Genetically Programmed Regioselective Immobilization of Enzymes in Biosilica Microparticles

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Diatoms are single-celled microalgae that produce a large variety of hierarchically porous, silica-based microparticles as cell wall material. The presence of genetically encoded silica nanopatterns endows the biosilica with favorable properties for a wide range of applications including catalysis, chemical sensing, photonics, and drug delivery. Enhancing the performance of diatom biosilica requires i) a better understanding of the structure–property relationship in this material, and ii) methods that enable the manipulation of the biosilica structure and properties in a targeted manner. Here, genetic engineering of the diatom *Thalassiosira pseudonana* is employed to immobilize enzymes (glucose oxidase and horseradish peroxidase) into structurally distinct regions of the biosilica, which are termed valves and girdle bands. Remarkably, glucose oxidase in girdle bands exhibits >3-fold higher catalytic activities compared to its location in valves. It is demonstrated through enzyme accessibility studies, protein engineering, and genetic engineering of biosilica morphology that the divergent enzyme activities are caused by the differences in the inherent silica nanopatterns of valves and girdle bands. This work highlights the importance of silica nanoscale architecture for the activity of immobilized enzymes and provides unprecedented tools for the biotechnological production of silica microparticles with tailored catalytic activities and anisotropic functionalities.

1. Introduction

A large variety of organisms in all domains of life are able to produce inorganic materials (i.e., biominerals) that exhibit unique structures and superior properties compared to geologically or synthetically produced minerals.[1–3] Recognition of the biominerals’ extraordinary features has spurred intensive research in the areas of Biomimetic Materials and Bionanotechnology. These research areas aim to utilize biological design principles or biological machineries to achieve unprecedented control over the 3D nano- to microscale architectures of inorganic materials. It is envisioned that such achievements will enable the environmentally benign production of novel functional materials with advanced properties. Diatoms, a large group of single-celled microalgae, have been a main resource for developing novel, biominaler-based functional materials.[4,5] Diatoms produce silica-based cell walls that have species-specific architectures with hierarchically porous nano- and micropatterns. The diatom biosilica microparticles are amorphous, have open 3D structures with relatively thin walls (typically between 0.1 and 1 µm), and yet are extremely robust toward mechanical stresses.[6,7] Due to the presence of orderly arranged pores, diatom biosilica exhibits optical and photonic properties that can be utilized for optical filters, light waveguides, and lenses.[8–10] The biosilica can be further functionalized by the controlled deposition of inorganic materials, or the attachment of organic molecules including DNA and proteins. The resulting materials exhibit enhanced performance in chemical sensing, chemoselective adsorption, drug delivery, and catalysis.[11–22]

Diatom biosilica has proven to be a highly suitable support material for the immobilization of catalysts. The oxidation of glucose by gold nanoparticles (AuNPs) and by the enzyme glucose oxidase (GOx) immobilized on biosilica from three different diatom species (*Thalassiosira pseudonana*, *Stephanopyxis turris*, and *Eucampia zodiacus*) was similar or even better than those of AuNPs and GOx immobilized on synthetic silicas (mesocellular foam, silica nanoparticles).[19,21] It was speculated that the open 3D architecture of diatom biosilica with thin silica walls and a relatively high fraction of >20 nm sized mesopores enabled efficient reactant diffusion through the material and thus high activity of the immobilized catalysts.[21,23] However, the morphologies of diatom biosilicas that were previously used for enzyme immobilization studies were quite different from each other, and within each species different regions of biosilica nano- and micromorphology exist. Therefore, it has been
extremely difficult to identify the structural features of biosilica that are particularly beneficial for the activities of immobilized catalysts. To further investigate this question, it would be necessary to immobilize the catalysts in selected regions of the biosilica rather than distributing it homogenously over the entire microparticle surface. Such regioselective immobilization of molecules or nanoparticles is difficult to achieve in vitro, because conventional chemical modification methods are different to biosilica nanomorphology. The only prior example of regioselective in vitro modification of diatom biosilica involved the arrangement of arrays of specific types of biosilica fragments (so-called valves) from the diatom S. turriss using a micromanipulator.[24] Due to a serendipitous wetting behavior of the S. turriss valves, a characteristic honeycomb pattern in each valve could be selectively functionalized with CdTe nanoparticles.[24] The catalytic activity of the regioselectively immobilized CdTe nanoparticles has not been reported, and selective functionalization of other parts of the biosilica was not achieved with this method.

As an alternative to in vitro methods for enzyme immobilization, we have previously developed a genetic engineering-based method (termed living diatom silica immobilization, LiDSI), which allows for the generation of T. pseudonana strains that incorporate a desired enzyme into the biosilica during cell wall biosynthesis.[25,26] This method relies on introducing into the T. pseudonana genome a recombinant gene that encodes a fusion of the desired enzyme with the cell wall protein silaffin-3 (or a fragment thereof). So far, fusion proteins immobilized through LiDSI were located in all parts of the cell wall and regioselective immobilization had not been achieved.[18,25,26] However, we reasoned that it should be possible to modify the LiDSI method for regioselective immobilization of enzymes by genetically fusing them to T. pseudonana cell wall proteins that are naturally confined to specific regions of the biosilica.

As seemingly suitable candidate fusion partners we identified i) silaffin-1 (Sil1), which is located only in the valve region, and ii) members of the cingulin protein family (CinY1, -2, -3, -4, and CinW1, -2, -3) whose locations are restricted to the girdle band region (Figure 1). [27-29] Provided that fusion with the enzyme molecule does not interfere with intracellular targeting of the cell wall protein, the fusion proteins would be expected to be located exclusively in the valves (for Sil1-enzyme fusions) or the girdle bands (for cingulin-enzyme fusions). Valves represent the top and bottom plates of the cylindrically shaped T. pseudonana biosilica cell wall, while girdle bands are overlapping rings that connect both valves in a gapless fashion. Both, the valves and the girdle bands, are perforated by numerous ≈20 nm sized (diameter) pores. On the size scale of tens to hundreds of nanometers, the surfaces of girdle bands appears rather smooth, whereas the surface of each valve is highly corrugated due the presence of patterns of ridges (termed costae and areola walls) and tube-like structures (termed fultopor-tulae; Figure 1).[30]

Based on these considerations, we aimed in the present work at establishing a modified LiDSI procedure that enables the regioselective incorporation of enzymes into T. pseudonana biosilica. Such achievement would then enable us to determine whether the specific catalytic activity of an enzyme (i.e., reaction rate per enzyme molecule) is influenced by the nanomorphology of the surrounding biosilica.

2. Results and Discussion

2.1. Regioselective Immobilization of Enzymes

As model enzymes for this study GOx and horseradish peroxidase (HRP) were selected, because both enzymes can be functionally expressed in T. pseudonana and can also be used for establishing a coupled enzymatic reaction.[26,31] To enable localization as well as quantification of the enzyme molecules, the GOx gene was fused to the yellow fluorescent protein (YFP) gene, yielding the fusion gene GOxYFP. The HRP gene was fused with the mTurquoise2 (mT2) gene resulting in the fusion gene HRPmT2. To attempt regioselective incorporation of the enzymes into biosilica, Sil1 and CinY2 were explored as targeting tags for the fusion proteins. Previously, it was shown through GFP tagging in T. pseudonana that Sil1 is exclusively located in the valve, whereas the CinY2 is restricted to girdle bands.[27-29] Therefore, four enzyme fusion genes were constructed that were intended to direct incorporation of GOx and HRP into the valve region (v-GOxYFP, v-HRPmT2) or the girdle band region (g-GOxYFP, g-HRPmT2) of the biosilica (Figure 2). Each fusion gene was introduced into the T. pseudonana genome by a previously described method.[12] Transfomant clones were then screened by fluorescence microscopy for the presence of YFP or mT2 fusion proteins.

Live cells as well as isolated biosilica from positive clones were imaged by confocal fluorescence microscopy to determine the location of each fusion protein (Figure 2). As expected, cells expressing v-GOxYFP or v-HRPmT2 exhibited YFP or mT2 fluorescence, respectively, in the valve region of the biosilica (Figure 2A,C). The selective presence of v-GOxYFP within the valve was clearly visible also in live cells (Figure 2A). In contrast, valve localization of v-HRPmT2 was barely discernible in live cells, because they contained numerous spherical structures with strong mT2 fluorescence throughout the cell and particularly close to the cell periphery (Figure 2C). Only after removal of the intracellular contents through extraction with detergent (i.e., biosilica isolation) was it possible to visualize the relatively weak mT2 fluorescence in the valve (Figure 2C). The same situation was observed in g-HRPmT2 expressing cells. They contained strongly mT2 fluorescent particles throughout the cell (Figure 2D), and girdle band located mT2 was visible only in isolated biosilica (Figure 2D). In contrast, YFP fluorescence was easily discernible both in live cells and isolated biosilica from clones expressing g-GOxYFP (Figure 2B).

From this analysis, we draw the following conclusions. First, regioselective immobilization of GOx and HRP fusion proteins both into the valve biosilica and the girdle band biosilica was achieved, because no mislocated YFP and mT2 fluorescence was observed in the isolated biosilica materials. Second, the GOx and HRP fusion proteins were tightly incorporated into the biosilica as they remained associated after treatment with 1% SDS at 55 °C (i.e., conditions for biosilica isolation). Third, the numerous cyan-fluorescent intracellular particles may represent HRP-mT2 loaded, acidic transport vesicles on their way to the silica deposition vesicle (SDV). Such acidic vesicles would be visible only in cells expressing mT2 fusion proteins rather than YFP fusion proteins, because of the much higher quantum yield of mT2 at low pH compared to YFP.[33,34]
2.2. Specific Activities of the Regioselectively Immobilized Enzymes

Enzyme assays confirmed that the GOx and HRP fusion proteins were catalytically active in the biosilica materials that were isolated from the four different transformant strains (Figure 3). Biosilica from *T. pseudonana* wild type (WT) cells did not exhibit GOx or HRP activity. The following reactions were catalyzed by the GOx and HRP fusion proteins, respectively

GOx catalysis: $\text{D-glucose} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{D-gluconic acid} + \text{H}_2\text{O}_2$ (1)

HRP catalysis: $3\text{H}_2\text{O}_2 + 2\text{pyrogallol} \rightarrow \text{purpuragallin} + \text{CO}_2 + 5\text{H}_2\text{O}$ (2)

To allow for a comparison of the enzyme activities in their different biosilica environments, their specific activities had to be determined. This required quantifying the abundance of the enzymes in the biosilica, which we intended to achieve via measuring the fluorescence intensities of their YFP and mT2 tags. To synthesize fluorescent biosilica standards, we produced in *Escherichia coli* and purified via nickel-nitrilotriacetic acid (Ni-NTA) chromatography the recombinant fluorescent proteins $\text{H}_6\text{-YFP-R}_6$ and $\text{H}_6\text{-mT2-R}_6$. Both proteins carried a hexahistidine-tag ($\text{H}_6$) on the N-terminus and six arginine residues ($\text{R}_6$) on the C-terminus. The $\text{H}_6$-tag was used for the purification of the proteins, and the $\text{R}_6$-tag is silica binding thus enabling tight immobilization of the proteins on diatom biosilica in
Figure 2. Localization of the enzyme fusion proteins in live *T. pseudonana* cells and isolated biosilica using confocal fluorescence microscopy. For each transformant strain, different individual cells and biosilica microparticles are shown in girdle view and in valve view. Schematics of the constructs encoding fusion proteins A) v-GOx<sup>YFP</sup>, B) g-GOx<sup>YFP</sup>, C) v-HRP<sup>mT2</sup>, and D) g-HRP<sup>mT2</sup> are shown above the corresponding confocal fluorescence microscopy images (z-projections). Yellow, cyan, and red colors indicate the YFP fluorescence, mT2 fluorescence, and chloroplast autofluorescence, respectively. The fluorescence images of live cells show overlays of the YFP or mT2 fluorescence with chloroplast autofluorescence. The corresponding bright field images of live cells and isolated biosilica microparticles are shown next to their fluorescence images. Scale bars: 2 μm.

Figure 3. Specific enzyme activities of the regioselectively immobilized A) GOx or B) HRP fusion proteins isolated from two independent transformant clones each (C1, C2). An activity of 1.0 U corresponds to 1.0 μmol purpurogallin produced per minute. Each bar represents the average value from the analyses of three independent experiments, and the standard deviation is indicated.
vitro\[35\]. The concentrations of $H_6$-YFP-R$_6$ or $H_6$-mT2-R$_6$ were quantified in solution using the molar extinction coefficients for YFP and mT2, and subsequently varying amounts of the proteins were bound to biosilica from $T. pseudonana$ wild type cells. The fluorescence intensity of YFP or mT2 bearing biosilica increased linearly with the amount of fluorescent protein that was bound (Figure S1, Supporting Information). Therefore, these materials were used as standards to determine the quantity of the regioselectively immobilized enzyme fusion proteins in genetically engineered biosilica, which allowed for the determination of the specific catalytic activities of the regioselectively immobilized enzyme fusion proteins in the biosilica. This revealed that enzyme molecules located in the valve region exhibited drastically lower catalytic activities than those located in the girdle band region (Figure 3). Girdle band located GOx or HRP, respectively, were about 3.1-fold and 2.3-fold higher in catalytic activity than the valve located enzymes. These activity differences exceeded those previously observed for GOx that was immobilized in vitro on biosilica from three different diatom species ($T. pseudonana$, $S. turris$, and $E. zodiacus$), which exhibited between 1.3- and 1.9-fold differences in specific activities.\[21\]

We reasoned that one of the following factors or a combination of these may be responsible for the consistently observed differences in the specific activities of regioselectively localized GOx and HRP:

i) The fluorescence intensities of YFP and mT2, and thus the determination of enzyme concentration, may be affected by the cell wall protein fusion partner and/or by the biosilica location (enzyme quantification).

ii) Material transport to the enzymes may depend on biosilica location (enzyme accessibility).

iii) The biosilica targeting tags may directly affect enzyme folding and stability (targeting tag).

iv) The nanoscale structure of the silica that surrounds the enzyme may affect its activity (silica morphology).

In the following, we have investigated which of the four factors may contribute to the observed differences between the valve and girdle band localized enzymes.

2.2.1. Enzyme Quantification

Determination of the specific activities of the enzyme fusion proteins is based on fluorescence intensity measurements (see above). The underlying assumption is that the fluorescence properties of the YFP and mT2 are not influenced by their location in the biosilica. Otherwise, the higher specific activities of girdle band located GOx and HRP could simply be due to an underestimation of the amount of the enzyme fusion proteins in the girdle band. To investigate this, we have compared the fluorescence properties of YFP and mT2 located in the valves with those in girdle bands in the isolated biosilica of $T. pseudonana$. The fluorescence intensity of a fluorophore is given by the quantum yield (Equation (3))\[36\]

$$\phi = \frac{\text{number of emitted photons}}{\text{number of adsorbed photon}}$$  \hspace{1cm} (3)

The quantum yield is linearly correlated with the fluorescence lifetime $\tau$ through Equation (4) ($k_i$ is the radiative rate constant)\[37,38\]

$$\phi = k_i \cdot \tau$$  \hspace{1cm} (4)

Fluorescence lifetime imaging microscopy (FLIM) was performed for YFP and mT2 in the isolated biosilica microparticles from one clone each of the four transformants described above (Figure S2, Supporting Information). The FLIM data for each sample exhibited a monoeponential decay curve from which the average fluorescence lifetime $\tau$ of the fluorophores was determined (Table 1). The analysis revealed within error identical $\tau$ values for YFP or mT2 located in girdle bands and valves. This result demonstrated that the observed activity differences between valve and girdle band located enzymes are not due to regiospecific differences in fluorescence intensity.

2.2.2. Enzyme Accessibility

The rate of substrate transport through the solid support material is generally regarded as an important factor for the catalytic activity of an immobilized enzyme.\[39,40\] Diatom biosilica is an inorganic-organic hybrid material ($\approx$90% inorganic silica by weight; $\approx$10% organic biomolecules), and the organic components may be more or less buried inside the silica or covered by other organic biomolecules.\[41–43\] Therefore, the material transport to a biosilica immobilized enzyme can be influenced by the degree of enzyme embedment inside the silica, and by the degree of coverage of the enzyme by layers of organic biomolecules.\[29,40,44,45\] It is to be expected that the rate of material transport to the enzyme, and thus its catalytic activity, will be the lower the more the enzyme is covered by silica and/or other organic biomolecules. Therefore, we investigated by accessibility assays whether potential differences in surface exposure contributes significantly to the activity differences between valve located and girdle band located enzymes.

In the first assay, the resistance of the biosilica embedded fusion proteins toward pronase (i.e., a mixture of proteases) was tested. After 15 min incubation, the activities of valve located GOx and HRP, respectively, were reduced down to 13 $\pm$ 3% and 24 $\pm$ 3% compared to their activities in the absence of pronase. The activities of girdle band located GOx and HRP, respectively, were reduced much less yielding 47 $\pm$ 4% and 60 $\pm$ 3% relative to the pronase free controls. This result seems to indicate that the HRP and GOx fusion proteins in

| Strain         | $\tau$ [ns] |
|----------------|-------------|
| v-GOxYFP       | 2.27 ± 0.17 |
| g-GOxYFP       | 2.09 ± 0.12 |
| v-HRPmT2       | 2.46 ± 0.15 |
| g-HRPmT2       | 2.49 ± 0.29 |

Table 1. Fluorescence lifetimes $\tau$ of valve or girdle band located YFP and mT2 fluorophores determined through FLIM. The values for $\tau$ and the standard errors were calculated using the SPCM64 software and represent the average value from at least 10 biosilica microparticles.

$$\phi = k_i \cdot \tau$$
the valves were substantially more exposed on the biosilica surface than the girdle band located enzymes. However, it might be possible that the result reflects differences in the kinetics of proteolytic cleavage of the biosilica targeting tags Sil1 and CinY2 rather than access of the protease to the fusion protein. Therefore, an antibody-based accessibility assay was performed using a previously established method. First, indirect immunolabeling of v-GOxYFP or g-GOxYFP bearing biosilica was performed with anti-GFP (also binds to YFP) as a primary antibody and an AlexaFluor647 (AF647) labeled secondary antibody (Figure S3, Supporting Information). Subsequently, the AF647 fluorescence intensity (excitation: 647 nm, emission: 525/45 nm) were quantified for both labeled biosilica materials using epifluorescence microscopy as previously described. The YFP fluorescence intensity \( I_{YFP} \) is a measure for the total amount of enzyme fusion protein that is present in the biosilica, whereas the AF647 fluorescence intensity \( I_{AF647} \) is a measure for the amount of enzyme fusion protein that is accessible for the anti-GFP antibody. Therefore, the higher the intensity ratio \( I_{AF647}/I_{YFP} \) the more enzyme fusion protein molecules are exposed on the biosilica surface. In v-GOxYFP bearing biosilica the ratio \( I_{AF647}/I_{YFP} \) was 2.4 ± 0.5 and 1.2 ± 0.35 in g-GOxYFP bearing biosilica. This result is consistent with the pronase accessibility studies above, thus indicating that the enzyme fusion proteins in the valve are more exposed on the biosilica surface than the girdle band located enzyme fusion proteins. Therefore, the rate of substrate transport can be ruled out as the main factor for determining the activity of biosilica immobilized enzymes, because it would favor the much more exposed valve located enzymes, which is not the case.

2.2.3. Targeting Tag

It is conceivable that the silaffin and cingulin parts of the fusion proteins may exert a stabilizing or destabilizing effect on the native conformation of GOx or HRP, thereby enhancing or decreasing their catalytic activities. YFP and mT2 cannot be a significant factor in this regard, because the activity differences between girdle band located and valve located enzymes were observed for enzymes tagged with either fluorescent protein (see Figure 3). Furthermore, being GFP derivatives, the polypeptide sequences and 3D structures of YFP and mT2 are almost identical. In contrast, the polypeptides used for regioselective biosilica targeting, Sil1 and CinY2, are significantly different from each other. To investigate whether the biosilica targeting tags are main determinants for the activity differences in valve and girdle band located enzymes, we attempted to generate regioselectively immobilized GOx and HRP fusion proteins using other targeting tags. Previous work has shown that the expression of the genes Sil3-GFP and SiMat2-GFP under control their own promoters \( P_{Sil3}, P_{SiMat2} \) resulted in regioselective incorporation of the corresponding fusion proteins into the valve and girdle bands, respectively (N. Poulsen, S. Görlich, and N. Kröger, unpublished data). Sil3 and SiMat2 have two features in common with Sil1 and CinY2: i) they are predicted to be intrinsically disordered proteins (i.e., devoid of \( \alpha \) helices and \( \beta \) sheets), and ii) they each contain multiple KxxK motifs, which are essential for silica targeting. Despite these shared features, the sequences of the four proteins exhibit only rather distant relationships ranging from 17% to 38% sequence identity in pairwise comparisons.

To attempt regioselective biosilica targeting of GOx using Sil3 and SiMat2 as targeting tags, the fusion genes \( v2-GOx_{YFP} \) and \( g2-GOx_{YFP} \) were constructed (Figure 4) and placed under control of the \( P_{Sil3} \) (for \( v2-GOx_{YFP} \)) or \( P_{SiMat2} \) (for \( g2-GOx_{YFP} \)) promoters. After transformation of \( T. pseudonana \) with the expression plasmids, cells of YFP fluorescent clones were imaged by confocal fluorescence microscopy. As expected, the \( v2-GOx_{YFP} \) fusion protein was specifically located in the valve biosilica, and the \( g2-GOx_{YFP} \) fusion protein was present only in the girdle band biosilica (Figure 4). The specific GOx activity of \( g2-GOx_{YFP} \) bearing biosilica was 2.7-fold higher than that of biosilica containing \( v2-GOx_{YFP} \) (Figure 5). This value closely

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**Figure 4.** Localizations of the GOx-bearing fusion proteins with the targeting tags A) Sil3 and B) SiMat2 using confocal fluorescence microscopy. The confocal fluorescence images of live cells and isolated biosilica microparticles show z-projections. For each transformant strain, different individual cells and biosilica microparticles are presented in girdle view and in valve view. Schematics of the constructs encoding fusion proteins \( v2-GOx_{YFP} \) and \( g2-GOx_{YFP} \) are shown above the corresponding confocal fluorescence microscopy images. The fluorescence images of live cells show overlays of the YFP fluorescence (yellow) with chloroplast autofluorescence (red). The corresponding bright field images of live cells and isolated biosilica microparticles are shown next to their fluorescence images. Scale bars: 2 \( \mu m \).
Figure 5. Specific activities of regioselectively immobilized GOx fusion proteins v2-GOxYFP and g2-GOxYFP from two independent clones each (C1, C2). An activity of 1.0 U corresponds to 1.0 µmol purpurogallin produced per minute. Each bar represents the average value from the analyses of three independent experiments, and the standard deviation is indicated.

matched the activity ratio of 3.1 between biosilica immobilized g-GOxYFP and v-GOxYFP, which contained different biosilica targeting tags (see Figures 2 and 3). This result indicated that the drastic activity differences between valve and girdle band located enzymes are not caused by a direct influence of the silica targeting tags on enzyme activity.

2.2.4. Silica Morphology

It has previously been shown that nanoscale features of the silica (e.g., porosity and surface curvature) can influence the conformational flexibility and orientation of the enzyme molecules on the surface.\textsuperscript{[48–51]} We assume that such influences would affect the catalytic activity of the immobilized enzyme, and thus might be responsible for the activity differences between valve and girdle band located enzymes. To investigate this, we attempted to regioselectively immobilize GOx in the biosilica of the diatom *Cyclotella cryptica*. This species is closely related to *T. pseudonana*, yet in particular the valve exhibits distinct structural features (Figure 6A–C).\textsuperscript{[52]} Foremost these are the so-called wide costae that emanate from a central ring structure (Figure 6C). Wide costae radially spread to the rim of the valve, and always terminate at a fultoportula pore (Figure 6C). The central area of the *C. cryptica* valve displays a field of rather uniform pores (i.e., cribrum pores) that is interrupted by the central ring structure and the wide costae (Figure 6C). Wide costa, the central ring structure and the central uniform pore field are absent from *T. pseudonana* (see Figure 1).

Due to the close phylogenetic relationship between *C. cryptica* and *T. pseudonana*, we speculated that Sil1 from *T. pseudonana* may function also in *C. cryptica* as a tag for targeting enzymes into the valve. Therefore, the fusion gene v-GOxYFP (see above) was incorporated into a *C. cryptica* expression plasmid, and introduced into *C. cryptica* by particle bombardment. YFP expressing *C. cryptica* cells were imaged by confocal fluorescence microscopy, revealing, as anticipated, that the v-GOxYFP fusion protein was incorporated exclusively into the valve biosilica. Similar as in *T. pseudonana* (see Figure 2A), the v-GOxYFP fusion protein in *C. cryptica* was primarily located around the fultoportule near the rim of the valve (Figure 6D). This region is structurally different in *T. pseudonana* and *C. cryptica* (compare Figure 1D and Figure 6C). In *C. cryptica* the fultoportulae are surrounded by wide costae, which are largely devoid of pores, whereas *T. pseudonana* fultoportulae are surrounded by a hierarchical arrangement of areolae and cribrum pores.

The average specific activity of valve located GOx in biosilica from three independent *C. cryptica* transformants was 9.5 ± 0.9 U mg\(^{-1}\) (8.7 ± 0.8, 9.1 ± 0.6, and 10.9 ± 1.3 U mg\(^{-1}\) and thus two times lower than the average specific activity of GOx in *T. pseudonana* transformants (19.8 ± 0.8 U mg\(^{-1}\); see Figure 3A). The lifetime of the YFP fusion protein in *C. cryptica* biosilica (2.03 ± 0.21 ns) was, within error, identical to its lifetime in *T. pseudonana* biosilica (2.27 ± 0.17 ns). After pronase treatment of v-GOxYFP bearing *C. cryptica* biosilica for 15 min, 17 ± 0.2% of GOx activity remained, which closely matched the value obtained for *T. pseudonana* biosilica (13 ± 3%). These data demonstrated that the differences in specific catalytic activities of GOx in biosilica from the two diatom species were not caused by errors in determination of the GOx concentration or in differences of GOx accessibility.

The above result supports the hypothesis that the morphological differences between the biosilica materials from *T. pseudonana* and *C. cryptica* are responsible for the observed differences in the catalytic activities of the immobilized GOx molecules. However, although the identical Sil3-GOxYFP fusion protein sequence was produced in both diatom species, it may be exposed to differences in two additional factors other than silica nanomorphology. First, the total composition of valve-associated proteins is currently unknown, but is likely to differ in both diatom species. Presently, we cannot rule out the possibility that *C. cryptica* valve proteins in immediate vicinity to the GOx fusion protein might exert an inhibitory effect thus resulting in a lower specific activity compared to GOx in *T. pseudonana* valves. Second, Sil1, which was used as valve targeting tag here, is known to become post-translationally modified in *T. pseudonana* (e.g., phosphorylation, glycosylation, and polyamine modification).\textsuperscript{[47]} It is conceivable that Sil1 becomes endowed with different posttranslational modifications in *C. cryptica*, which might negatively affect GOx activity. To rule out such potential influences, we used a recently generated morphological mutant of *T. pseudonana* that was generated through knocking out the *SinI* gene. The mutant (Sin1KO1) exhibited a different silica structure than the wild type only in the valve region, which had a drastically reduced number of areolae pores and a strongly reduced z height of the costae.\textsuperscript{[53]} The silica structure of the girdle bands in Sin1KO1 appeared to be unchanged.\textsuperscript{[53]} Here, we generated transformant clones that expressed the v2-GOxYFP or
WT genetic background. This revealed a $55.6 \pm 0.2\%$ increase in the specific activity for GOx that was immobilized in valves from the Sin1KO1 mutant compared to GOx in valves from the control with wild type morphology ($36.9 \pm 3.0 \text{ U mg}^{-1}$ vs $23.7 \pm 1.9 \text{ U mg}^{-1}$; Figure 8). For the girdle band located GOx fusion proteins, the specific activity in the biosilica isolated from the Sin1KO1 mutant was relatively modestly reduced by $20.2 \pm 0.1\%$ compared to the biosilica from the control with wild type morphology ($58.6 \pm 2.2 \text{ U mg}^{-1}$ vs $73.6 \pm 3.1 \text{ U mg}^{-1}$; Figure 8).

In wild type *T. pseudonana* the Sin1 protein is located in all parts of the biosilica, and thus in the Sin1 knockout mutant this protein is completely lacking both in valves and girdle bands. The lack of Sin1 in valves cannot be responsible for the increase of specific activity of valve-located GOx, because Sin1 absence in girdle bands coincides with a slight decrease in specific activity of girdle band-located GOx. If Sin1 affects GOx activity at all (which is currently unknown), it can either have a stimulating or an inhibiting effect but not both. As only the Sin1 is gene affected in the Sin1KO1 mutant, it is reasonable to assume that the composition of all other biomolecules is identical in the biosilicas of Sin1KO1 mutants and wild type. This leaves the striking difference in silica nanomorphology as the most likely explanation for the substantially enhanced specific catalytic activity of the v2-GOxYFP fusion proteins that are located in the valves of the Sin1KO1 mutant.

### 2.3. Immobilization of Two Enzymes Catalyzing a Coupled Reaction

To further develop the tools for modification of biosilica, we investigated the possibility of simultaneously and regioselectively immobilizing GOx and HRP within the same diatom biosilica microparticle. Together the two enzymes catalyze the following reaction

$$3\text{D-glucose} + 3\text{O}_2 + 2\text{pyrogallol} \rightarrow 3\text{D-gluconic acid} + 2\text{H}_2\text{O} + \text{purpurogallin} + \text{CO}_2$$

(5)

Therefore, we attempted to introduce into a v-GOxYFP expressing *T. pseudonana* transformant clone also the g-HRPmT2 fusion gene. Cells of the resulting double transformant were expected to produce and target YFP-tagged GOx into the valves and mT2-tagged HRP into the girdle bands. For introduction of the second fusion gene a second antibiotic selection marker, ble, was used, which confers resistance to the antibiotic zeocin. *T. pseudonana* clones that were resistant to nourseothricin and zeocin were screened by fluorescence microscopy for the presence of YFP and mT2. Biosilica isolated from cells of the double transformant contained the v-GOxYFP and g-HRPmT2 fusion proteins, respectively, in the desired valve and girdle band location (Figure 9). This result demonstrated that valve and girdle band targeting of artificial fusion proteins within the same cell do not interfere with each. Encouraged by this observation, we attempted to generate additional three double transformants that produce biosilica with the remaining combinations of valve and girdle band targeted GOx and HRP fusion proteins. Starting with a v-GOxYFP or g-GOxYFP recipient...
strain the transformation and selection strategy described above yielded cells with the desired combinations of correctly targeted GOx and HRP fusion proteins (Figure 9).

We then tested two independent clones (C1, C2) from each of the four different biosilica materials for their capabilities to catalyze the coupled enzymatic reaction. Biosilica with both enzymes immobilized in girdle bands (g-GOxYFP/g-HRPmT2) exhibited the highest specific activity, whereas the lowest catalytic activity was obtained with the biosilica containing valve-located GOx and HRP fusion proteins (v-GOxYFP/v-HRPmT2; Figure 10). The two biosilica materials containing the two enzymes in different regions possessed intermediate levels of specific activity, which were essentially identical to each other (Figure 10). The activity of the girdle band located GOx–HRP enzyme pair was 2.6-fold higher than of the valve located enzyme pair, which closely matched the ratios observed for the individual enzymes (see Figure 3).

These data demonstrated that proximity of GOx and HRP molecules within the same region of the biosilica was not important for promoting the catalytic activity of the enzyme pair. Instead, the catalytic activity of the individual enzyme molecules is limiting for the speed of the coupled enzymatic reaction. This suggests that substrate channeling between proximal HRP and GOx molecules within the same biosilica microparticle is either not occurring or at least not a rate-limiting factor.

Figure 7. Confocal fluorescence microscopy images (z-projections) of transformant strains expressing A) v2-GOxYFP and B) g-GOxYFP in Sin1KO1 mutants of T. pseudonana. Yellow and red colors indicate YFP fluorescence and chloroplast autofluorescence, respectively. The fluorescence images of live cells show overlays of the YFP fluorescence with chloroplast autofluorescence. The corresponding bright field images of live cells and isolated biosilica microparticles are shown next to their fluorescence images. C,D) SEM images of biosilica valves from transformants that produce the v2-GOxYFP fusion protein in the genetic background of C) the Sin1 knockout mutant (Sin1KO1) and D) wild type (WT). The left parts of the SEM micrographs show a complete valve, and the right parts show a zoom-in image of the region highlighted with a yellow dotted square in the left parts. Scale bars: A,B) 2 µm; C,D) 1 µm (left part) and 500 nm (right part).

Figure 8. Specific activities of regioselectively immobilized GOx fusion proteins v2-GOxYFP and g-GOxYFP from two independent clones each (C1, C2). The fusion proteins were produced protein in the genetic background of the Sin1 knockout mutant (Sin1KO1) or the wild type (WT). An activity of 1.0 U corresponds to 1.0 µmol purpurogallin produced per minute. Each bar represents the average value from the analyses of three independent experiments, and the standard deviation is indicated.

We then tested two independent clones (C1, C2) from each of the four different biosilica materials for their capabilities to catalyze the coupled enzymatic reaction. Biosilica with both enzymes immobilized in girdle bands (g-GOxYFP/g-HRPmT2) exhibited the highest specific activity, whereas the lowest catalytic activity was obtained with the biosilica containing valve-located GOx and HRP fusion proteins (v-GOxYFP/v-HRPmT2, Figure 10). The two biosilica materials containing the two enzymes in different regions possessed intermediate levels of specific activity, which were essentially identical to each other (Figure 10). The activity of the girdle band located GOx–HRP enzyme pair was 2.6-fold higher than of the valve located enzyme pair, which closely matched the ratios observed for the individual enzymes (see Figure 3). These data demonstrated that proximity of GOx and HRP molecules within the same region of the biosilica was not important for promoting the catalytic activity of the enzyme pair. Instead, the catalytic activity of the individual enzyme molecules is limiting for the speed of the coupled enzymatic reaction. This suggests that substrate channeling between proximal HRP and GOx molecules within the same biosilica microparticle is either not occurring or at least not a rate-limiting factor.
3. Conclusions

The present work establishes regioselective functionalization of diatom biosilica microparticles with enzyme molecules. This enabled us to clearly demonstrate that silica nanomorphology has a crucial effect on the enzyme’s activity, whereas the speed of reactant transport through diatom biosilica appears to be irrelevant. Therefore, we conclude that the highly porous and thin wall structure of the biosilica readily exposes the immobilized enzyme molecules to the reactant solution. How silica nanomorphology influences enzyme activity remains speculative. Protein molecules bind to silica mainly via polar residues and positively charged residues of the protein surface, which establish hydrogen bonds and Coulomb interactions with the silanol Si–OH and silanolate −Si=O groups, respectively, on the silica surface. At a given pH, the density of surface charge increases as the curvature of the silica surface decreases. We assume that the negative charge density on *T. pseudonana* girdle band silica is higher than on valve silica, because valves have a more curved surface due to the presence of numerous struts and ridges. Why an increased surface charge would be beneficial for enzyme activity is currently unclear. We hypothesize that the silica surface charge and the associated ion layer may act as an allosteric effector thus remotely influencing the conformation of the active site and thus catalytic activity of bound enzymes. Shedding light on this question will require careful characterization of the surface properties of the biosilica of girdle bands and valves as well as atomistic modeling of the interactions of GOx and HRP with these surfaces.

The new methods for regioselective diatom engineering established in the present work provide possibilities for new applications. Diatom biosilica nanoparticles containing girdle-band located enzymes may be used as self-propelled microswimmers that convert the chemical energy of an enzyme catalyzed reaction into mechanical energy for directional propulsion. Such systems are under investigation for use in smart drug delivery. Furthermore, diatom biosilica microparticles are natural photonic
structures that have favorable properties not only as support material for catalysts, but also for optical sensors, for focusing and filtering light, and for optical switches. Assembling these particles into regular 2D and 3D arrays has therefore the potential to generate metamaterials with further enhanced or new properties compared to individual microparticles. The regioselective modification procedure established here can in principle also be used to incorporate protein-based receptors (e.g., the IgG binding GB1 domain and corresponding ligands into the valve and girdle band regions). This would result in silica microparticles with the propensity to interact in designed orientations, which might enable their assembly into regular 2D and 3D arrays.

4. Experimental Section

Chemicals, commercial enzymes and antibodies, confocal fluorescence microscopy, cloning, expression and isolation of H₆-YFP-R₆ and H₆-mT₂-R₆, antibody accessibility experiments, and scanning electron microscopy are described in detail in the Supporting Information.

Construction of Fusion Genes in Diatom Expression Vectors: All the cloning experiments in this section were performed using the NEBuilder HiFi DNA Assembly Cloning Kit by following the manufacturer’s instructions. The detailed procedures for the construction of g-GoxYFP⁺, g-HRP₄T₂, v-GoxYFP⁺, v-HRP₄T₂, v₂-GoxYFP⁺, and g₂-GoxYFP⁺ fusion genes are described in the Supporting Information.

Diatom Culture Conditions: T. pseudonana (Hasle et Heimdal clone CCMP1335) and C. cryptica (CCMP33) strains were grown in and artificial sweater medium (ESAW) according to the North East Pacific Culture Collection (http://www3.botany.ubc.ca/cccm/NEPCC/esaw.html) at 18 °C under a 12 h light (54 μmol photons m⁻² s⁻¹) and 12 h dark cycle.

Figure 10. Specific activities of the regioselectively immobilized Gox and HRP enzyme pair from two independent transformant clones each (C1, C2). The specific activities were calculated using the total mass of both enzymes in the biosilica. Each bar represents the average value from the analyses of three independent experiments, and the standard deviation is indicated.

Genetic Transformations of T. pseudonana and C. cryptica: The plasmids were introduced into T. pseudonana cells using the Biorad PDS-1000/He particle delivery system (BIORAD, Hercules, CA, USA) as described previously. Co-transformations were performed with pTpfcp/nat plasmid for the selection of transformants on agar plates containing 150 μg ml⁻¹ nourseothricin. Using the same strategy, the double transformants were generated by introducing the second fusion gene into the single T. pseudonana transformant along with the pTpfcp/nat plasmid, which confers resistance to the zeocin antibiotic. The double transformants were selected on agar plates containing 150 μg ml⁻¹ zeocin.

The genetic transformation of C. cryptica was performed as described for T. pseudonana, but using the plasmid pCFC/nat. To select transformants, the concentration of nourseothricin was 250 μg ml⁻¹, which was the amount required to kill the wild type cells when plated at 5 × 10⁶ cells per plate.

Isolation of Diatom Biosilica: For confocal fluorescence microscopy, cells were extracted at 55 °C for 1 h with lysis buffer (1% (w/v) SDS, 100 × 10⁻³ M EDTA, 100 × 10⁻³ M Tris-acetate pH 8.0, 1 × 10⁻³ M PMSF). After centrifugation for 3 min at 6000 × g, the pellet was washed with biosilica isolation buffer II (100 × 10⁻³ M Tris-acetate, 100 × 10⁻³ M EDTA, 1 × 10⁻³ M PMSF pH 8.0). Afterward, the isolated biosilica was resuspended in 100 × 10⁻³ M Tris acetate, pH 8.0 and used for confocal microscopy.

For all other experiments, the biosilica was isolated using the nonionic detergent octylphenyl-polyethylene glycol (Igepal CA-630). The cells were resuspended in biosilica isolation buffer II (50 × 10⁻³ M sodium phosphate pH 7.0, 1× protease inhibitor cocktail), and then lysed by vortexing (3 times, 30 s) with glass beads (diameter, 0.25–0.30 mm). The lysate was centrifuged (3 min, 6000 × g, 4 °C), and the pellet was extracted with biosilica isolation buffer II (100 × 10⁻³ M Tris-acetate, 100 × 10⁻³ M EDTA, 1 × 10⁻³ M PMSF pH 8.0). Afterward, the isolated biosilica was resuspended in biosilica isolation buffer II by centrifugation and resuspension. The isolated biosilica was resuspended in biosilica isolation buffer II, and used for further experiments.

In Vitro Immobilization of Fluorescent Proteins: Wild type T. pseudonana cells were extracted at 95 °C for 1 h using lysis buffer (1% (w/v) SDS, 100 × 10⁻³ M EDTA pH 8.0) and subsequently centrifuged at 3200 × g for 15 min. The pellet was washed by centrifugation and resuspension cycle five times with water, twice with acetone, and again three times with water. After freeze drying, the sample was calcined at 550 °C for 4 h, yielding organic-free biosilica. This biosilica material was used to immobilize known amounts of recombinantly expressed H₆-YFP-R₆ and H₆-mT₂-R₆ in vitro as described in the following. The biosilica (2 mg) was washed with 50 × 10⁻³ M sodium phosphate and resuspended in 100 × 10⁻³ M sodium phosphate pH 7.0 and incubated with varying amounts of H₆-YFP-R₆ or H₆-mT₂-R₆ for 1 h under constant gentle shaking at room temperature. Then, the suspensions were centrifuged, the pellets were washed three times with 50 × 10⁻³ M sodium phosphate pH 7.0, and resuspended in 600 μL of the same buffer. Afterward, around 16 μg of the biosilica (5 μL of the suspensions) containing variable amounts of the proteins (8–246 ng of H₆-YFP-R₆ and 8–326 ng of H₆-mT₂-R₆) in a total volume of 200 μL of 50 × 10⁻³ M sodium phosphate pH 7.0 used for the fluorescence measurements using a plate reader (BioTek, Synergy HT). For measurement of FTYF fluorescence an excitation wavelength of 500/18 nm and an emission wavelength of 530/18 nm was used. For quantification of mT₂ fluorescence, an excitation wavelength of 430/18 nm and an emission wavelength of 474/18 nm was used. The fluorescence of the YFP or mT₂ bearing biosilica was recorded and plotted against the amount of protein attached on the surface. No fluorescence was detected in the supernatant after the centrifugation in the prior step.

Enzyme Activity Assays—Glucose Oxidase: The activity of Gox was determined by incubating biosilica in 400 μL of assay buffer (90 × 10⁻³ M glucose, 50 × 10⁻³ M sodium acetate buffer pH 5.2) under constant shaking at room temperature for 20 min. After incubation, the reaction mixture was centrifuged, and then 55 μL of pyrogallol-HRP buffer (2.5 U horseradish peroxidase, 43 × 10⁻³ M pyrogallol, 0.1 M potassium...
phosphate buffer pH 6.0) was added to 300 µL of supernatant and incubated for 5 min at room temperature. Subsequently, the biosilica was pelleted by centrifugation (2 min, 16 000 × g), and the supernatant was used to measure the absorbance of the product, purpurogallin, at 410 nm.

Enzyme Activity Assays—Horseradish Peroxidase (HRP): To determine the activity of HRP, biosilica was incubated for 20 min at room temperature in 400 µL of reaction buffer (100 × 10⁻⁵ M potassium phosphate pH 6.0, 3.9 × 10⁻³ M H₂O₂ and 43 × 10⁻³ M pyrogallol). Subsequently, the biosilica was pelleted by centrifugation (2 min, 16 000 × g), and the supernatant was used to measure the absorbance of the product, purpurogallin, at 410 nm.

Enzyme Activity Assays—GOx–HRP Coupled Enzymatic Reaction: To determine the activity of the GOx–HRP enzyme pair, biosilica was mixed with 400 µL of reaction buffer (90 × 10⁻³ M glucose, 43 × 10⁻³ M pyrogallol, and 50 × 10⁻³ M sodium acetate buffer pH 5.2). After incubation for 20 min at room temperature, the reaction mixture was centrifuged (2 min at 16 000 × g) and the absorbance of the supernatant was measured at 410 nm.

Fluorescence Lifetime Imaging: Fluorescent biosilica isolated from transformant cells was imaged with a Zeiss alpha-Apochromat 63× (1.4 numerical aperture) oil DIC M27 objective. FLIM images of 512 × 512 pixels were obtained using a Zeiss LSM780 inverted microscope coupled with a Becker & Hickl time correlated single photon counting system (TCSPC). A Diode laser with wavelength of 473 nm (for YFP) or 440 nm (for mT2) and a repetition frequency of 50 MHz was used to excite the fluorescent proteins. The average fluorescence lifetime of YFP and mT2 was calculated for each pixel in the image by fitting a single exponential using the Becker & Hickl SPCImage software.

Protease Treatment: Isolated biosilica was incubated in 300 µL of pronase solution (0.5 µg mL⁻¹) in 50 × 10⁻³ M sodium phosphate pH 7.0) for 15 min at 30 °C under constant shaking. Subsequently, the biosilica was washed three times with 500 µL of 50 × 10⁻³ M sodium phosphate pH 7.0 and used for GOx or HRP activity assays.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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