Enzyme Shielding in an Enzyme-thin and Soft Organosilica Layer

M. Rita Correro,[a] Negar Moridi,[a] Hansjörg Schützinger,[a][b] Sabine Sykora,[a] Erik M. Ammann,[a] E. Henrik Peters,[a] Yves Dudal,[b] Philippe F.-X. Corvini,[a][c] and Patrick Shahgaldian*[a]

[a] School of Life Science, University of Applied Sciences and Arts Northwestern Switzerland, Gründenstrasse 40, Muttenz CH-4132 (Switzerland)
[b] INOFEA AG, Hochbergerstrasse 60C, Basel CH-4057 (Switzerland)
[c] School of the Environment
Nanjing University, 210093 Nanjing, (China)

Abstract: The fragile nature of most enzymes is a major hindrance to their use in industrial processes. Herein, we describe a synthetic chemical strategy to produce hybrid organic/inorganic nanobiocatalysts; it exploits the self-assembly of silane building blocks at the surface of enzymes to grow an organosilica layer, of controlled thickness, that fully shields the enzyme. Remarkably, the enzyme triggers a rearrangement of this organosilica layer into a significantly soft structure. We demonstrate that this change in stiffness correlates with the biocatalytic turnover rate, and that the organosilica layer shields the enzyme in a soft environment with a markedly enhanced resistance to denaturing stresses.

Keywords: Nanoparticles • Enzyme Catalysis • Self-assembly • Organosilica

Version: last version submitted

Published: Angew. Chem. Int. Ed. 2016, 55, 6285-6289; Angew. Chem. 2016, 21, 6393-6397

Link to published version: https://doi.org/10.1002/anie.201600590
Biocatalysis is a major driver of the chemical industry. However, the use of enzymes in industrial processes is limited by their significant fragility and fast aging in non-physiological environments. In addition to genetic engineering techniques to improve enzyme stability, a variety of chemical approaches have been developed to immobilize and shield enzymes on solid carriers. Enzyme immobilization on solid supports is a valuable approach to address enzyme stability, and has the additional benefit of allowing the biocatalyst to be retained for continuous operations.

A large number of bio-conjugation strategies have been developed to allow the immobilization and protection of enzymes on a variety of carrier materials, such as (bio-)polymers, zeolites, noble metals, and metal- or metalloid oxides (e.g., silica). Recently, sophisticated approaches where enzymes were confined and protected in materials such as metal-organic frameworks or virus-like particles have been successfully developed.

Silica is a material of choice to immobilize and protect enzymes as it has advantages such as low production cost and high thermal and mechanical stability. Sol-gel methods have been extensively developed to embed enzymes in silica matrices. Reetz et al. reported on the entrapment of a lipase in chemically modified silica gels. The enzyme was immobilized in a sol-gel matrix containing a mixtures of tetramethyl orthosilicate and alkylsilanes. A strong dependence of the silane composition of the matrix on the enzymatic activity was demonstrated. However the main focus of sol-gel methods to protect enzymes has been on macroscopic systems that do not allow controlling the three-dimensional structure of the material produced. Consequently, most techniques are not amenable to the production of discrete functional nanoparticles that can be dispersed into fluids, hence severely limiting their use in biomedical applications. Additionally, silica is inherently negatively charged and cannot create a shell closely surrounding the whole surface of the protein. We expect that the presence in these silica-based materials of additional functional groups will increase the number of interaction points with the protein surface. This should allow for better protection of the enzyme, owing to a better chemical complementarity between the surface of the enzyme and the protective material. Furthermore, the environment shielding the enzyme should be designed such that it does not hinder the enzyme’s conformational dynamic mobility, which is crucial for its biocatalytic activity. This issue has been often neglected in the design of enzyme protection systems.
We have recently demonstrated that a virus can serve as a template to grow an organosilica layer on its capsid surface in purely aqueous conditions. Herein, we report a synthetic strategy to grow a protective layer of controlled thickness at the surface of immobilized enzymes; cf. Scheme 1. The method consists of a sequential reaction of i) immobilization of an enzyme at the surface of silica particles; ii) controlled self-assembly and subsequent polycondensation of silanes, resulting in the growth of an organosilica layer at the surface of the particles.

Scheme 1. Principle of enzyme protection. (a) Step 1: enzyme immobilization at the surface of SNPs (in black); Step 2 & 3: silane self-assembly and polycondensation.

As carrier material, we chose amino-modified silica nanoparticles (SNPs) produced with a diameter of 266 ± 1 nm, as measured by field-emission scanning electron microscopy (FE-SEM). The amine functions allowed the further covalent anchoring of the target enzyme, β-galactosidase (β-gal) from \textit{K. lactis}, using glutaraldehyde as a homobifunctional cross-linker. The enzymatic activity, which was measured using the conventional o-nitrophenyl-β-galactoside (ONPG) assay, showed that less than 1% of enzyme was left free in the supernatant, a value that was consistent with the total protein concentration that was under the limit of detection of the method (Supporting Information). The enzyme activity assay showed a loss of 60% of the initial activity, which could be explained by the unfavorable orientation of a fraction of the immobilized enzymes or a partial denaturation upon immobilization. The particles were subsequently incubated with a mixture of (3-aminopropyl)triethoxysilane (APTES, 19 mg mL\(^{-1}\)) and tetraethylorthosilicate (TEOS, 80 mg mL\(^{-1}\)) in order to grow an organosilica layer at the surface of the enzyme. β-gal from \textit{K. lactis} is a large tetrameric enzyme comprising a dimer of dimers with two biocatalytic centers located at the interface within each dimer. This three-dimensional structure can be approximated as a tri-axial ellipsoid with dimensions of 15.9 × 9.3 × 5.3 nm. Assuming that the immobilization strategy used in the present work did not favor any specific orientation of the protein with regard to the surface, the protective layer would need a thickness of at least 16 nm to fully shield the enzyme. The
organosilane polycondensation reaction on the SNPs with surface-immobilized β-gal (SNP_{ENZ-OS}) was monitored over time (Figure 1).

![Figure 1. Microscopy study. (a) Kinetics of layer growth (mean ± s.e.m), measured on FE-SEM micrographs, at the surface of the SNPs with (SNP_{ENZ-OS}, white square) or without (SNP_{OS}, black squares) surface-immobilized β-gal. Both systems show a linear diameter increase of 1.2 nm h⁻¹ showing that the presence of the enzyme at the surface of the SNPs did not significantly influence the kinetics of layer growth (b,c) FE-SEM micrographs of SNP_{ENZ-OS} (b, c) with a protective organosilica layer of 17 nm. In (b) is shown a particle when the protective layer is damaged; the rounded edge of this layer suggests a soft material. Scale bars represent 100 nm.

The evolution of particle diameter over time was found to be linear, with a linear increase of 1.2 nm h⁻¹. In the last sample collected after 15 hours of polycondensation, the thickness of the organosilica layer (17 ± 0.6 nm) was sufficient to shield the whole enzyme regardless of its orientation to the SNP surface. All the particles present a fairly homogeneous and flat surface. There were only a few sporadic cases where the layer was partially broken and its edges did not appear sharp, suggesting that this organosilica layer was soft.

When measuring the enzymatic activity of the shielded β-gal (SNP_{ENZ-OS}), we noticed that the enzymatic activity was low when freshly produced SNP_{ENZ-OS} was measured right after synthesis, while the same sample measured after 12 hours of storage at 25°C had a significantly higher activity. Indeed, before the layer growth, the activity measured was 73 mU mg⁻¹ and dropped after the layer growth down to 21 mU mg⁻¹. After 12 hours of storage at 20°C, the activity was found to be 50 U mg⁻¹ which corresponds to a recovery of 68% of the initially immobilized activity. From our experience with virus-imprinted particles, we knew that the organosilica layer was not mechanically stable after the synthesis and had to be stored at room temperature for 12 hours to gain stability.\[9a\]

We decided to investigate this phenomenon further and to assess possible changes in the nanomechanical properties of the protective layer by means of atomic force microscopy (AFM). The AFM experiments were carried out by measuring force-distance curves on different SNPs of the same sample (Figure 2).
Figure 2. Nanomechanical properties assessment. (a) Force-distance curve measurements carried out on bare silica nanoparticles (black), SNP<sub>OS</sub> (---) and SNP<sub>ENZ-OS</sub> (— • —) after 12 hours storage. (b-h) Stiffness distribution histograms for bare SNPs (b), SNP<sub>ENZ-OS</sub> (c, e and g) and SNP<sub>OS</sub> (d, f and h) after 2 (c, d), 5 (e, f) and 12 (g, h) hours of curing.

As expected, bare SNPs were stiff, with a stiffness value of 34 ± 0.11 N m<sup>-1</sup> (Figure 2). At the beginning of the curing reaction, the SNP<sub>ENZ-OS</sub> were also stiff with an average value of 14 ± 0.02 N m<sup>-1</sup>. After 5 hours of curing, the stiffness value dropped to 6 mN m<sup>-1</sup> with a moderately broader distribution. The softening effect of the organosilica-protein layer continued until the SNP<sub>ENZ-OS</sub> reached a value as low as 0.5 mN m<sup>-1</sup> after 12 hours, which then remained constant for several days. By contrast, the SNP<sub>OS</sub> did not exhibit such a trend. The organosilica layer in these reference particles was soft, with a value of 0.28 N m<sup>-1</sup> after termination of the layer growth reaction, which did not change significantly over the time period of the curing.

The formation of covalent siloxane (Si-O-Si) bonds first requires the hydrolysis of the ethoxy functions of the silanes into the corresponding silanols, which further undergo a condensation
reaction. In the present case, one could assume that the initial stiff layer was predominantly stabilized by hydrogen bonds (H-bonds) and ionic interactions of the silanes of short polysiloxanes with the surface of the protein; this layer became softer through the formation of Si-O-Si bonds.

Regarding the change of enzymatic activity, two hypotheses could explain the recovery of enzyme activity during the curing/softening of the organosilica layer. The first one was that an increase in porosity of the protective layer resulted in a higher mass transfer of the substrate to the active site of the enzyme. The second hypothesis was that the soft environment of the organosilica layer allowed the protein to acquire a sufficient conformational mobility, known to be of crucial importance for the catalytic activity of the enzyme. To better understand the recovery of enzymatic activity during the curing phase, we performed a kinetic study of the enzymatic activity of freshly produced samples submitted to the curing reaction at 25°C (Figure 3).

Although the maximum velocity of SNP\textsubscript{ENZ-OS} increased over curing time, the apparent Michaelis-Menten constant $K_{m}^{app}$ remained relatively unchanged with values averaging 4 mM, similar to that of the native enzyme (Figure 3). These results allowed us to rule out the possibility that the recovery of enzyme activity was due to an increase in the porosity of the protective organosilica layer. Indeed, in that case, the $K_{m}^{app}$ values would have varied while the $V_{m}^{app}$ should have remained constant. Therefore, our results provide clear evidence that the recovery of enzyme activity was due to a favorable change in enzyme conformation enabled by the softness of its protective layer.

![Figure 3](image)

Figure 3 Recovery of enzymatic activity during the curing period. (a) SNP\textsubscript{ENZ-OS} catalyzed ONPG hydrolysis reaction velocity; the results obtained for longer curing durations are similar to that obtained after 480 min, and are omitted for clarity of the figure. (b) Apparent maximum velocity values ($V_{m}^{app}$) were extracted from the kinetic data using the Lineweaver-Burk plot as a function of the curing time; the insert shows the evolution of the $K_{m}^{app}$ values measured for the same ONPG concentrations.

The recyclability of SNP\textsubscript{ENZ-OS} was tested by repetitive cycles of centrifugation/re-suspension in fresh buffer. No relevant loss of activity was measured after 10 repetitive cycles. We also applied
a series of stress conditions to SNP\textsubscript{ENZ-OS} (Figure 4). The biocatalytic hydrolytic activity of SNP\textsubscript{ENZ-OS}, SNP\textsubscript{ENZ} and free β-gal were tested at increasing reaction temperatures. The activity of the SNP\textsubscript{ENZ} and β-gal exhibited a similar trend, with more than 20% of activity loss at 45°C; 53% at 50°C, 79% at 55°C and as much as 96% at 60°C (Figure 4). In contrast, the behavior of SNP\textsubscript{ENZ-OS} was very different: the biocatalytic activity increased to 118%, 123% and 114% at 45°C, 50°C and 55°C, respectively. Moreover, SNP\textsubscript{ENZ-OS} preserved 88% of its catalytic activity at 60 °C. This gain of activity could be explained by a protective effect of the organosilica layer shielding the enzyme, along with an increase in the kinetic energy of the substrate molecules, resulting in an overall increase in the biocatalytic turnover rate.

Figure 4. Physical, chaotropic and biochemical stress tests. (a) Relative activity of SNP\textsubscript{ENZ-OS} (black squares), soluble β-gal (black triangles) and SNP\textsubscript{ENZ} (white triangles) measured at increasing reaction temperatures. (b) Relative activity of SNP\textsubscript{ENZ-OS}, soluble β-gal and SNP\textsubscript{ENZ} measured after increasing incubation time at 50°C. (c) Relative activity of SNP\textsubscript{ENZ-OS} (grey bars), soluble β-gal (black bars) and SNP\textsubscript{ENZ} (white bars) after freeze-thaw cycles. (d) Relative activity of SNP\textsubscript{ENZ-OS}, soluble β-gal and SNP\textsubscript{ENZ} after ultrasound treatment at increasing durations. (e) Relative activity of SNP\textsubscript{ENZ-OS}, soluble β-gal and SNP\textsubscript{ENZ} measured after incubation in solutions of different pH value. (f) Relative activity of SNP\textsubscript{ENZ-OS}, soluble β-gal and SNP\textsubscript{ENZ} measured in presence of 6 M urea or (g) 1% SDS. (h) Relative activity of SNP\textsubscript{ENZ-OS}, soluble β-gal and SNP\textsubscript{ENZ} after 1 hour protease treatment.

To confirm this hypothesis and to gain further insights into the thermal stability of SNP\textsubscript{ENZ-OS}, we incubated them at 50°C for increasing durations of time and measured their biocatalytic activity at 40°C. Whereas both SNP\textsubscript{ENZ} and β-gal lost more than 94% and 97% of activity after only 10 min
and 20 min of incubation, respectively, the decay in the biocatalytic activity of SNP$_{ENZ-OS}$ was much slower, with only 25% and 40% of activity loss after 10 and 20 min of incubation, respectively; more than 25% activity was preserved even after 60 min of incubation. To investigate further this thermal protective effect, we submitted the SNP$_{ENZ}$, SNP$_{ENZ}$ and β-gal to freezing-thawing cycles. The results showed that while SNP$_{ENZ}$ and β-gal had already lost 45% and 25% of their activity after the first cycle, respectively, the loss for SNP$_{ENZ-OS}$ was only 5%. While both SNP$_{ENZ}$ and β-gal experienced a gradual loss of biocatalytic activity with each cycle, reaching values of 15% and 38%, respectively, after 5 cycles, SNP$_{ENZ-OS}$ remained relatively unchanged with a biocatalytic activity of 95%.

As an additional physical stress test, we submitted the particles to ultrasound. The particles were incubated in phosphate buffer in an ultrasonic bath at 25°C for increasing durations of time. Although the biocatalytic activity of both SNP$_{ENZ}$ and β-gal decayed quite rapidly over time, the loss of activity of SNP$_{ENZ-OS}$ was very moderate; it conserved 88% of activity after 40 minutes of treatment (18% activity was conserved for β-gal and 5% for SNP$_{ENZ}$) (Figure 4).

To assess the resistance of the SNP$_{ENZ-OS}$ to pH variation, they were incubated for 15 min at different pH values. For values lower than the optimum pH value of the enzyme (6.5), SNP$_{ENZ}$ and β-gal have a similar trend with a loss of more than 40% activity at pH 5 and 100% at pH 4. By contrast, SNP$_{ENZ-OS}$ was more stable and lost only 29% and 71% of activity for the same pH values. When stored at pH 3, SNP$_{ENZ-OS}$, SNP$_{ENZ}$ and β-gal all lost more than 97% of their initial activity. At pH values higher than the optimum value, SNP$_{ENZ}$ lost more than 45% and 75% of activity at pH 7 and pH 8, respectively, while SNP$_{ENZ-OS}$ lost only 10 and 15% of activity. At these pH values, the most stable system is the soluble β-gal, which retained 100% of activity. At pH 9 and pH 10, SNP$_{ENZ}$ and β-gal both lost more than 95% of activity, whereas SNP$_{ENZ-OS}$ lost only 19% and 64%, respectively. For higher pH values, the hydrolytic activity of SNP$_{ENZ}$ and β-gal was reduced to 0%, whereas SNP$_{ENZ-OS}$ retained 7% of activity. Overall, these results clearly demonstrate that the organosilica layer had a beneficial effect on the resilience of the enzyme to pH changes.

We also studied the effect of the organosilica layer against chaotropic stress using urea (6M) and sodium dodecylsulfate (1%). The activity was measured directly in the denaturing conditions. In the case of urea, neither SNP$_{ENZ}$ and β-gal exhibited any biocatalytic activity but 12% of activity
could be measured for SNP<sub>ENZ-OS</sub>. Urea acts as a potent H-bond donor and acceptor, it can also act as an enzyme inhibitor by forming H-bonds with important residues located in the active site of the enzyme. In the present case, one of the effects of the protective layer could be to conserve the active conformation of the protein; the loss in enzyme activity may be due in part to inhibition caused by the high concentration of urea.

In the case of SDS treatment, we showed that, as expected, the free β-gal was completely inactive in these conditions. While the activity of SNP<sub>ENZ</sub> was as low as 7%, SNP<sub>ENZ-OS</sub> maintained 45% of activity. The final assay that was performed was the stability against protease digestion. As expected, after incubation with Proteinase K for 60 min, no more activity could be measured for β-gal and a loss of 30% of activity was measured for SNP<sub>ENZ</sub>, suggesting that the accessibility of the immobilized enzyme partially hindered protease digestion. In the case of SNP<sub>ENZ-OS</sub>, no loss of biocatalytic activity was measured, confirming that the shielded enzyme is not accessible to the protease.

In order to test the efficiency of the developed nanobiocatalysts in a real matrix; we tested the hydrolytic activity of the shielded β-galactosidase for its natural substrate, lactose, in skim milk using <sup>14</sup>C-radioactively-labeled lactose. The experimental results showed that the shielded enzyme catalyzed the conversion of lactose into glucose and galactose 30% more efficiently than the soluble β-gal (Supporting Information).

In order to assess the versatility of the developed shielding strategy, we tested different enzymes; i.e. acid phosphatase, laccase, alcohol dehydrogenase, glutamate-oxalate transaminase (Table 1 and Supporting Information).

Table 1: Immobilization and shielding of different enzymes

| Enzyme                  | Enzyme size (nm) | Shell thickness (nm) | Specific activity* |
|-------------------------|------------------|----------------------|-------------------|
| Acid phosphatase        | 4.0 x 6.0 x 7.5  | 12                   | 23.0<sup>1</sup>  |
| Laccase                 | 5.7 x 11.0 x 16.5| 19                   | 11.0<sup>2</sup>  |
| Alcohol dehydrogenase   | 10.5 x 10 x 5.5  | 13                   | 1.5<sup>3</sup>   |
| Aspartate aminotransferase | 10.5 x 6.3 x 5.4 | 18                   | 75.0<sup>4</sup>  |

*Absolute activity (mU) per mg of nanoparticles measured using the following substrates: *1*-p-nitrophenyl-phosphate; *2*-2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); *3*-amino-benzyl alcohol, NAD*; *4*-L-aspartate, 2-oxoglutarate.

For all tested enzymes, the shielding strategy turned out to be successful. More specifically, another hydrolase enzyme, namely acid phosphatase, was protected with an organosilica shell of
12 nm sufficient to cover the whole enzyme. It showed a relevant biocatalytic activity of 23 mU/mg of SNPs. The activity of a shielded laccase, which is a copper-containing oxidase that requires oxygen as second substrate, demonstrated that the shielding organosilica layer does not prevent oxygen diffusion and that the protected enzyme is active after protection. In the case of a nicotinamide adenine dinucleotide (NAD\(^+\))-dependant alcohol dehydrogenase, the activity measured when the enzyme is fully shielded in the protective organosilica layer showed that this shell does not hinder the diffusion of the cofactor. Finally, the activity recovered for an aspartate transaminase demonstrated that the amine exchange between aspartic and oxoglutaric acids is not hampered in the protective organosilica shell.

In summary, we developed a versatile strategy to produce nanobiocatalysts with enhanced stability that imparts to the enzyme resistance to a large set of denaturing stresses.

Acknowledgements

Financial supports from the Swiss Nanoscience Institute (SNI) through the NanoArgovia program (NanoZyme project), the Swiss commission for technology and innovation (grant agreement 16437.1 PFEN-NM) the EU-Eurostar program (grant agreement EI6894) and the Swiss State Secretariat for Education, Research and Innovation (EU-H2020 INMARE project) are gratefully acknowledged.

References

[1] P. Grunwald, CRC Press, Boca Raton, 2015.

[2] a) M. Goldsmith, D. S. Tawfik, *Method Enzymol.* 2013, 523, 257-283; b) S. G. Peisajovich, D. S. Tawfik, *Nat. Methods.* 2007, 4, 991-994.

[3] a) U. Hanefeld, L. Gardossi, E. Magner, *Chem. Soc. Rev.* 2009, 38, 453-468; b) F. Secundo, *Chem. Soc. Rev.* 2013, 42, 6250-6261; c) U. T. Bornscheuer, *Angew. Chem., Int. Ed.* 2003, 42, 3336-3337; *Angew. Chem.* 2003, 115, 3458-3459.

[4] a) M. Misson, H. Zhang, B. Jin, *J. R. Soc. Interface* 2015, 12, 20140891; b) L. Cao, *Carrier-bound immobilized enzymes: principles, application and design*, John Wiley & Sons, Weinheim, 2006.

[5] K. Liang, R. Ricco, C. M. Doherty, M. J. Styles, S. Bell, N. Kirby, S. Mudie, D. Haylock, A. J. Hill, C. J. Doonan, P. Falcaro, *Nat. Commun.* 2015, 6, 7240.

[6] D. P. Patterson, B. Schwarz, R. S. Waters, T. Gedeon, T. Douglas, *ACS Chem. Biol.* 2014, 9, 359-365.
[7] a) C. Sanchez, P. Belleville, M. Popall, L. Nicole, *Chem. Soc. Rev.* **2011**, *40*, 696-753; b) R. Ciriminna, A. Fidalgo, V. Pandarus, F. Béland, L. M. Ilharco, M. Pagliaro, *Chem. Rev.* **2013**, *113*, 6592-6620; c) M. T. Reetz, A. Zonta, J. Simpelkamp, *Angew. Chem., Int. Ed.* **1995**, *34*, 301-303; *Angew. Chem. 1995*, *107*, 373-376; d) M. T. Reetz, A. Zonta, J. Simpelkamp, *Biotechnol. Bioeng.* **1996**, *49*, 527-534.

[8] a) E. Z. Eisenmesser, D. A. Bosco, M. Akke, D. Kern, *Science* **2002**, *295*, 1520-1523; b) K. A. Henzler-Wildman, M. Lei, V. Thai, S. J. Kerns, M. Karplus, D. Kern, *Nature* **2007**, *450*, 913-916.

[9] a) A. Cumbo, B. Lorber, P. F. X. Corvini, W. Meier, P. Shahgaldian, *Nat. Commun.* **2013**, *4*, 1503; b) S. Sykora, A. Cumbo, G. Belliot, P. Pothier, C. Amal, Y. Dudal, P. F. X. Corvini, P. Shahgaldian, *Chem. Commun.* **2015**, *51*, 2256-2258.

[10] A. Pereira-Rodriguez, R. Fernandez-Leiro, M. I. Gonzalez-Siso, M. E. Cerdan, M. Becerra, J. Sanz-Aparicio, *J. Struct. Biol.* **2012**, *177*, 392-401.

[11] J. Almarza, L. Rincon, A. Bahsas, F. Brito, *Biochemistry* **2009**, *48*, 7608-7613.
Supporting Information

Content

General ................................................................................................................................. 13
β-galactosidase purification and SDS-PAGE ........................................................................ 13
Enzyme shielding .................................................................................................................. 13
Enzymatic assay and kinetic studies .................................................................................... 13
Atomic Force Microscopy .................................................................................................... 13
Scanning electron microscopy and particle size measurement .............................................. 14
Enzymatic assay of the shielded lactase in skim milk .......................................................... 14
Physical, chaotropic and biochemical stress tests ............................................................... 14
Additional enzyme shielding and activity assays ................................................................. 15
Table 1: Immobilization and shielding of different enzymes. Enzymes features, catalytic reactions, reaction conditions and organosilica layer thickness of the different enzymes. ................................................................. 15
Figure S1 ............................................................................................................................. 15
References ............................................................................................................................ 16
General

Tetraethyl orthosilicate (TEOS, ≥99%), (3-aminopropyl)triethoxysilane (APTES, ≥ 98%), ammonium hydroxide (ACS grade, 28-30%), ethanol (ACS grade, anhydrous), glutaraldehyde (Grade I, 25% in water), β-galactosidase (β-gal) from Kluyveromyces lactis and Proteinase K from Trirachium album were purchased from Sigma-Aldrich (Switzerland). All chemicals were used without further purification. Nanopure water (resistivity ≥ 18 MΩ cm) was produced with a Millipore Synergy purification system. Silica nanoparticles (SNPs) were prepared using the reported procedure.[7]

β-galactosidase purification and SDS-PAGE

10 mL β-gal were dialysed against 2 L dialysis buffer (100 mM phosphate, 5 mM MgCl₂, 100 mM NaCl, pH 6.5) using a SnakeSkin dialysis tube (10K MWCO) at 4°C for 24 h. The dialyzed enzyme was analysed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). As reported in the literature, the commercial enzyme solution shows the main protein band at ca. 100 kDa and additional bands at ca. 50 and 30 kDa. These additional bands have been shown to react with anti-β-gal antibodies and have been attributed to fragmentation products of the native enzyme.[2] From this analysis, one can assume that the enzyme represents 100% of the protein content.

Enzyme shielding

All reactions were carried out in 20 mL glass vials under moderate magnetic stirring (400 rpm). To 18 mL of a suspension of 3.2 mg mL⁻¹ of SNPs, at 20°C, were added 11 µL (0.047 mmol) of APTES. After 90 min of reaction, the suspension was centrifuged at 3220 g for 10 min, the resulting pellet was suspended in water. This operation (hereinafter called “washing cycle”) was repeated twice. The so-modified SNPs were then incubated during 30 min in 18 mL of 0.1% (v/v) aqueous glutaraldehyde and subsequently submitted to two washing cycles in water. The resulting pellet was suspended in 18 mL of MES buffer (10 mM MES, 5 mM MgCl₂, pH 6.2) and reacted, at 20°C, with β-gal (at 0.035 mg mL⁻¹, corresponding to 601 µM mL⁻¹) for 1 h to produce SNP<sub>ENZ</sub>. Subsequently and without intermediate washing, 86 µL (0.386 mmol) of TEOS were added to the reaction mixture and allowed to react for 1 h. Consequently, 21 µL APTES (0.088 mmol) were added. Aliquots of 1 mL (SNP<sub>ENZ-OS</sub>) were collected at increasing reaction durations (3, 6, 9, 12 and 15 h after the addition of APTES), washed in nanopure water, suspended in 1 mL of nanopure water and imaged by FE-SEM. In parallel, a reference sample (SNP<sub>OS</sub>), was prepared following the same synthetic procedure but omitting the enzyme addition. The silane polycondensation reaction was stopped after 15 h by washing the SNP<sub>ENZ-OS</sub> and the SNP<sub>OS</sub> in activity buffer (AB) containing 100 mM potassium phosphate, 5 mM MgCl₂, pH 6.5. SNPs were then suspended in AB, maintained at 25°C for 24 h and finally stored at 4°C. Particle sizes were measured on micrographs taken at a magnification of 150,000 X using the Olympus analySIS software package. The catalytic activity of SNP<sub>ENZ-OS</sub> was determined as described in the following section.

Enzymatic assay and kinetic studies

In a typical β-gal activity assay, o-nitrophenyl-β-D-galactopyranoside (ONPG) was used as substrate. The assay was performed at 40°C under shaking at 650 rpm in AB. 190 µL of ONPG (21 mM) in AB were equilibrated for 5 min in a Thermomixer (Eppendorf) at 40°C. 10 µL of SNP<sub>ENZ-OS</sub>, soluble β-gal or SNP<sub>ENZ-REC</sub> were added to the ONPG solution and the reactions mixtures were maintained under shaking (650 rpm) at 40°C for 5 min. The ONPG hydrolysis was stopped by the addition of 200 µL of 1 M Na₂CO₃ solution. After centrifugation at 16100 g for 90 sec, the amount of the produced o-nitrophenol (ONP) was determined by measuring the absorbance at 420 nm (OD 420) of 200 µL solution in a 96 well-plate using a Synergy H1 (BioTek). The catalytic activities were calculated using the molar extinction coefficient of o-nitrophenol (ε<sub>420nm</sub> = 4300 M⁻¹ cm⁻¹) measured by preparing a standard curve using the same buffer. The apparent catalytic parameters (V<sub>app</sub> and K<sub>app</sub>) of SNP<sub>ENZ-OS</sub>, were measured performing a typical activity assay with increasing ONPG quantities (at final concentrations 0.5-20 mM respectively). V<sub>app</sub> and K<sub>app</sub> were extracted from the double reciprocal (Lineweaver-Burke) plot.

Atomic Force Microscopy

A solution of SNPs (3.2 mg mL⁻¹) in nanopure water was prepared and a volume of 2 µL was spread on freshly cleaved mica. Imaging was performed in contact mode in air using a NTEGRA Prima (NT-MDT) system equipped with gold-coated silicon rectangular cantilevers (length 95 µm, width 30 µm, NT-MDT). Cantilever spring constants were determined experimentally performing thermal fluctuations measurements. Force-distance spectroscopy was obtained by measuring cantilever deflection as a function of the scanner z-piezo tube extension. Stiffness was measured as the slope of the linear part of force-displacement curve.
Scanning electron microscopy and particle size measurement

Particles were imaged using a Zeiss SUPRA® 40VP scanning electron microscope. A 2 µL drop of each sample was placed on freshly cleaved mica substrates, dried under ambient conditions, and sputter-coated with a gold-platinum alloy for 15 s at 10 mA. Secondary electron micrographs were acquired using the InLens mode with an accelerating voltage of 20 kV. Particle sizes were measured on micrographs acquired at a magnification of 150,000 X using the ®AnalySIS software package. 100 measurements were carried out for each sample.

Enzymatic assay of the shielded lactase in skim milk

27 µL of radioactive lactose stock (3.7 MBq Lactose [D-glucose-1,14C], Hartmann Analytic GmbH, Germany) were added to 428 µL of skim milk and equilibrated at 40°C for 30 min in a shaker at 500 rpm (Thermomixer Comfort, Eppendorf AG, Germany). 45 µL of shielded enzyme (1.44 U mL-1) or soluble β-gal (1.44 U mL-1) or buffer solution (100 mM K2HPO4, 5 mM MgCl2, pH 6.5) were added and incubated at 40°C in a shaker at 500 rpm. 25 µL aliquots were collected every 60 min for 9 hours and after 20 and 24 hours and added to a volume of 50 µL acetonitrile in order to precipitate the soluble proteins. After centrifugation at 21,500 g for 10 min (Himac CT 15RE, Hitachi Koki Co., Ltd Japan) the supernatant was transferred into GC-inlet vials (Infochroma AG, Switzerland) for high-performance liquid chromatography (HPLC) measurement. HPLC measurements of the hydrolysed lactose were performed using an Agilent Technologies HPLC 1200 Series coupled to a radioisotope ©Ramona Star detector (Raytest GmbH, Germany). The separation was carried out on a GRACE Prevail Carbohydrate ES 5u column (250 mm x 4.6 mm). Elution was performed using acetonitrile/water 75:25 (v/v) and a flow rate of 1.0 ml min⁻¹. The experiment was performed in duplicates and the results are shown in Figure S1.

Physical, chaotropic and biochemical stress tests

Activity assay at increasing temperatures - 190 µL of ONPG (21 mM) in AB were equilibrated for 5 min at 45, 50, 55 and 60°C respectively. 10 µL of SNP_ENZ-OS, soluble β-gal or SNP_ENZ-REF were added to the ONPG solution and the reactions mixtures were maintained under shaking (650 rpm) at the appropriate temperature for 5 min. The reaction was consequently stopped with 1M Na2CO3 and measured as described above.

Enzyme stability at 50°C - 200 µL of SNP_ENZ-OS, soluble β-gal and SNP_ENZ-REF were incubated at 50°C for 60 min under shaking at 650 rpm. 10 µL aliquots were collected every 10 min, added to 190 µL of ONPG (21 mM) in AB and measured for hydrolytic activity at 40°C as described above.

Freeze-thaw cycles - 100 µL of SNP_REF-OS, soluble β-gal and SNP_ENZ-REF were stored at -20°C for 60 min. Subsequently, the samples were warmed at 25°C for 30 minutes; 10 µL aliquots were collected and the ONPG hydrolysis was measured as described above. Four additional freeze-thaw cycles were repeated.

Ultrasonic treatment - 100 µL of SNP_ENZ-OS, soluble β-gal and SNP_ENZ-REF were incubated at 25°C in an ultrasonic bath (Elma, 37 kHz) for 40 min. 10 µL aliquots were collected every 10 min, added to 190 µL of AB and measured for ONPG hydrolysis as described above.

Incubation in different pH solutions - 30 µL of SNP_ENZ-OS and 50 µL of SNP_ENZ-REF were centrifuged at 16100 g for 90 sec and 400 g for 3 min respectively [NB: a faster centrifugation of the SNP_ENZ-REF induced the formation of a hard, compact pellet that could not be suspended with a mild mixing and was not suitable for SNPs suspension]. The resulting pellets were suspended in 30 and 50 µL respectively of solutions at different pH values (100 mM citrate buffer, 5 mM MgCl2 for pH values 3-5; 100 mM phosphate buffer, 5 mM MgCl2 for pH values 6-8; 100 mM ethanolamine buffer, 5 mM MgCl2 for pH values 9-11). After 15 min incubation at 25°C under shaking at 650 rpm, samples were centrifuged and suspended in 30 and 50 µL respectively of AB. 10 µL aliquots were thus added to 190 µL ONPG and catalytic activities were measured as described above. Similarly, 10 µL of β-gal were incubated with 90 µL of solutions at different pH values (3-11) for 15 min at 25°C under shaking at 650 rpm. After a 1:100 dilution, the ONPG hydrolysis was measured [NB: with such dilution, the effects on the activity assay of pH and composition of the buffer used for the stress assay was neglected]. The catalytic activities were calculated using the molar extinction coefficient of α-nitrophenol (ε280nm = 4300 M⁻¹ cm⁻¹).

Activity assay in presence of 6 M urea or 1% SDS - 190 µL of ONPG (21 mM) in AB containing 6.6 M urea or 1.1% SDS respectively, were equilibrated for 5 min at 40°C. 10 µL of SNP_ENZ-OS, soluble β-gal or SNP_ENZ-REF were added and the catalytic activity was measured as described above. The catalytic activities were calculated using the molar extinction coefficient of α-nitrophenol (ε280nm = 4300 M⁻¹ cm⁻¹).

Proteinase treatment - 250 µL of SNP_ENZ-OS or SNP_ENZ-REF were centrifuged at 16100 g for 90 sec and 400 g for 3 min respectively and resulting pellets suspended in 250 µL of digestion buffer (10 mM potassium phosphate, 5 mM MgCl2, pH 7.5) containing 1 mg mL⁻¹ Proteinase K. Similarly, β-gal was incubated with the proteinase solution. The protease digestion was allowed during 60 min at 37°C under shaking at 650 rpm. The catalytic activities of 10 µL SNP_ENZ-OS, SNP_ENZ-REF and soluble β-gal were measured.
Additional enzyme shielding and activity assays

Different enzymes (acid phosphatase, laccase, alcohol dehydrogenase and aspartate transaminase (AST)) were immobilized on silica nanoparticles and shielded with an organosilica layer following the general procedure previously described (representative micrographs are showed in Figure S2). The catalytic activities of the shielded enzymes were measured by means of established catalytic assays where the reaction products were spectrophotometrically measurable at different wavelengths (Table 1)[3-6].

Table 2: Immobilization and shielding of different enzymes. Enzymes features, catalytic reactions, reaction conditions and organosilica layer thickness of the different enzymes.

| Enzyme (EC; organism) | Enzyme Features (weight, size) | Catalyzed reaction | Reaction conditions | $\varepsilon_{\text{product}}$ (mM$^{-1}$cm$^{-1}$) | Shell (nm) |
|-----------------------|--------------------------------|--------------------|--------------------|-----------------------------------------------|------------|
| Acid phosphatase (EC 3.1.3.2) *S. tuberosum* | Homo-dimer 472 kDa 4.0 x 6.0 x 7.5 nm | $p$-nitrophenyl-phosphate $\rightarrow p$-nitrophenol$^3$ | 25 mM acetate pH 5.2; 37°C | 16'825$^a$ | 12 |
| Laccase EC 1.10.3.2 *T. arenaria* | Homo-dimer 160 kDa 5.7 x 11.0 x 16.5 nm | ABTS + O$_2$ $\rightarrow$ ABTS$^{+}$ + H$_2$O$^4$ | 80 mM citric acid, 40 mM Na$_2$HPO$_4$, pH 3; 25°C | 16'700$^a$ | 19 |
| Alcohol dehydrogenase EC 1.1.1.1 *S. cerevisiae* | Homo-tetramer 146 kDa 10.5 x 10 x 5.5 nm | 2-amino-benzyl alcohol $+$ NAD$^+$ $\rightarrow$ 2-amino-benzaldehyde $+$ NADH$^5$ | 21 mM Na$_2$H$_2$PO$_4$, 0.33 mM Na$_2$HPO$_4$, pH 8.8; 25°C | 3'826$^b$ | 13 |
| AST* EC 2.6.1.1 *S. scrofa* | Homo-dimer 92.7 kDa 10.5 x 6.3 x 5.4 nm | L-aspartate $+$ 2-oxoglutarate $\rightarrow$ oxaloacetate $+$ L-glutamate$^6$ | 80 mM Na$_2$HPO$_4$, pH 7.5; 37°C | 920$^d$ | 18 |

* Aspartate aminotransferase; reactions measured spectrophotometrically at: $^a$405 nm; $^b$420 nm; $^c$360 nm; $^d$460 nm

Figure S1

Figure S1: Lactose hydrolysis in skim milk catalysed by the shielded β-gal (black square) and the soluble enzyme (black triangle)
Figure S2: FE-SEM micrographs of shielded SNPs. A): Acid phosphatase; layer: 12 nm. B): Laccase; layer 19 nm; C): Alcohol dehydrogenase; layer 13 nm; D): glutamic oxaloacetic transaminase; layer 18 nm. Scale bars represent 100 nm.

References

[1] A. Cumbo, B. Lorber, P. F. X. Corvini, W. Meier, P. Shahgaldian, Nat. Commun. 2013, 4, 1503.
[2] D. F. M. Neri, V. M. Balcao, M. G. Cameiro-Da-Cunha, L. B. Carvalho, J. A. Teixeira, Catal. Commun. 2008, 9, 2334-2339.
[3] Y. Sugiura, H. Kawabe, H. Tanaka, S. Fujimoto, A. Ohara, J. Biol. Chem. 1981, 256, 664-670.
[4] H. Cabana, J. P. Jones, S. N. Agathos, Eng. Life Sci. 2007, 7, 429-456.
[5] J. H. R. Kagi, B. L. Vallee, J. Biol. Chem. 1960, 235, 3188-3192.
[6] R. Katoch, Analytical techniques in biochemistry and molecular biology, Springer, New York, 2011