Recent developments in biotransformation processes have been driven by the increasing demand for sustainable, energy-saving, and environmentally friendly synthetic strategies [1, 2]. Although enzymes are biocatalysts with high efficiency and excellent selectivity, their widespread application in industrial processes is often limited due to poor reusability and low operational stability [3]. Many immobilization strategies have been developed to improve biocatalyst properties [4–7]. Maximum retention of the enzymatic activity has been the primary concern, whereas less attention has been paid to the biocatalyst structure and its changes during immobilization and use under process conditions. This is surprising considering...
that the number and size of immobilized biocatalysts, distribution of the biocatalyst particles in the immobilization matrix, properties of matrix, e.g., particle size and porosity, are all expected to have a crucial effect on the final activity [8, 9]. Further, the selection of the most suitable immobilization method for the intended application under given conditions, e.g., in organic solvents or at increased temperature, is usually carried out by empirical screening [10]. Rational identification is mostly impossible, as the microscopic structure and molecular basis of enzyme immobilization have not yet been fully elucidated [11]. Structural analysis can simplify this process by enabling preliminary evaluation of the selected immobilization method or matrix under given conditions.

Previous studies on the relationship between the structure and catalytic properties of immobilized biocatalysts have mostly investigated carrier-free immobilization by employing bright-field and electron microscopy [12–16]. Immobilization within a matrix limits the number of techniques that can be applied. However, fluorescence confocal microscopy has been employed to give a three-dimensional visualization of a biocatalyst’s distribution within an immobilization matrix and provide an understanding of the diffusional restrictions [17].

In the present study, three different microscopy techniques, i.e., bright-field, confocal and electron microscopy, were used in combination to systematically investigate the structural behavior of an enzyme during immobilization and under typical process conditions. Haloalkane dehalogenase LinB from Sphingobium japonicum UT26 [18] was selected as a model enzyme owing to its wide application potential, e.g., in biocatalysis, bioremediation, decontamination of warfare agents, biosensing and protein tagging [19]. This enzyme was immobilized in cross-linked enzyme aggregates (CLEAs) [20] and its mechanical properties were further enhanced by entrapment into lens-shaped particles of polyvinyl alcohol (lentikats) [21]. Comparison of structural microscopic data with catalytic activity data revealed important information about the underlying structure–activity relationships.

2 Materials and methods

2.1 Materials

Recombinant histidine-tagged haloalkane dehalogenase LinB from S. japonicum UT26 [18] was produced in Escherichia coli BL21(DE3) and purified as described previously [22]. Prepared LinB was lyophilized using an ALPHA 1-2 LD freeze dryer (Martin Christ, Germany). Ammonium ferric sulfate, N,N-dimethylformamide, polyethylene glycol with a molecular weight of 600, 1000, 4000, or 6000 and potassium dihydrogen phosphate were all purchased from Fluka (Switzerland). Ampicillin sodium salt and iso-propyl-β-D-thiogalactopyranoside were obtained from Duchefa (The Netherlands). Nitric acid was purchased from Lach-Ner (Czech Republic). Polyvinyl alcohol was kindly provided by LentiKat’s (Czech Republic). Acetone, diethyl ether, ethanol, isopropanol and methanol were purchased from Chromservis (Czech Republic). All other chemicals were purchased from Sigma-Aldrich (USA). All reagents used were of analytical grade.

2.2 Enzyme immobilization

2.2.1 Preparation of dehalogenase CLEAs

Haloalkane dehalogenase LinB (38.4 mg), lyophilized in 50 mM phosphate buffer, and bovine serum albumin (38.4 mg) were dissolved in 9.6 mL of water in a centrifuge tube. Dissolved proteins were precipitated by adding 28.8 mL of saturated ammonium sulfate (pH 8.0) and allowing it to react for 45 min under stirring. Next, the solution was mixed with 3.1 mL of dextran polyaldehyde [23] and stirred for a further 45 min. The precipitation and cross-linking steps were performed in an ice bath. After cross-linking, the suspension was subjected to centrifugation at 4000 g for 20 min at 4°C. The supernatant, containing residual ammonium sulfate and free enzyme, was subsequently withdrawn and the resulting CLEAs were resuspended in 30.7 mL of saturated sodium hydrogen carbonate. Sodium borohydride (61.4 mg) was added to the solution and the mixture was allowed to react for 30 min at 4°C under stirring. Dehalogenase CLEAs were washed three times with 50 mM phosphate buffer (pH 7.5) and separated by centrifugation at 4000 g for 10 min at 4°C. Optimization of the dehalogenase CLEAs is described in detail in the Supplementary Material.

2.2.2 Preparation of dehalogenase lentikats

Immobilization of the dehalogenase CLEAs into lentikats was performed as described previously [24]. Polyvinyl alcohol and polyethylene glycol were heated in distilled water until the polyvinyl alcohol particles had dissolved completely. The mixture was then cooled to 35°C. A dispersion of dehalogenase CLEAs (0.5, 1.0, or 1.5 g) was slowly added to the polymeric mixture to give a final CLEA content of 10, 20, or 30 wt%, respectively. After thorough mixing, small droplets of the resulting mixture were dripped onto a smooth plastic plate, which was then dried at 30°C. After drying, the resulting lenses were soaked in 0.1 M sodium sulfate for 40 min to re-swell. Prepared lentikats were washed with 400 mL of 50 mM phosphate buffer (pH 7.5) and stored at 4°C [21].

2.3 Activity assay

The enzymatic activity of free and immobilized dehalogenase LinB was determined in 10 mL of 100 mM glycine buffer (pH 8.6) at 37°C by colorimetry [25]. Unless otherwise stated, 1,2-dibromoethane was added as substrate to a final concentration of 8.7 mM, determined using a
operated at 20 kV. The temperature program began with an isothermal period at 40°C for 1 min, after which the temperature was increased to 170°C at rate of 20°C per min. The reaction was initiated by addition of dehalogenase to the reaction mixture and terminated by mixing with 35% v/v nitric acid. The concentration of reaction products (bromide ions) was monitored spectrophotometrically at 460 nm using a Sunrise spectrophotometer (Tecan, Switzerland) following addition of mercuric thiocyanate and ferric ammonium sulfate. The concentration of reaction products was determined from a calibration curve prepared using sodium bromide as a standard solution. Characterization of the dehalogenase lentikats is described in detail in the Supporting Information.

2.4 Microscopic analysis

2.4.1 Bright-field optical microscopy

Bright-field optical microscopy was used to study CLEAs dispersed in 50 mM phosphate buffer (pH 7.5) in a ratio of 1:1 (v/v). The CLEA dispersion was spread over a glass slide and visualized using an Olympus BX50 microscope (Olympus, Japan) equipped with a 10× objective lens. Images were captured using an Olympus E-410 digital camera (Japan) mounted on the microscope. A size analysis of CLEAs was performed on 1000 particles of each type in three replicate measurements using the software ImageTool 3.0 (Department of Dental Diagnostic Science, University of Texas, USA). CLEAs were classified into different distribution groups according to their Feret’s diameter.

2.4.2 Scanning electron microscopy

Scanning electron microscopy was performed on both CLEAs dispersed in deionized water and CLEAs entrapped in a polymeric matrix. The aqueous CLEA dispersion was spread over a glass slide (diameter of 10 mm) and dried at 22°C overnight. The CLEAs entrapped in lentikats were fixed in 3% v/v phosphate buffered glutaraldehyde for 20 min at 22°C. The lentikats were washed three times with 50 mM phosphate buffer (pH 7.5) for 10 min and then postfixed in 1% v/v osmium tetroxide for 30 min at 22°C. Lentikats were washed a further three times with 50 mM phosphate buffer (pH 7.5) for 10 min and then dehydrated with different grades of ethanol (30, 50, 70, and 100% v/v, 10 min each step). The dehydrated lentikats were dried overnight in air at 22°C.

Both samples of CLEAs were sputter coated with gold for 3 min using an SCD040 system (Baltec, Liechtenstein) and current of 30 mA. Electron microscopy images were obtained with a Vega TS 5136 XM scanning electron microscope (Tescan, Czech Republic) operated at 20 kV.

2.4.3 Confocal microscopy

The preparation of samples of polyvinyl alcohol particles with and without CLEAs for confocal microscopy followed different staining procedures.

The polyvinyl alcohol matrix of lentikats incubated in phosphate buffer or organic solvents was visualized using the fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid. The dye (5.2 mg) was first dissolved in 20 mL of 50 mM phosphate buffer (pH 7.5) and then lentikats were added. After 30 min incubation under shaking at 22°C, the lentikats were washed with 200 mL of 50 mM phosphate buffer (pH 7.5). The prepared samples were stored in the same buffer prior to microscopy analysis. The CLEAs entrapped in lentikats were labelled using fluorescein 5(6)-isothiocyanate (Invitrogen, USA) dissolved in dimethyl sulfoxide (final concentration of 10 mg/mL). The fluorescent dye (40 μL) was added to 20 mL of carbonate/bicarbonate buffer (15 mM sodium carbonate, 30 mM sodium bicarbonate, pH 9.5) together with lentikats. Staining was performed for 60 min under shaking at 22°C. Subsequently, the lentikats were washed with 50 mL of 0.05 M Tris sulfate buffer (pH 8.2) and frozen in tissue freezing medium Jung (Leica Microsystems, Germany) at −26°C using a Cryocut 1800 cryostat (Leica Microsystems, Germany). The frozen samples were sectioned vertically and horizontally with thickness of 5 μm using the microtome incorporated in the cryostat, then mounted on glass slides with mounting medium Mowiol 40-88 (Sigma-Aldrich, USA). Both samples of particles were examined using an Olympus Fluoview 500 confocal microscope (Olympus, Japan) equipped with 10×, 20×, and 40× objectives. Images were digitized and analyzed using the software Fluoview (Olympus, Japan) and ImageTool 3.0 (Department of Dental Diagnostic Science, University of Texas, USA).

3 Results and discussion

3.1 Dehalogenase CLEAs

Dehalogenase CLEAs were successfully prepared by co-aggregation of haloalkane dehalogenase LinB and bovine serum albumin, followed by cross-linking with a mild cross-linker, dextran polyaldehyde, and final centrifugation. The activity retention of CLEAs prepared in this first trial was 35.5 ± 3.6% [26]. The effects of different precipitants, concentrations of cross-linker and addition of protective ligand were tested and optimized (Fig. 1). Ammonium sulfate at concentrations higher than 70% and 3.1 mL of dextran polyaldehyde were found to be the most appropriate reagents for preparation of the dehalogenase CLEAs (see Supporting Information, Fig. S1–S3).

The size distribution of the optimized CLEAs separated by centrifugation was determined by bright-field microscopy (Fig. 2). The dispersion of dehalogenase CLEAs
showed dissimilar trends (Fig. 2) as demonstrated by the CLEAs separated by centrifugation and decantation and activity measurements. The size distribution of investigated by a combination of bright-field microscopy the effects of both centrifugation and decantation were the particle size and subsequent catalytic activity. Thus, pared CLEAs was expected to have a significant effect on molecules to the catalytic sites [27].

The technique used for final separation of the prepared CLEAs was expected to have a significant effect on the particle size and subsequent catalytic activity. Thus, the effects of both centrifugation and decantation were investigated by a combination of bright-field microscopy and activity measurements. The size distribution of CLEAs separated by centrifugation and decantation showed dissimilar trends (Fig. 2) as demonstrated by the significantly different mean particle size for the two approaches: 49.5 ± 8.6 and 134.5 ± 28.4 μm for centrifuged and decanted CLEAs, respectively (median particle size of 30.2 ± 2.7 and 88.7 ± 29.9 μm, respectively). Whereas the majority of CLEAs separated by centrifugation exhibited particle sizes of <25 μm, decantation resulted in partial loss of these small-sized particles, which can be attributed to their resistance in moving through the liquid as given by Stokes’ law [28]. The highest proportion of decanted CLEAs (approximately 27%) had particle sizes ranging between 25 and 50 μm. In addition, the number of larger CLEAs with sizes from 150 to 1000 μm was significantly higher (up to 17%) for the decanted compared to centrifuged CLEAs. Unexpectedly, CLEAs obtained by centrifugation had comparable activities to those generated by decantation (26.7 ± 3.2% and 29.4 ± 1.7%, respectively; Fig. 3), suggesting that the particle size was not the only determinant of catalytic activity. Therefore, scanning electron microscopy was used to obtain detailed images of any morphological differences between the CLEAs separated by the two methods (Fig. 3). The structure of CLEAs separated by centrifugation was significantly affected by the centrifugation force. The particles were clearly less structured compared to more porous CLEAs separated under the influence of gravitational force during decantation. The particle-size distribution and morphological differences observed for the decanted CLEAs suggest that two factors are important: (i) reduction of the enzymatic activity due to diffusional limitations of bulki-er particles; and (ii) increased enzymatic activity due to the higher specific surface area of the decanted CLEAs. The balance between these two effects determines the resulting activity of the decanted CLEAs.

Because the activity of the decanted and centrifuged CLEAs was similar, only centrifugation was used to prepare CLEAs in the subsequent experiments for practical reasons, primarily the shorter separation time. The partial loss of enzymatic activity as a result of morphological changes of CLEAs during centrifugation has been shown to be recovered by using vortexing and the FastPrep cell disruption system [13, 29]. In the present study, we tested whether treatment of centrifuged CLEAs by sonication would change the particle-size distribution and/or morphology of CLEAs, and thus increase their catalytic activity. Bright-field microscopy revealed similar particle-size distributions for the centrifuged and sonicated CLEAs (Fig. 2), with mean particle size of 49.5 ± 8.6 and 57.3 ± 14.7 μm, respectively (median particle size of 30.2 ± 2.7 and 37.2 ± 7.5 μm, respectively). Several significant morphological changes that occurred during sonication of CLEAs were clearly evident in the scanning electron micrographs (Fig. 3). The aggregates additionally processed by sonication were more structured and less compressed compared to the unsonicated CLEAs. The large surface area and many cavities of the sonicated CLEAS would allow substrate molecules to readily access

![Figure 1](image-url)
the enzyme active site, whereas enzyme molecules in the interior of the less porous centrifuged CLEAs would have a lower probability of reacting with the substrate molecules, resulting in lower catalytic activity. The structural changes observed by electron microscopy strongly correlated with activity measurements: the sonicated CLEAs exhibited a significantly higher activity (41.3 ± 3.0%) than the centrifuged CLEAs (26.7 ± 3.2%, Fig. 3). These results are in agreement with observations by Garcia-Garcia et al. [13] and Montoro-Garcia et al. [29].
3.2 Dehalogenase lentikats

A highly concentrated heterogeneous mixture of dehalogenase CLEAs was immobilized in a polyvinyl alcohol matrix to generate dehalogenase lentikats. To the naked eye, the resulting lentikats were white, elastic, lens-shaped particles of diameter 3–5 mm. Entrapment of optimized CLEAs into the polymer was accompanied by a slight decrease in the enzymatic activity (29.5 ± 2.0%) [26]. Therefore, confocal and scanning electron microscopy (Fig. 4) were used to evaluate the factors affecting the catalytic properties of the immobilized enzyme, e.g., internal structure of the matrix defined by the matrix homogeneity, pore size distribution, mode of pores, CLEA dispersion and loading. Microscopic analysis of microtomed horizontal sections through the lentikats revealed that the polyvinyl alcohol had a heterogeneous structure, with significant differences between the center and edge of particles. Whereas the pore-size distribution at the edge was from 0 to 10 μm (mean of 4.5 ± 1.5 μm), pores from 4 to 28 μm (mean of 12.5 ± 4.1 μm) were predominantly observed in the center of the lenses (Fig. 5). The pores were spherical with an area of 72.3 μm² and a circularity of 0.9 (a value of 1.0 indicates a perfect circle, Table 1). The interconnection of pores was clearly visible in both the confocal and electron micrographs. This is important to facilitate the flow of substrate to CLEAs located in the inner structure of the lens-shaped particles. Microscopy of microtomed vertical sections through the lentikats showed that pores were densely distributed over the whole scanned area except for the upper surface of the lenses, where a separate zone without pores was formed. Assessment of CLEAs in lentikats (Fig. 4) revealed broad distributions for abundance (133.4 ± 101.0 aggregates per mm² of matrix) and area fraction (6.9 ± 4.5%), suggesting a non-uniform distribution of CLEAs through the lenses (Supporting Information, Table S1). Investigation of the size of the entrapped CLEAs showed that there were significant differences between aggregates dispersed in the polyvinyl alcohol matrix. The size of aggregates influenced their incorporation into porous matrix: CLEAs significantly larger than the pores appeared to protrude through a couple of pores (Fig. 4), whereas small-sized CLEAs were partially embedded in the polymeric matrix and located in single pores. The mean CLEA size (16.6 ± 36.6 μm) was in agreement with the measured par-
Table 1. Pore parameters of lentikats incubated in phosphate buffer, dioxane or tetrahydrofuran for 1 week

<table>
<thead>
<tr>
<th>Pore properties</th>
<th>Phosphate buffer (pH 7.5)</th>
<th>25% dioxane</th>
<th>50% dioxane</th>
<th>25% tetrahydrofuran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (μm²)</td>
<td>72.3 ± 18.6</td>
<td>73.1 ± 56.0</td>
<td>21.2 ± 12.1</td>
<td>59.5 ± 37.3</td>
</tr>
<tr>
<td>Feret’s diameter (μm)</td>
<td>12.0 ± 4.9</td>
<td>12.9 ± 3.0</td>
<td>6.9 ± 1.4</td>
<td>10.7 ± 2.6</td>
</tr>
<tr>
<td>Circularity</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

a) Median (bold) and mean ± standard deviation were determined from confocal micrographs by image analysis of 200 pores in the center of the lentikats.

b) Feret’s diameter – the longest distance between any two points along the selection boundary.
c) Circularity – shape descriptor: 4π*area/perimeter². A value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape.

ticle-size distribution of dispersed CLEAs used for the preparation of lentikats (Fig. 2), suggesting that the entrapment was efficient with no or negligible leaching of aggregates. The observed microscopic structure of entrapped CLEAs, particularly their porosity, was similar to that observed for dispersed CLEAs treated by sonication (Fig. 3). The findings regarding the size and structure of immobilized CLEAs were also consistent with their measured enzymatic activity, which was only slightly diminished compared to that of dispersed CLEAs.

The results indicated that the enzymatic activity of the lentikats could be improved by optimizing the CLEA loading. In particular, the microscopic inspection of the CLEA distribution in the lentikats suggested that the loading capacity of the polyvinyl alcohol matrix might be even higher than used here. Therefore, the effect of CLEA loading on the activity of lentikats was tested whilst simultaneously assessing the quality of immobilization by confocal microscopy (Fig. 4). The abundance and area fraction of the CLEAs determined by image analysis were proportional to the amount of CLEAs used for the preparation of the lentikats (Supporting Information, Table S1). The loading had an obvious influence on the activity of the lentikats. However, the differences were rather small and not proportional to the content of CLEAs (Fig. 4). The highest enzyme loading resulted in the highest activity, but probably not all the loaded CLEAs remained active. Too low loading of CLEAs resulted in inefficient utilization of the polymeric matrix. However, the access of substrate molecules to individual CLEAs was simpler than in more loaded lentikats. To investigate the effect of diffusional limitations on the activity of differently loaded lentikats, the lenses were sliced and their activity was determined (Fig. 4). Even though the activity was slightly increased for all types of lentikats, the enzymatic activity still did not directly correlate with the extent of enzyme loading, which is similar to observations by Ursoiu et al. for immobilized lipase [30]. Further experiments were performed with lentikats containing 1 g of CLEAs (biocatalyst represents 20 wt% of the lens), following the recommendation of Schlieker and Vorlop [31].

The individual properties of the matrix and enzyme are not the only determinants of activity of immobilized biocatalysts [9]. The availability of a substrate is governed by its concentration and solubility, which can be improved by addition of an organic cosolvent. In the present study, hydrophobic bromocyclohexane was used as a model substrate for haloalkane dehalogenase. The use of 10% tetrahydrofuran and 40% dioxane improved the solubility of the substrate by approximately 4-fold compared to aqueous buffer (Supporting Information, Fig. S6). Further increasing the solvent concentration did not increase the concentration of dissolved substrate.

The suitability of the polyvinyl alcohol matrix for application in organic solvents was studied by confocal microscopy and compared with the behavior in aqueous solution (Fig. 5, Table 1). Lentikats stored in phosphate buffer were elastic lens-shaped particles with spherical pores. The pore area and diameter exhibited broad distributions with mean values of 98.5 ± 81.6 and 12.8 ± 4.8 μm, respectively (Fig. 5, Table 1). Incubation in 25% tetrahydrofuran did not affect the overall shape of the lentikats. However, minor disruption of the structure in the center of lenses was observed. The pore area and diameter in tetrahydrofuran (62.2 ± 37.3 and 10.6 ± 2.6 μm, respectively) were smaller of lenses in phosphate buffer (Fig. 5, Table 1). The catalytic properties of the free dehalogenase and dehalogenase lentikats (Fig. 5) differed in tetrahydrofuran. Free dehalogenase showed good activity in 5 and 10% tetrahydrofuran, whereas 20% tetrahydrofuran had a deleterious effect on the catalytic activity; the free dehalogenase was not active under the latter concentration, whereas the dehalogenase lentikats retained a catalytic activity of 21%.

Tetrahydrofuran is often substituted by dioxane in some processes because of the latter’s lower toxicity. A negligible effect on the structure of lenses was observed in the case of 25% dioxane (pore area and diameter of 71.4 ± 56.0 and 12.8 ± 3.0 μm, respectively, Fig. 5, Table 1), whereas 50% dioxane caused a reduction in the elasticity and higher incursion of the lenses. The observed pores were significantly smaller in the presence of 50% dioxane (pore area and diameter of 21.6 ± 12.1 and 7.0 ± 1.4 μm, respectively) compared to lenses incubated in phosphate buffer (Fig. 5, Table 1). The catalytic activities of both free dehalogenase and dehalogenase lentikats in 5 and 10% dioxane were comparable to the activity in glycine buffer (Fig. 5). Activities of approximately 90% were observed in...
20% dioxane for both free dehalogenase and dehalogenase lentikats. The dehalogenase lentikats performed better in 30, 40, and 50% dioxane than the free enzyme. This was particularly evident in the case of 50% dioxane, in which the free enzyme was completely inactive, whereas the dehalogenase lentikats exhibited an activity of 20%. These results are consistent with data measured by Wilson et al. [32], who observed that penicillin G acylase CLEAs entrapped in lentikats showed a higher activity than the free enzyme in 75% dioxane. The authors suggested that the hydrophilic matrix surrounding the CLEAs protected the enzyme from inactivation in organic media. This is supported by our microscopic analysis. The narrow pores of the matrix could help to protect the biocatalyst by hindering access of the cosolvent molecules to the CLEAs, thus lowering the deleterious effect of the cosolvent on aggregates in the interior of lenses. This hypothesis is also supported by obvious correlation between activity of free enzyme and CLEAs incubated in 25% tetrahydrofuran and 50% dioxane and pore size of polymeric matrix incubated in both cosolvents compared to incubation in buffer.

4 Concluding remarks

Bright-field and electron microscopy were used to provide an insight into the distributional and structural changes induced during optimization of a catalyst’s immobilization. Confocal and electron microscopy enabled the structure of a biocatalyst entrapped in a porous matrix and quality of immobilization to be analyzed. Microscopic evaluation of the internal structure of the matrix exposed to different organic cosolvents suggested an explanation for the different catalytic behavior of the free and immobilized biocatalyst. The results demonstrated that microscopic techniques are useful tools obtaining information about an immobilized biocatalyst, which can help to optimize the immobilization procedure.

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5 References