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Influence of silica architecture on the catalytic activity of immobilized glucose oxidase

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Immobilizing enzymes on solid support materials is of great interest for many applications as it improves enzyme lifetime and enables its recycling. Synthetic mesoporous silica materials are widely used as supports for enzyme immobilization due to their high enzyme-loading capacity and their excellent mass transport properties through interconnected channels of mesopores. Much less is known about the enzyme support properties of naturally occurring mesoporous silica materials. These are readily available through the growth of diatoms, which biosynthesize hierarchically porous silica microshells with pore diameters ranging from 10 to 1000 nm. Here the authors investigated the performance as enzyme support materials of biosilica from three diatom species in comparison to synthetic silica materials. Using glucose oxidase (GOx) as a model enzyme, the authors determined the enzyme-loading capacities and the specific catalytic activities of the GOx-bearing (bio)silicas both under suspension conditions and in a flow-through setting. Remarkably, the specific activity of GOx was strongly dependent on the type of diatom biosilica material and in the best case was as high as on synthetic mesoporous silica. Porosity analysis of the materials points to an important role of large mesopores (diameters >20 nm) for supporting a high specific activity of (bio)silica-immobilized GOx.

1. Introduction

Enzymes are versatile tools for a variety of applications, including the synthesis of medicinal drugs, food processing, biomass degradation for energy harvesting and chemical sensors.^{1–5} The catalytic activity of an enzyme is strictly dependent on its native three-dimensional (3D) structure, which is prone to denaturation during the application process. Immobilizing enzymes in a solid material has frequently been shown to enhance the lifetime of enzyme activity.^{6,7} However, the solid support constitutes an impediment to the diffusion of the reactants and thus often slows down the reaction rate compared to the free enzyme in solution.⁸ To alleviate this problem, high-surface-area mesoporous materials – for example, the synthetic mesoporous silica particles MCM-41 and SBA-1 – have been used, which provide numerous liquid channels for reactant diffusion.^{9,10} The pore sizes of such materials are primarily in the range of 2 to 50 nm dia.¹¹ and are thus large enough to allow for the incorporation of protein molecules or even protein complexes. Mesoporous silica materials are available also from natural sources through the biosynthetic capabilities of diatoms, which are a large group of microalgae. The pore diameters in diatom biosilica typically range from ~10 nm up to several hundreds of nanometers and are generally arranged in a hierarchical fashion.¹²

The architecture of diatom biosilica is species specific, and as there are tens of thousands of diatom species known,¹³ this biological material offers a much wider range of mesoporous silica structures than what is available through chemical synthesis. Furthermore, diatom biosilica is a renewable material whose production requires only sunlight, carbon dioxide (CO₂) and seawater.

Previously, it has been shown that the biosilica from natural sediments of dead diatoms, called diatomaceous earth,^{14–16} and freshly prepared biosilica from recent diatoms^{17,18} are suitable materials for enzyme immobilization. It was hypothesized that enzymes immobilized in diatom biosilica may exhibit particularly high catalytic activities (particularly in flow-through systems) due to the hierarchical porosity and open 3D structure of the material.¹⁴ However, to date, no systematic analysis has been published that has addressed this hypothesis. Here the authors report the catalytic activities of the enzyme GOx-PA (i.e. glucose oxidase derivatized with the silica-binding peptide protamine)¹⁹ after immobilization in biosilica microparticles from three diatom species and in two different synthetic silica materials. For each material, the activity of the immobilized GOx-PA was determined both in a suspension and under flow-through conditions.

Additionally, the pore size distributions (PSDs) in the biosilica and synthetic silica materials were characterized, which revealed a striking correlation between the presence of 20–50 nm sized mesopores in (bio)silica and the activity of immobilized GOx-PA.

2. Results and discussion

2.1 Porosities and enzyme-loading capacities of biosilica and synthetic silica materials

To investigate the influence of biosilica morphology on enzyme catalysis, biosilica microparticles were prepared from three rather structurally different diatom species: *Stephanopyxis turris*, *Thalassiosira pseudonana* and *Eucampia zodiacus* (Figures 1(a)–1(c)). *S. turris* biosilica (Figure 1(a)) consists of dome-shaped particles with a double layer, each containing circular pores. One layer contains hexagonally arranged large areola pores (pore diameters between 1 and 1.5 μm), and the other layer contains much smaller cribrum pores (pore diameters between 50 and 80 nm), which are also arranged in a hexagonal pattern (Figure 1(f)). *T. pseudonana* biosilica contains circular plate-like structures (Figure 1(b)) with seemingly randomly arranged ~ 20 nm sized pores (Figure 1(g)). Groups of such pores are bordered by ridges (Figure 1(g)), which constitute a cross-connected dendritic structure that radiates throughout the plate (Figure 1(b)). *E. zodiacus* biosilica has an elongated structure with two conically shaped ends (Figure 1(c)). It is composed of a single layer of silica that contains a square pattern of rectangular pores (100–200 nm long, 80–150 nm wide; Figure 1(h)). Each pore is subdivided into four to six almost equally sized pores by silica cross-bars (Figure 1(h)). As synthetic silicas, mesocellular silica foam (MCF) and Stöber silica beads were prepared. The MCF silica has an aerogel-like structure containing uniform, ink-bottle-like, interconnected mesopores, with diameters in the range from 20 to 50 nm (Figures 1(d) and 1(i)). The silica beads consisted of spherical particles with diameters in the range of 100–200 nm, which did not exhibit any pores when imaged with scanning electron microscopy (SEM) (Figures 1(e) and 1(k)).

Native diatom biosilica was isolated by an established extraction procedure using a detergent (sodium dodecylsulfate (SDS)) and a metal-ion-chelating agent (ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)).²⁰ This material still contains tightly associated biomolecules that are incorporated into the biosilica.^{20–22} As the authors intended to use the biosilica for enzyme attachment, it was desirable that this material has a high surface area, particularly in the range of mesopores that are sufficiently large to be accessible to enzyme molecules. It was previously demonstrated that calcination of isolated biosilica from the diatom *Pinnularia* sp. at temperatures up to 550°C increased the specific surface area (SSA) of the material presumably due to the removal of the biosilica-associated biomolecules (i.e. proteins, polysaccharides and long-chain polyamines).²³ The authors investigated whether this effect was also observed for the biosilica that they had isolated from different diatom species, and they also included a detailed analysis of the distribution of mesopores (Table 1, Figures 2 and 3).

The nitrogen adsorption isotherm of *E. zodiacus* biosilica is presented in Figure 2. It shows the typical shape for large-pore materials with a relatively low uptake of nitrogen at low relative pressures due to the limited micropore volumes and a distinct increase in the adsorbed volume at higher relative pressures of $p/p_0 > 0.8$ due to the adsorption in large mesopores and macropores of the diatom biosilica. The slight hysteresis over the entire range of relative pressure is associated with the low overall nanoporosity of the samples. However, adsorption and desorption branches of all isotherms are still comparable and the given porosity values are well reproducible. The SSAs and specific pore volumes (SPVs) of *E. zodiacus* biosilica increased from 21 to 112 m^2/g and 0.11 to 0.3 cm^3/g , respectively, after calcination. Increases in pore volumes upon calcination were also observed for the other two diatom biosilica materials (Table 1). The non-local solid density functional theory (NLDFT) PSDs of *E. zodiacus* biosilica (Figure 2) show that the increase in the porosity is mainly associated with the generation of additional

Figure 1. SEM images of biosilica and synthetic silica materials that were used in the present study; (a, f) *S. turris*; (b, g) *T. pseudonana*; (c, h) *E. zodiacus*; (d, i) mesocellular silica foam; (e, k) silica beads

Table 1. SSAs, SPVs and maximum GOx-PA-loading capacity of (bio)silica samples

Sample	SSA: m ² /g		SPV: cm ³ /g		GOx-PA loading: × 10 ⁻³ g enzyme/g silica
	Native	Calcined	Native	Calcined	
<i>S. turris</i>	24	56	0.11	0.15	17.7 ± 2.4
<i>T. pseudonana</i>	30	97	0.18	0.40	35.5 ± 1.6
<i>E. zodiacus</i>	21	112	0.11	0.30	20.1 ± 2.3
MCF	n.d.	677	n.d.	2.43	26.6 ± 1.3
Silica beads	n.d.	34	n.d.	0.15	2.4 ± 0.4

The maximum amount of GOx-PA that could be loaded on the materials (only for calcined samples) was determined through activity measurements of the unbound enzyme. n.d., not determined

Figure 2. Characterization of the porosity of the biosilica from the diatom *E. zodiacus* after SDS/EDTA extraction (native biosilica) and after heating at 550°C (calcined biosilica). Nitrogen adsorption/desorption isotherms at -196°C (a), as well as cumulative (b) and differential (c) pore size distributions

mesopores of diameters in the range of 5 to 20 nm. The distinct increase in pore volume during the calcination step correlates with the proposed removal of proteins and other biomolecules from the silica during this heat treatment. For the sake of comparability, the same cleaning procedure was also applied to the synthetic silica materials. Table 1 summarizes the SSAs and SPVs of the three

biosilica samples and the two synthetic silica materials after the described cleaning procedure.

The SSAs of calcined biosilica microparticles were different for the three diatom species (Table 1). The SSAs of *T. pseudonana* and *E. zodiacus* biosilica were almost twice that of *S. turris*

Figure 3. Distribution of mesopore sizes expressed as relative pore volumes in percentage of total pore volume. Note that the pore size ranges between 2 and 20 nm were chosen to overlap

biosilica. The SSA of silica beads was only 30% the SSA of *E. zodiacus* biosilica, while the SSA of MCF silica was approximately six times higher. The SPVs of the biosilica and synthetic silica materials followed a very similar trend except that the SPV of *T. pseudonana* biosilica was higher than that of *E. zodiacus* biosilica.

Consistent with its low porosity, SB silica beads exhibited also the lowest enzyme-loading capacity of the five (bio)silica materials that were tested here (Table 1). The enzyme that was loaded on the (bio)silica materials was GOx-PA, which is GOx (from the fungus *Aspergillus niger*) derivatized with the silica-binding peptide PA.¹⁹

Although the MCF had by far the highest porosity (Table 1), it did not exhibit the highest GOx-PA-loading capacity, which was ~25% lower than that of *T. pseudonana* biosilica, and only ~24 and ~50% higher than the enzyme-loading capacities of *E. zodiacus* and *S. turris*, respectively (Table 1).

To investigate the reason for the unexpectedly low GOx-PA-binding capacity of MCF silica, the authors determined the distribution of mesopore sizes in MCF silica and the other (bio)silica materials (Figure 3). This analysis revealed that the majority of pores in MCF silica are sufficiently large to accommodate GOx-PA, which has a hydrodynamic diameter of ~20 nm¹⁹ (see data for 10–20 nm and 20–50 nm pores in Figure 3). MCF silica has a higher proportion of large mesopores (10–20 and 20–50 nm pore diameter) and a much higher SSA than *T. pseudonana* biosilica. Therefore, the authors assume that the discrepancy between the extremely large surface area of MCF and its only moderate enzyme-loading capacity is likely a consequence of its void-window-type pore structure,²⁴ which may not allow the GOx-PA molecules to access all >20 nm sized pores. Additionally,

the incubation time for GOx-PA binding (2 h) might have been insufficient to allow for penetration of the enzyme into the interior pores of the MCF material. In contrast to MCF, diatom biosilica has open pores and rather thin walls ($\leq 1 \mu\text{m}$) that allow the enzyme to access rapidly the silica surfaces throughout the material.

2.2 Suspension and flow-through catalysis by immobilized GOx-PA

The authors then investigated whether the specific activity of the immobilized GOx-PA (i.e. the catalytic activity per enzyme molecule) is dependent on the type of silica material. This question was addressed using two different experimental setups: a stirred suspension of the enzyme–silica material in a glucose-containing solution (‘suspension condition’) and a packed column of the enzyme–silica material exposed to a constant flow of a glucose containing solution (‘flow-through condition’). The five silica materials were loaded with saturating amounts of GOx-PA, and the specific enzyme activities were determined. Under flow-through conditions, the specific enzyme activity was by far the highest for GOx-PA attached to silica beads, whereas the enzyme immobilized on the other silica materials exhibited only between 15–26% of the GOx-PA activity on silica beads (Figure 4, gray bars). The same trend, albeit with less drastic activity differences, was observed under suspension conditions (Figure 4, black bars). For each GOx-PA-loaded silica material, the specific activity was higher under suspension conditions compared to flow-through conditions (Figure 4). This result indicated that the diffusion of substrates to the immobilized enzyme molecules was less efficient when the (bio)silica particles were tightly packed in a column compared to a loose dispersion of the particles under suspension conditions. The reduction in specific catalytic activity was most drastic for GOx-PA immobilized on MCF silica (5.1-fold reduction) and much less pronounced for GOx-PA immobilized on diatom biosilica (1.5–2.5-fold reduction).

Figure 4. Specific activities of saturating amounts of GOx-PA immobilized on the (bio)silica materials under suspension conditions (black bars) and flow-through conditions (gray bars)

The preceding results seemingly indicated that biosilica microparticles were less efficient than the synthetic silica materials in supporting high specific enzyme activity. However, it was conspicuous that in both the suspension and the flow-through setups, the specific enzyme activity was lowest for the material with the highest enzyme loading (*T. pseudonana* biosilica), whereas the material with the lowest enzyme loading (silica beads) exhibited the highest specific enzyme activity. Therefore, the authors suspected that the relatively low specific activity of GOx-PA on enzyme-saturated biosilica is due to enzyme overloading, which has also been observed with other immobilized enzymes. Enzyme overloading of a support material leads to partial blocking of the active sites of a substantial fraction of the enzyme molecules due to molecular crowding.²⁵ To investigate whether molecular crowding caused the relatively low specific activity of GOx-PA on biosilica, the authors determined for each (bio)silica material the catalytic activity as a function of the amount of immobilized enzyme. These experiments were performed under flow-through conditions. For each support material, the catalytic activity increased linearly at low enzyme loading and already reached a plateau at enzyme loadings that

were below the maximum-loading capacity (Figure 5). Within the linear regime of catalytic activity against enzyme loading, each newly added enzyme molecule makes the same contribution to the total catalytic activity as the enzyme molecules that were already bound to the (bio)silica material. In contrast, in the plateau regime where a higher amount of enzyme molecules is already present on the (bio)silica, binding of additional enzyme molecules does not lead to any or substantially lower increase in the total catalytic activity than in the linear regime. This indicates that at high enzyme loading, the immobilized enzyme molecules exert a mutually inhibitory effect (i.e. overcrowding).

Here the authors aimed to assess the influence of silica structure on catalytic activity under optimal conditions for the enzyme. Therefore, they eliminated the effects of overcrowding by loading the (bio)silica material with low amounts of GOx-PA. In each case, the amount of immobilized GOx-PA was within the linear regime of the activity against the enzyme-loading diagram. The specific catalytic activities of the materials were determined for both flow-through and suspension conditions. For a given GOx-PA-bearing material, the specific catalytic activities of flow-through and suspension conditions were very similar (Figure 6). However, substantial differences in specific catalytic activities were observed between the different materials (Figure 6). The highest specific activities were observed for GOx-PA immobilized on *T. pseudonana* biosilica and on MCF silica, which were essentially identical. These values were about twice the specific activities of GOx-PA immobilized on *E. zodiacus* biosilica and on silica beads. GOx-PA loaded on *S. turris* biosilica had an intermediate specific activity (Figure 6). These data demonstrate that in the absence of overcrowding effects, the physical structure of the (bio)silica support material exerts an influence on the activity of the immobilized enzyme molecules.

Figure 5. Activity of GOx-PA immobilized on the (bio)silica materials as a function of GOx-PA loading on 0.3 ml column volume (flow-through system). Each data point shows the activity during the first hour of catalysis. The arrows and neighboring numbers indicate the enzyme loading (in % of the maximum possible enzyme loading) at which the activity plateau was reached

Figure 6. Specific activities of enzyme-loaded (bio)silica materials bearing low amounts of GOx-PA. The measurements were performed under suspension conditions (black bars) and flow-through conditions (gray bars)

3. Materials and methods

3.1 Materials

Milli-Q purified water (H_2O) (resistivity: $18.2 \text{ M}\Omega \text{ cm}$) was used throughout. GOx from *A. niger*, horseradish peroxidase (HRP), PA sulfate from salmon, *o*-phenylenediamine dihydrochloride (OPD), tris-(hydroxymethyl)-aminomethane (Tris) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) were purchased from Sigma-Aldrich. Sodium chloride (NaCl), sodium hydroxide (NaOH), SDS, EDTA, hydrochloric acid (HCl) and acetic acid were purchased from Merck. Bis (sulfosuccinimidyl) suberate (BS3) was purchased from Thermo Scientific. Glucose was purchased from Alfa Aesar. Dibasic and monobasic sodium phosphates and sodium acetate (NaAc) were purchased from Carl Roth. Citric acid and sulfuric acid (H_2SO_4) were purchased from VWR. Columns for flow-through experiments (2.5 ml volume, $10 \mu\text{m}$ filter pore size) were purchased from Mobitech.

3.2 Diatom cell culture

T. pseudonana was grown in enriched artificial seawater medium according to the protocol from Canadian Center for the Culture of Microorganisms. Cells were grown at 18°C in 20 l polycarbonate bottles (round bottom) under constant illumination, constant aeration with compressed air and constant stirring with a magnetic stirring bar. *S. turris* and *E. zodiacus* were grown in artificial seawater medium according to the protocol of the North East Pacific Culture Collection in 20 l polycarbonate vessels (Nalgene). A Rumed 1301 light thermostat (18°C , 12 h/12 h day/night cycle, c. 1000 lux) provided constant growing conditions. Adjustment of the rising pH to 8.0–8.2 was carried out with 2.5 mol/hydrochloric acid. The cells were harvested by consecutive filtration of the culture medium with a $1 \mu\text{m}$ nylon mesh (Stockhausen Sieb- und Filtererzeugnisse).

3.3 Preparation of biosilica and synthetic silica materials

Diatom cells were extracted at 95°C for 1 h with a lysis buffer (1% (w/v) SDS, 0.1 mol/l EDTA adjusted to pH 8.0 with hydrochloric acid) and subsequently centrifuged at $3200g$ for 10 min. The pellet was washed with water by five centrifugation and resuspension cycles, then was washed twice with acetone by resuspension and centrifugation and was finally dried at room temperature, yielding pure biosilica. The dried material was calcined at 550°C for 4 h, yielding organic-free biosilica.

MCF²⁴ and silica beads²⁶ were synthesized according to published protocols. The resulting silica materials were subjected to the same extraction, washing and calcination procedures as described for biosilica earlier to ensure comparable surface chemical properties.

3.4 Synthesis of GOx-PA

GOx was derivatized with PA using the homobifunctional cross-linker BS3 (Scheme 1). For a typical reaction, 2 ml of 1 g/l GOx,

Scheme 1. Derivatization of GOx with PA using BS3 as a cross-linker. Note that GOx contains several accessible lysine residues and thus is likely modified with more than one PA residue¹⁹

80 μl Hepes pH 7.5, 120 μl 4 M sodium chloride and 200 μl 40 g/l PA were mixed followed by addition of 50 μl 20 g/l BS3. The reaction mixture was incubated for 1 h at room temperature on a rotator mixer and subsequently stopped by the addition of Tris-hydrochloric acid pH 7.5 to a final concentration of 0.1 M. The solution was then dialyzed at 4°C against three changes of 0.05 M NaAc pH 5.1 (12–14 kDa molecular weight cutoff). The GOx-PA concentration in the dialysate was determined by measuring the flavin adenine dinucleotide (FAD) adsorption at 450 nm (molar extinction coefficient $\epsilon = 11\,300 \text{ M}^{-1} \text{ cm}^{-1}$), and the activity of GOx-PA was determined as described in Section 3.5). GOx-PA preparations were stored at 4°C for up to 2 months without any noticeable decline in specific activity.

3.5 GOx-PA activity assay

The assay involved two reaction steps (Scheme 2). In the first step, the GOx-PA was incubated with glucose, and in the second step the hydrogen peroxide (H_2O_2) produced during the first step is quantified using the enzyme HRP. The first assay step was performed differently depending whether the GOx-PA was present in solution or immobilized on silica particles.

Scheme 2. Reactions catalyzed by GOx and HRP

3.5.1 For GOx-PA in solution

For the first step of the assay, GOx-PA-containing solutions were diluted to 177 μl with water, 5 μl 2 M NaAc pH 5.1 was added and the reaction was started by adding 18 μl 1 M D-glucose. The reaction was incubated for 20 min under constant shaking at 1400 revolutions per minute (rpm) in a thermomixer (Eppendorf) at 23°C. Subsequently, varying amounts of volume (5–100 μl) were quickly removed from the reaction mixture, adjusted to 100 μl with water and added to 100 μl of a solution containing 2 $\times$ phosphate-buffered saline (PBS) (40 mM sodium phosphate pH 7.5, 300 mM sodium chloride), 10 μg HRP and 200 μg OPD. The reaction was incubated for 20 min at room temperature and stopped by adding 100 μl of 9 M sulfuric acid. The absorbance of 200 μl of the reaction mixture was measured at 492 nm on a Synergy H1 plate reader (Biotek). One unit (U) of GOx produces 1.0 μmol of hydrogen peroxide per min at pH 5.1. The amount of hydrogen peroxide was determined through the reaction with OPD catalyzed by HRP. The resulting product, 2,3-diaminophenazine (DAP) was quantified through its molar extinction coefficient of 38 000 $\text{M}^{-1} \text{cm}^{-1}$ at 492 nm.²⁷ One mol of DAP is equivalent to 2 mol of glucose.

3.5.2 For immobilized GOx-PA

Setup and incubation of the first assay step are identical as described for GOx-PA-containing solutions earlier. The reaction mixture was then filtered through a multiwell filter plate (0.2 μm pore size, Pall Corporation) by applying a vacuum. Varying amounts of the filtrate volume (2–10 μl) were adjusted to 100 μl with water, and the amount of hydrogen peroxide was determined as described earlier.

3.6 Determining the GOx-PA-binding capacity of (bio)silica materials

(Bio)silica materials (5 mg) were washed with 0.05 M Tris–hydrochloric acid pH 7.0 by three centrifugation and resuspension cycles. The washed (bio)silica materials were resuspended in aliquots of 5 mg in 0.05 M Tris–hydrochloric acid pH 7.0 and incubated with varying amounts of GOx-PA (10–300 μg) in a total volume of 400 μl . The suspensions were shaken at 1400 rpm in a thermomixer (Eppendorf) for 2 h at 23°C. Subsequently, the suspensions were centrifuged (2 min, 16 000g) and the GOx activity in each supernatant was determined (see Section 3.5), and the mass of GOx-PA in the supernatant was calculated using the previously determined specific activity of GOx-PA ($0.053 \pm 0.006 \text{ U}/\mu\text{g}$).^{19,28} For samples containing GOx-PA in the supernatant, the mass of bound GOx-PA was calculated by subtraction of the calculated mass of GOx-PA in the supernatant from the total mass of GOx-PA that was applied to the silica sample.

3.7 Measuring catalysis under flow-through and suspension conditions

3.7.1 Flow-through catalysis

The bottom filter (10 μm pore size) of a 2.5 ml column was overlaid with a nylon membrane (0.2 μm pore size) and then

packed with hydrated and GOx-PA-loaded (bio)silica materials in 100 mM NaAc pH 5.1 to a final volume of 300 μl (*S. turris*: 15 mg; *T. pseudonana*: 14 mg; *E. zodiacus*: 11 mg; MCF: 36 mg; silica beads: 90 mg). The top of the drained hydrated gel bed was covered with a second filter (10 μm pore size) to protect the gel bed. At a flow rate of 4.5 ml/min, a 50 ml solution of 10 mM D-glucose in NaAc buffer pH 5.1 was circulated through the packed column. Periodically, 50 μl aliquots were withdrawn from the circulating solution.

3.7.2 Suspension catalysis

GOx-PA-loaded (bio)silica microparticles (30 mg dry weight) were resuspended in 50 ml 100 mM NaAc pH 5.1 containing 10 mM d-glucose, and the suspension was constantly stirred with a magnetic bar. Periodically, 50 μl aliquots were withdrawn from the suspension and filtered through a multiwell filter plate (0.2 μm pore size, Pall Corporation) by applying a vacuum.

The glucose concentrations in the samples were determined by a coupled GOx-HRP enzyme assay. For a typical assay, varying amounts of the samples (10–40 μl) were mixed with water to a final volume of 200 μl , and 20 μl 10 $\times$ PBS buffer (0.2 M sodium phosphate pH 7.5, 1.5 M sodium chloride) and 5 μl 4 g/l HRP were added. The reaction was started by adding 100 μl 2 g/l OPD, incubated for 20 min at room temperature and stopped by adding 100 μl of 9 M sulfuric acid. The absorbance of 200 μl of the reaction mixture was measured at 492 nm on a Synergy H1 plate reader (Biotek). The GOx-PA activity was calculated as described in Section 3.5.

3.8 Porosity measurements

Nitrogen physisorption isotherms were measured at -196°C on a Quadrasorb apparatus (Quantachrome Instruments). SSAs were calculated using the multipoint Brunauer–Emmett–Teller equation ($p/p_0 = 0.05\text{--}0.2$). Total pore volumes were calculated at $p/p_0 = 0.99$. PSDs were calculated using the NLDFT method for nitrogen at -196°C on silica with cylindrical pore geometry.

3.9 Electron microscopy

SEM analyses were performed on a Zeiss DSM 982 Gemini field-emission scanning electron microscope. Droplets of water suspensions of the samples were put on sample holders equipped with carbon tabs.

4. Conclusion

In the present study, the performance of diatom basillia as enzyme support material in direct comparison to synthetic silicas has been systematically investigated for the first time. This study revealed that diatom biosilica can be loaded with enzyme amounts that are comparable to or even better than the enzyme loading of MCF, a high-surface-area synthetic mesoporous silica material that is commonly used for enzyme immobilization. Most importantly, the authors demonstrate that in the absence of overcrowding effects, the catalytic activity of each enzyme molecule is dependent on the type of (bio)silica material to which

it is immobilized. Remarkably, the enzyme activity differences do not only exist between diatom biosilica with synthetic silica, but also among the three biosilica materials. It can be excluded that the activity differences were caused by differences in biosilica composition, because the species-specific organic components of the native biosilica had been completely removed by heat treatment. Therefore, it appears evident that the GOx-PA activity was influenced by the structure of the biosilica. When comparing the distributions of mesopores sizes, it is striking that *E. zodiacus* biosilica, which supports the lowest GOx-PA activity, contains a lower percentage of large mesopores (>20 nm) and correspondingly a higher percentage of small mesopores (≤15 nm) compared to the other two biosilica materials. In contrast, *S. turris* and *T. pseudonana* biosilicas, which both support very similar high GOx-PA activities, contain very similar percentages of small and large mesopores. Therefore, the authors hypothesize that mass transport through large mesopores is an important factor for the activity of enzymes that are immobilized on diatom biosilica. For the synthetic silica materials, the correlation between mesoporosity and support of enzyme activity is less evident. GOx-PA immobilized on silica beads exhibit only about half the specific activity as GOx-PA on MCF silica, yet for both materials, about 60% of the porosity volume is constituted by large mesopores (>20 nm). Presumably, the small mesopores in the void-window-type structure significantly slow down the mass transport through the MCF material, whereas on the silica beads, the large mesopores may be readily accessible on the external surface. Nevertheless, there is a clear difference regarding the pore volume contribution of <20 nm mesopores, which is dominated by mid-sized mesopores (10–20 nm) in MCF and small mesopores (2–10 nm) in silica beads. The correlation between a low volume fraction of small mesopores and the support of high enzyme activity in synthetic silica materials is consistent with the porosity–activity correlation in diatom biosilica-immobilized GOx-PA.

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