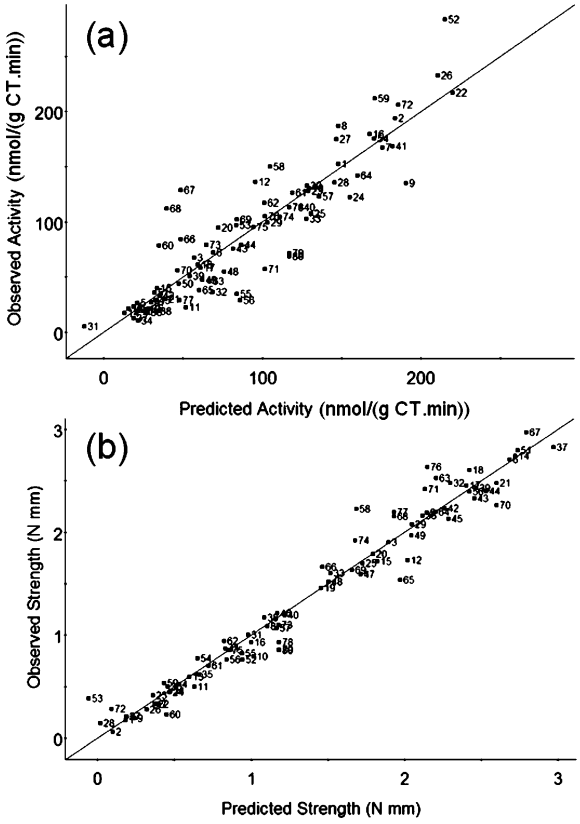

Figure 3. Plots showing agreement of observed and predicted values for (a) catalytic activity and (b) mechanical strength for 80 composites prepared according to the DoE strategy employed in the production of this library. The linear correlation suggests that both models may be used predictively, although the model for strength shows significantly less scatter and is of higher quality than the model for activity.

reflected by the fact that PEG and strength are strongly anticorrelated in the PLS loading plot (Figure S6).

A plot of strength versus activity is shown in Figure 4 for all 80 of the materials in the library plus the free lyophilized enzyme (with no support material). Three basic conclusions can be drawn from an initial visual analysis of this data. First, it is clear that many of the composite materials in the library (>90%) are significantly stronger, more catalytically active, or superior in both respects to the free lyophilized enzyme. Second, the distribution of data does not suggest obvious clusters of materials, but it does indicate that none of the materials gave rise to points in the upper right-hand quadrant in this plot, that is, with highest activity and highest strength. Third, one can infer from this distribution that strength and activity are on the whole negatively correlated for this particular combination of support materials, that is, the contributions of the various support components to the strength and activity are likely to be essentially additive and there is no evidence for strong positive synergies in the mixtures.

Optimization of Support Materials. The most significant test of the validity of any model is to use it predictively: in this case, that involved predicting a series of optimized support materials and gauging how the properties of these materials agreed with the model predictions. To this end, we used the model to suggest compositions for a series of nine composite support materials and then produced these

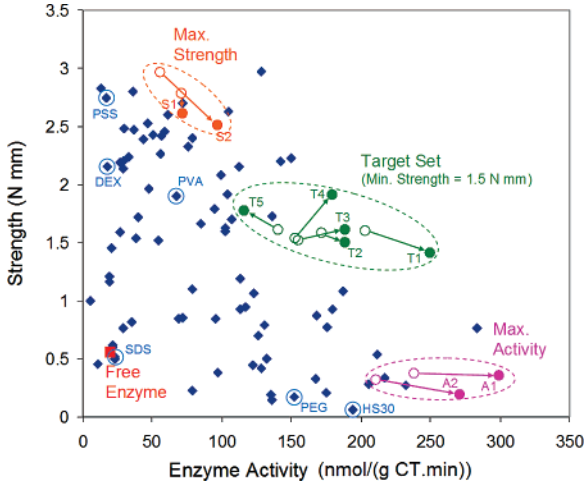

Figure 4. Plot of strength vs enzyme activity for all of the composite materials produced in this study (the complete dataset is presented in Table 1 and Table S1): blue diamonds, library of 80 composites prepared from DoE strategy (single-component composites are circled and labeled as such); orange circles, composites optimized for strength only using DoE model; pink circles, composites optimized for enzyme activity only using DoE model; blue circles, target set of composites optimized for both activity and strength. In all cases, open symbols denote predicted values, and closed symbols denote measured values.

materials in accordance with the predictions (Table 1). This was done using the "optimizer" routine available in MODEE 6.0 (see statistical notes, Supporting Information). Three sets of materials were produced. Two compositions were predicted where activity was optimized and strength was ignored (Table 1, entries A1 and A2). Similarly, two compositions were predicted where strength was optimized and activity was ignored (Table 1, entries S1 and S2). Finally, a set of five target compositions was predicted where activity was maximized but with a requirement of a minimum strength of 1.5 N mm (Table 1, entries T1−T5). This strength value was chosen since it was found that the beads with strengths below a threshold value of around 1 N mm were mechanically weak, harder to reuse, and unlikely to be suitable for applications such as continuous-flow reactors. All optimizations were made without any constraints on the values for the percentage inclusions of the various components, that is, each component was free to have any weight fraction between 0 and 1, providing that the total of all of the weight fractions was unity.

Figure 5 shows the fit between the predicted and observed strength and catalytic activity for the nine optimized materials. In general, the predictive power of the model appears to be reasonably good, particularly for the composite strength. The model tends to slightly under-predict the enzyme activity for this set of samples (Figure 5a), although it is not suitable to draw too many interpretations here for a set of only nine data points. The scatter in the data for catalytic activity (Figure 5a) means that the predictive power of the model is lower for this response. Overall, both catalytic activity and mechanical strength responses are distributed relatively close to the regression line, and this suggests that the model can be used predictively at a moderate level of precision for both properties.

The data obtained for the three sets of optimized materials are also shown on the plot in Figure 3. Both the predicted values (open circles) and the observed values (closed circles) are shown for each of the three sets. From this plot it is clear that the model is useful for the production of composites with mechanical strengths and catalytic activities that fall within specified targeted areas of the parameter space, even if the predictions for activity are less precise than those for strength, as evidenced by the flattened oval distribution of the three groupings shown in Figure 3. The composites designed for maximum strength were found to be in the top 10% of the distribution of materials studied and were found to have significantly higher catalytic activity than materials prepared from the neat strong support materials (PSS and DEX, see Figure 4), even though catalytic activity was excluded from the optimization process for materials S1 and S2. Of the materials optimized for only activity, one sample (A1) was found to be more active, within the precision of the measurement, than *any* of the materials produced in the initial library of 80 composites. This illustrates the power of DoE to predict improved second-generation materials in applications such as this. Figure 6 shows formulation−space prediction contour plots for both strength and activity for sample A1; it is clear that the model has optimized activity (Figure 6a) but at the expense of mechanical strength which falls in the low (blue) region of Figure 6b.

Perhaps most interesting materials are the target set of materials, T1−T5. Within this set, we discovered materials which had a desirable combination of properties for practical enzyme catalysis in organic media. Composite T1, for example, displayed good mechanical properties, was much easier to handle in organic media than the neat enzyme, and was found to have an enzyme activity that was around 12 times higher than unsupported α-CT. This compares favorably with reports concerning α-CT supported on acrylic copolymer beads where recyclability was demonstrated although with much more modest activity enhancement (approximately 2 times higher) over the free enzyme.[20,27] The contour plots in Figure 7 illustrate how the dual optimization procedure has maximized the activity for this formulation, while retaining acceptable (although not maximized) mechanical strength (Figure 7b).

Reuse of Optimized Composites. One of the main aims of this optimization study was to produce beads that were not only catalytically active but also mechanically strong enough to be reused. Although the *strength* of each bead type can be assayed, this does not in itself provide a definitive measure of reusability. Three of the nine sets of optimized beads were studied in terms of reusability. One set of these beads (sample A1) was optimized for maximum catalytic activity with no regard to mechanical strength. Two sets were optimized for maximum activity with a targeted "minimum" mechanical strength value of 1.5 N mm (samples T3 and T4). To assess the mechanical stability of the beads, each set was suspended in hexane and shaken for the equivalent of more than 100 cycles of use (based on the procedure for the enzyme activity assay). SEM images were captured before and after this procedure.

Table 1. Optimized Compositions for Supported Enzyme Beads[a]

	predicted activity (nmol mg^{-1} CT min^{-1})	predicted strength (N mm)	weight fraction PEG	HS30	PVA	SDS	DEX	PSS	observed activity (nmol mg^{-1} CT min^{-1})	observed strength (N mm)
A1	239	0.37	0.59	0.23	0.180	0	0	0	300	0.36
A2	210	0.32	0.67	0	0	0.33	0	0	272	0.19
S1	70	2.78	0	0.15	0.10	0	0.33	0.42	72	2.61
S2	55	2.95	0	0.15	0	0	0.33	0.52	97	2.51
T1	203	1.60	0.19	0.20	0.50	0	0	0.11	250	1.41
T2	172	1.58	0.12	0.14	0.70	0	0	0.035	189	1.50
T3	155	1.51	0.14	0.20	0.40	0	0.20	0.060	189	1.61
T4	153	1.53	0.20	0.14	0.19	0	0.41	0.063	179	1.91
T5	140	1.61	0.002	0.31	0.63	0.012	0.013	0.040	117	1.77

[a] Samples A1 and A2 were optimized for catalytic activity only. Samples S1 and S2 were optimized for mechanical strength only. Samples T1−T5 were optimized for enzyme activity with a threshold minimum practical mechanical strength target of 1.5 N mm.

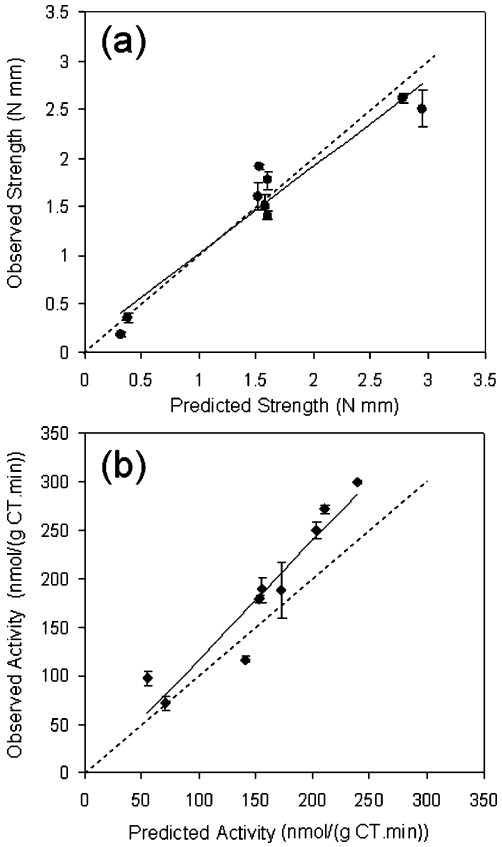

Figure 5. Plots illustrating validity of DoE model predictions for (a) strength and (b) catalytic activity. The best fit (solid line) and ideal correlation (dashed line) is shown in both cases.

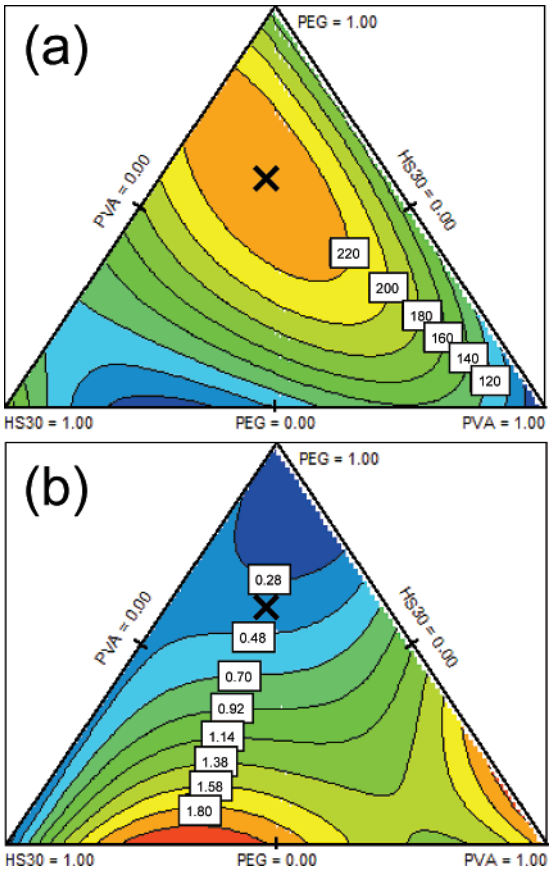

Figure 6. Contour prediction plots for (a) catalytic activity and (b) strength for three-component composites prepared from PEG, HS30, and PVA. The composition used to generate optimized sample A1 (maximize activity, ignore strength) is marked as X.

Sample A1 was optimized for maximum activity with no regard for mechanical strength. As observed by SEM images (Figure S9), the smooth skin of the unused beads has been stripped away by the collisions experienced with the reaction vessel and the other beads during the shaking assay, thus exposing the subsurface. Large cracks also appeared, through which the "herringbone"[26] interior structure is visible. The major component of these beads was PEG (59% w/w), which was shown to be the weakest of the materials studied.

Sample T4 was designed for maximum catalytic activity with a targeted mechanical strength value of 1.5 N mm. SEM imaging (Figure S9) showed that there was very little damage to the surface of the bead, which appears to have the characteristically smooth skin common to most of the composite preparations. The major component of this blend was DEX (41% w/w), which was shown to be one of the stronger materials. Similar results were observed for sample T3 where PVA was the dominant component. This shows that mechanical fracture of the beads and the formation of fines can be largely avoided through appropriate choice of the support material.

The catalytic activity of the enzyme-loaded beads was studied over several cycles of use. The initial activities of the three sets of composites were found to be very similar to the values predicted by the model. After ten cycles of use, the beads retain on average around 60% of the initial

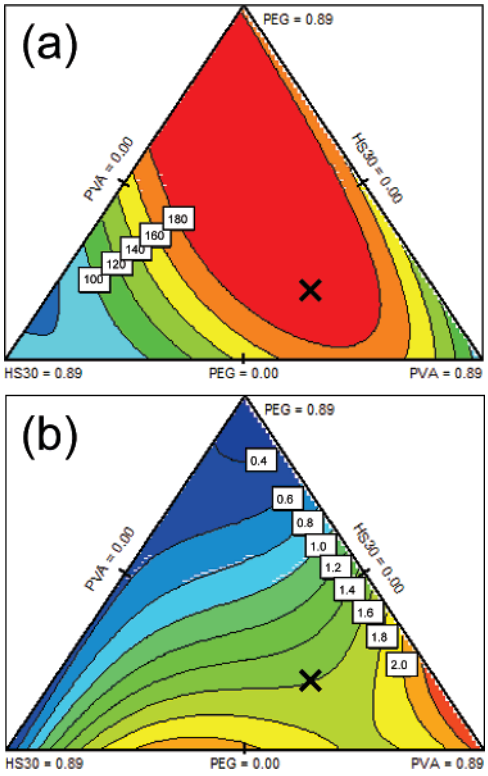

Figure 7. Contour prediction plots for (a) catalytic activity and (b) strength used to generate optimized sample T1 (maximize activity, minimum threshold strength). The predicted best composition used to produce T1 is marked as X. The PSS loading was set as a constant (0.11) to generate these 2D plots.

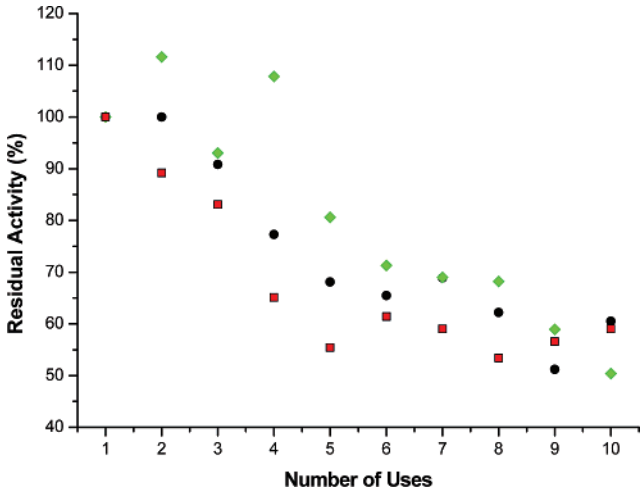

Figure 8. Residual catalytic activity of optimized composites after a number of uses: diamonds, A1; circles, T1; squares, T3.

catalytic activity (Figure 8). By comparison, an equivalent unsupported preparation of lyophilized enzyme lost virtually all of its activity after just one use. It should be noted that the catalytic activity for the supported materials was observed to decrease at approximately the same rate as a function of usage, regardless of the bead composition (Figure 8). This suggests that the enzyme deactivation is not, in this case, strongly influenced by the mechanical stability of the support matrix. Nonetheless, the preparation of mechanically stable preparations is still of significant advantage for separation and recycling.

Conclusions

The main aim of this research was to develop a methodological approach to optimizing not just enzyme activity but also the mechanical strength of immobilized enzyme preparations. In addition, we were interested in exploring an original approach to the development of biocatalytic materials. Hence, in this paper, we have presented a methodological approach to allow us to systematically prepare active yet strong enzyme preparations. We have achieved this by synthesizing 80 rather than hundreds of different materials with incremental changes in composition.

The catalytic activities reported here are much lower than the best available preparations; however, alternative methods for the preparation of highly active enzyme tend to focus only on activity rather than finding a compromise between activity and strength. It is unlikely that the activities exhibited by these materials are high enough for practical utilization; however, this new methodology opens up the prospect of further development.

The use of experimental design methodology has allowed the efficient screening of a variety of enzyme-containing composites produced by our freeze-drying route. The information obtained from this screen was used to construct a model which represents this complex six component system. The model has been shown to be accurate in the prediction of the experimental responses for any combination of these six "building block" materials. Beads have been produced that possess three times the mechanical strength and up to fifty times the catalytic activity of the control materials. In addition to this, the best preparations were shown to be reusable, retaining useful activity after more than 10 consecutive uses. The beads were also shown to be physically strong enough to be used in more than 100 cycles of the enzyme reaction.

In general, it was found that the materials which produced the strongest beads were also associated with the lowest catalytic activity. Arguably, the best material produced in this study (sample T4) was composed as follows: PEG 20% w/w, HS30 14% w/w, PVA 19% w/w, SDS 0% w/w, DEX 41% w/w, and PSS 6.3% w/w. This illustrates how DoE approaches may identify relatively complex formulations that are nonintuitive but are nonetheless, we believe, the near-optimal solutions based on the base set of six building blocks employed in this study.

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Supporting Information Available. Tables listing the enzyme activities and strengths for the library and the design summary for the DoE approach and figures showing the scaled and centered coefficient plots for mechanical strength, the residuals *N* plot for mechanical strength, the scaled and centered coefficient plots for enzyme activity, the PLS loading scatter plots, and SEM images of a fractured composite bead, of the bead surface, and of the composite materials before and after 100 uses, and statistical notes on

the optimization routine. This material is available free of charge via the Internet at http://pubs.acs.org.

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