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METHODS AND PROTOCOLS

Sorption-assisted surface conjugation: a way to stabilize laccase enzyme

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Abstract Enyzme immobilization on solid surfaces is one of the most relevant methods to improve enzyme activity and stability under harsh conditions over extended periods. A typically interesting application is the immobilization of laccases, multicopper enzymes oxidizing aromatic compounds, to solid surfaces in order to develop valuable tools for the elimination of micropollutants in wastewater. Laccase of the white-rot fungus *Coriolopsis polyzona* has been successfully immobilized on fumed silica nanoparticles using a novel method. It consists in the sorption of the enzyme to amino-modified silica nanoparticles and the subsequent covalent cross-linking using glutaraldehyde as a homobifunctional linker. The so-produced nanoparticulate material has been characterized by means of scanning electron microscopy and Brunauer–Emmett–Teller surface area analysis revealing modifications of the surface structure and area during the coupling procedure. Laccase immobilization on spherical nanoparticles produced according to the method of Stöber has been shown to be much less efficient than on fumed silica nanoparticles. Long-term stability assays revealed that the novel developed method allows a drastic stabilization of the enzyme. In real wastewater, 77% of the laccase activity remained on the nanoparticles over 1 month, whereas the activity of free laccase dropped to 2.5%. The activity loss on the nanoparticles resulted from partial inactivation of the immobilized enzymes and additional release into the surrounding solution with subsequent fast inactivation of the free enzymes, since almost no activity was found in the supernatants.

Keywords Laccase · Silica nanoparticles · Enzyme immobilization · ABTS

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Introduction

To date, aromatic compounds such as phenol derivatives are still an unresolved problem in wastewater treatment. Huge amounts of those chemicals are discharged every year into the environment by petroleum, resin, plastic, textile, pulp, paper, dye, and pharmaceutical industries (Husain and Ulber 2011). As an example, through its widespread use and release into the environment, bisphenol A is often detected in drinking water, surface water, effluent water from wastewater treatment plants, sediment samples, and tissues from aquatic animals (Garoma et al. 2010). Such micropollutants are often toxic for aquatic organisms or act as endocrine-disrupting chemicals (EDCs) influencing organisms at low doses (Cirja et al. 2008). As a matter of fact, the elimination of phenolic micropollutants remains a critical challenge for wastewater treatment.

Among the enzymes of interest for industrial applications, laccases are versatile biocatalysts active for the degradation of a wide range of compounds including EDCs and further pollutants in industrial effluents (Nicell et al. 1993; Baldrian 2006; Auriol et al. 2007; Cabana et al. 2007a; Martin et al. 2007; Majeau et al. 2010). These ligninolytic enzymes, produced by a variety of organisms such as fungi, are multicopper oxidases capable of oxidating phenols which subsequently lose their xenobiotic properties (Durán et al. 2002; Cabana et al. 2007b). A laccase from the white-rot basidiomycete *Coriolopsis polyzona* (*C. polyzona*) has been extensively studied during the last few years and was shown to efficiently degrade such EDCs (Cabana et al. 2007c, 2009a).

In order to facilitate the use of enzymes in continuous processes in terms of separation, recycling, and reuse, a number of carrier-bound immobilized enzymes has been developed (Cao et al. 2003). Nevertheless, the applications of enzymes for the treatment of effluents at large scale have been hindered by high enzyme prices combined with unsatisfying enzyme stability caused by unfavorable conditions, e.g., inadequate pH, temperature, or salt concentration (Hildén et al. 2009; Majeau et al. 2010). One possible approach to meet this challenge is the immobilization of enzymes on solid supports improving enzyme activity and stability (Wandrey et al. 2000; Sheldon et al. 2005).

Laccase immobilization has been addressed as a way to intensify and stabilize biocatalytic activity (Sheldon 2007). Due to the high specific surface (over 300 $m^2\ g^{-1}$) of nontoxic nanostructured support material, fumed silica nanoparticles (fsNP) are expected to be of interest for the immobilization of laccases (Corvini and Shahgaldian 2010). Furthermore, these systems are relatively cheap and are produced at industrial scale (Pratsinis 1998). First studies on the immobilization of this laccase from *C. polyzona* on spherical nanoparticles (spNP) produced by the Stöber method (Stöber et al. 1968) revealed promising laccase activity for the enzymatic degradation of bisphenol A (Galliker et al. 2010).

In the present study, we report on the coupling of a laccase of *C. polyzona* to the surface of fsNP. Different coupling procedures have been tested in order to obtain the highest loads of laccase activity on the nanostructured material keeping the laccase waste as low as possible. The coupling procedure has been compared to the previously reported spNP-laccase conjugates (Galliker et al. 2010). The produced fsNP–laccase conjugates have been characterized by scanning electron microscopy (SEM) and Brunauer–Emmett–Teller (BET) surface area measurements. Finally, the laccase stability in both phosphate buffer and wastewater has been assessed.

Materials and methods

General

Fumed silica nanoparticles (fsNP; surface area 390± 40 $m^2\ g^{-1}$; aggregates of particles with a size of 7 nm) and chemicals were purchased from Sigma-Aldrich (Switzerland). Laccase of *C. polyzona* was purchased from Wetlands Engineering SPRL (Belgium) and was always one time precipitated with acetone to remove possible impurities before applying to an experiment. Sörensen phosphate buffer (PB; 30 mM NaH_2PO_4, 40 mM Na_2HPO_4; pH 7) was used to suspend the nanoparticles. All experiments were carried out at room temperature. Protein contents were determined according to the Lowry method (Lowry et al. 1951) using a plate-reader (Synergy™ 2 Multi-Mode Microplate Reader, BioTek Instruments, Inc., Switzerland) and the Gen5 1.08 Data Analysis Software (BioTek Instruments, Inc., Switzerland) for detecting the absorbance of the emerging molybdenum blue at 750 nm. 2,2′-Azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) was used as substrate to determine laccase activity (Johannes and Majcherczyk 2000). Fifty microliters of laccase or fsNP–laccase conjugates containing sample (diluted in McIlvaine buffer 80 mM citric acid, 40 mM Na_2HPO_4; pH 3) were added to 150 μL of 0.267 mM ABTS (in McIlvaine buffer; pH 3) in 96-well plates. The ABTS oxidation was monitored by measuring the absorbance at 420 nm over 195 s (6 s intervals). The value of $\varepsilon_{[414\ nm]}$=36,000 L $mol^{-1}\ cm^{-1}$ (Childs and Bardsley 1975) as molar extinction coefficient of $ABTS^{\bullet+}$ was adjusted to $\varepsilon^*_{[420\ nm]}$=30,800 L $mol^{-1}\ cm^{-1}$ as interferences of remaining unreacted ABTS with the $ABTS^{\bullet+}$ can lead to an underestimation of enzyme activity (dependent on ABTS concentration) (Johannes and Majcherczyk 2000). One unit [U] was defined as the amount of laccase capable of oxidizing 1 μmol ABTS per minute. An analytical balance and a pH electrode from Mettler-Toledo GmbH, Switzerland, shakers from Edmund Bühler GmbH, Germany, and centrifuges from Vaudaux-Eppendorf AG, Switzerland were used for all experiments.

Synthesis of fsNP–laccase conjugates

Five hundred milligrams of fsNP were suspended in 25 mL PB. The suspension was sonicated (60 W, 45 kHz, continuous operation; USC 200–2,600, VWR International GmbH, Germany) for 10 min. Five hundred microliters of 3-aminopropyltriethoxysilane (APTES) were added, and the solution was incubated under gentle agitation (100 rpm) for at least 12 h. The unreacted APTES was washed away by seven centrifugation/re-suspension steps at 4,500×*g* for 3 min. The APTES content in the washing solutions was monitored as follows: 50 μL of a 5.3 mM glutaraldehyde

solution was added to 150 μL supernatant of the washing solution. The yellow coloration due to the imine bond resulting from the chemical reaction of APTES with glutaraldehyde was measured spectrophotometrically at 435 nm (limit of detection 1.8 μM). After seven washing steps, no APTES was longer detected.

The coupling procedure was performed using two different methods. The first approach is based on a previously developed method (Galliker et al. 2010), which was slightly optimized concerning washing steps (protocol method, P-M). One milliliter of glutaraldehyde (50% in water) was added to the amino-functionalized fsNP and the solution was incubated under agitation (100 rpm) for 1 h. The excess of glutaraldehyde was washed away by five centrifugation/re-suspension steps at 4,500×*g* for 3 min. The decreasing concentration of glutaraldehyde during the washing steps was monitored using the same colorimetric principle as for APTES (glutaraldehyde reacts with APTES and turns yellow), except that a 2.1 mM APTES solution was used as a reagent (limit of detection 4.3 μM). 7.5 U laccase per milligram fsNP were added and the solution was incubated under agitation (100 rpm) for at least 12 h. The excess and not stable bound enzymes were exhaustively washed away after six centrifugation/re-suspension steps at 4,500×*g* for 10 min. The supernatants containing free laccase and 0.5 mL of the fsNP–laccase conjugate suspensions (pellets re-suspended in the initial volume of PB) were collected after every washing step for further analysis.

As a second method (sorption-assisted immobilization, SAI), 7.5 U laccase per milligram fsNP were directly added to the amino-functionalized fsNP (after the APTES reaction) and the solution was incubated under agitation (100 rpm) for at least 1 h. One milliliter of glutaraldehyde (50% in water) was added dropwise to the mixture of fsNP and laccase and the solution was incubated under agitation (100 rpm) for at least 12 h. The unreacted glutaraldehyde and the excess and not stable bound enzymes were washed away by six centrifugation/re-suspension steps at 4,500×*g* for 10 min. The supernatants containing free laccase and 0.5 mL of the fsNP–laccase conjugates suspensions (pellets re-suspended in the initial volume of PB) were collected after every washing step for further analysis.

In order to compare the efficiency of the two methods to simple sorption of laccase to fsNP, 20 mg of fsNP were suspended in 1 mL PB. 7.5 U of laccase per milligram fsNP were added, and the suspension was incubated under agitation (100 rpm) for at least 12 h. The enzyme remaining in the supernatant was washed away by six centrifugation/re-suspension steps at 5,000×*g* for 3 min. The supernatants containing free laccase and 0.2 mL of the fsNP–laccase conjugates suspensions (pellets re-suspended in the initial volume of PB) were collected after every washing step for further analysis.

Comparison of APTES-activation procedures applied to both spNP and fsNP

Spherical nanoparticles (spNP) were produced according to the Stöber method (Stöber et al. 1968). Briefly, 9.12 mL ammonia (25% in water) were first poured into 129.83 mL EtOH (100%) and 11.05 mL tetraethyl orthosilicate (100%) were added to the mixture. The solution was incubated under moderate stirring at 20°C for 24 h before it was washed three times with EtOH and three times with water. For the APTES-activation step, 40 mg of fsNP, as well as spNP, were suspended on the one hand in 2 mL PB, and on the other hand, in 2 mL solution consisting of acetone and water (99:1 *v*/*v*) as previously described (Cabana et al. 2009b). After seven centrifugation/re-suspension washing steps at 5,000×*g* for 3 min, both series of APTES-activated nanoparticles were re-suspended in PB and the coupling procedure as described above was applied using corresponding amounts of laccase and glutaraldehyde.

Optimization of applied laccase amount

FsNP–laccase conjugates were produced following the above-described SAI method. Four different amounts of laccase were tested, i.e., 7.5, 6.0, 4.0, and 3.0 Umg^{-1} fsNP. The subsequent reaction with laccase and glutaraldehyde lasted 96 h.

Scanning electron microscopy studies

The following fsNP samples were analyzed by SEM after sonication: fsNP suspended in PB, fsNP suspended in aerobically treated effluent from the wastewater treatment plant Birsfelden, Switzerland (chemical oxygen demand, COD=24 mg L^{-1}; pH 8.2), fsNP with sorbed laccase, fsNP after APTES modification, fsNP after modification with laccase and glutaraldehyde according to P-M and SAI methods. The SEM analyses were carried out as previously described (Galliker et al. 2010) using a Supra 40 V system (Carl Zeiss, Switzerland) with accelerating voltages of 15 and 20 kV, respectively.

Surface area measurements by Brunauer–Emmett–Teller analysis

Between 6 and 50 mg of the following samples were sonicated for 10 min and lyophilized at 0.008 mbar (Gamma 2–16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Germany)—unsuspended fsNP, fsNP suspended in PB, fsNP suspended in effluent from the wastewater treatment plant Birsfelden, Switzerland, fsNP with sorbed laccase, amino-functionalized fsNP (after the APTES modification), and fsNP–laccase conjugates obtained using the P-M and the novel SAI methods

as well as spNP with and without APTES modification. Subsequently, the nanoparticles were flushed with nitrogen in a sample degassing system for at least 3 h (FlowPrep 060, Micromeritics GmbH, Germany). The surface area of the nanoparticles (cooled with liquid nitrogen) was determined by measuring the amount of helium binding over time to the nanoparticles using a surface area and pore size analyzer (Gemini V, Micromeritics GmbH, Germany).

Stability assay in PB

One-milliliter aliquots of fsNP–laccase conjugates suspended in PB, produced according to the two different methods described above, were incubated under agitation at 230 rpm for 32 days. Samples were taken at 11 different dates for measurements of remaining enzyme activity; to ensure reproducibility the experiments were carried out in triplicates. Samples were centrifuged at 5,000×*g* for 3 min and the pellets re-suspended in 1-mL PB. The supernatants were centrifuged a second time at 16,100×*g* for 3 min to pelletize remaining traces of nanomaterials. The laccase activity associated to both re-suspended pellets and supernatant fractions was determined.

To compare the stability of the immobilized enzyme with that of its soluble form, free laccase was dissolved in PB at an enzymatic activity of approximately 10 $U mL^{-1}$ in triplicates. The mixtures were incubated under agitation (230 rpm). Samples were taken at seven different dates over 29 days.

Stability assay in wastewater

The enzyme stability assay in wastewater was carried out similarly to that performed in PB. The fsNP–laccase conjugates produced according to the novel developed SAI method were suspended in effluent water from the wastewater treatment plant Birsfelden, Switzerland. Samples were taken at 11 different dates over 32 days.

To compare the stability of the immobilized enzyme with that of its soluble form, a control incubation experiment with the solubilized enzyme was carried out as for the stability test in PB. Additionally, the stability of sorbed laccase to fsNP was tested as in the previously described stability assay.

Results

Synthesis of fsNP–laccase conjugates

Laccase-modified nanoparticles have been produced by means of two different methods. On the one hand, laccase was coupled on fsNP as previously described (Galliker et al. 2010) with slight optimizations (P-M). After modification of the fsNP with APTES and glutaraldehyde, laccase was coupled to the activated fsNP surface. On the other hand, a novel procedure was developed called SAI method. In this procedure, glutaraldehyde is added dropwise to the mixture of APTES-modified fsNP and laccase.

Producing fsNP–laccase conjugates by applying 7.5 U laccase per milligram fsNP led to different results when comparing the two different coupling methods. Whereas 29.8±0.8% of the laccase activity was bound to fsNP according to P-M method, more than the double amount of laccase activity (61.4±1.8%) remained on fsNP when applying the SAI (Table 1) method for the preparation of the fsNP–laccase conjugates. The residual activities (difference to 100%) did not bind or sorb to the fsNP but remained in solution. The laccase activity load of 2.67±0.08 $U mg^{-1}$ fsNP was as well almost twice as high than that obtained using the previously developed P-M method (1.47±0.04 $U mg^{-1}$ fsNP). The specific activity was slightly higher (7.70±0.22 $U mg^{-1}$ protein) on SAI fsNP than on P-M ones (6.34±0.17 $U mg^{-1}$ protein). As a comparison element, the simple sorption of laccase to the nanomaterial was also assessed by mixing fsNP with lacccase without addition of any chemical substances. In this case, 32.8±0.9% of the laccase activity remained associated to the fsNP, which resulted in an enzymatic load of 1.50±0.04 U mg^{-1} fsNP and a specific activity of 7.23±0.20 $U mg^{-1}$ protein.

Comparison of APTES-activation procedures applied to both spNP and fsNP

For benchmarking, the coupling procedure of laccase was applied to fsNP as well as with spNPs. In addition, it was tested whether carrying out the APTES reaction in acetone could lead to any improvement of the enzyme load compared to APTES-activation step in PB. The specific laccase activity was not significantly different (11.36±0.48 compared to 10.14±0.78 $U mg^{-1}$ protein) when carrying out the APTES modification step of the fsNP coupling procedure in acetone compared to PB. Inversely, the laccase activity load on fsNP prepared in PB was much higher (3.29±0.25 compared to 2.44±0.10 $U mg^{-1}$ fsNP) than when organic solvent was used (Table 2). The binding efficiency (percentage of applied laccase activity finally coupled on fsNP) of the laccase activity to the fsNP was noticeably higher (63.0±4.9%) when applying the APTES modification in PB than in acetone (48.5±2.1%). These differences were hardly observed when spNP were used as support material. Using spNP led to a quite low binding efficiency in both cases (4.2±0.2% in PB compared to 4.0±0.1% in acetone). The enzyme activity loads on spNP (0.23±0.01 in PB and 0.24±0.01 $U mg^{-1}$ spNP in acetone)

Table 1 Comparison of sorbed laccase to fumed silica nanoparticles (fsNP) and fsNP–laccase conjugates produced using two different methods

	Binding efficiency[a] [%]	Enzyme load[b] [U mg^{-1} fsNP]	Specific activity[c] [U mg^{-1} protein]
Sorption	32.8±0.9	1.50±0.04	7.23±0.20
P-M[d]	29.8±0.8	1.47±0.04	6.34±0.17
SAI[e]	61.4±1.8	2.67±0.08	7.70±0.22

[a] Binding efficiency was defined as the laccase activity bound to fsNP in percent [%] after exhaustive washing relative to the initially applied laccase activity (100%). The difference to 100% remained in solution

[b] Laccase activity load was defined as the activity remaining associated to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) applied fsNP

[c] Specific activity was defined as the laccase bound to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) protein

[d] Optimized protocol method (Galliker et al. 2010)

[e] Novel-developed sorption-assisted immobilization method

were rather low. The laccase binding efficiency as well as the laccase activity load on fsNP APTES-modified in PB compared to spNP in the same buffer was 15 times higher.

Optimization of applied laccase amount

In order to produce fsNP–laccase conjugates with optimized enzyme loads and as well to develop an efficient binding of the enzyme laccase, the coupling procedure was optimized concerning the applied laccase amount. The binding efficiency was increased from 75.1±6.4% to 98.2±4.3% when decreasing amounts of laccase (from 7.5 to 3.0 Umg^{-1} fsNP) were applied (Table 3). The laccase activity load on the fsNP as well as the specific activity of the laccase was markedly augmented when decreasing amounts of enzymes were used for the last step of the coupling. The load and efficiency of binding were the highest (2.92±0.15 Umg^{-1} fsNP; 11.3±0.6 Umg^{-1} protein) when 4.0 Umg^{-1} fsNP were applied.

Scanning electron microscopy studies

Differently treated fsNP samples were analyzed by SEM to detect changes of the surface during the coupling procedure of laccase to fsNP. Visualization of the fsNP with low magnifications (×20,000 and ×57,050) revealed the high tendency of these fsNP to aggregate. The fsNP were forming some large aggregates with a size of more than 1 μm in suspension (Fig. 1). In turn, these aggregates were further clumping together to form bigger aggregates when applying the samples on the mica surfaces for SEM analysis. The size of these aggregates was in the order of several micrometers.

Microscopic observations carried out at a higher magnification (×150,000) revealed some differences at the surface of the various fsNP samples. FsNP aggregates in PB (Fig. 2), in wastewater (not shown), with sorbed laccase (not shown) and after modification with APTES (Fig. 2) consisted of tiny beads forming cauliflower-like shapes.

Table 2 Comparison of APTES-activation procedures applied to both spherical nanoparticles (spNP) and fumed silica nanoparticles (fsNP)

	Binding efficiency[a] [%]	Enzyme load[b] [U mg^{-1} fsNP]	Specific activity[c] [U mg^{-1} protein]
fsNP			
In PB[d]	63.0±4.9	3.29±0.25	10.14±0.78
In acetone	48.5±2.1	2.44±0.10	11.36±0.48
spNP			
In PB	4.2±0.2	0.23±0.01	8.20±0.30
In acetone	4.0±0.1	0.24±0.01	9.10±0.09

[a] Binding efficiency was defined as the laccase activity bound to fsNP in percent [%] after exhaustive washing relative to the initially applied laccase activity (100%)

[b] Laccase activity load was defined as the activity remaining associated to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) applied fsNP

[c] Specific activity was defined as the laccase bound to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) protein

[d] Phosphate buffer (pH 7)

Table 3 Comparison of fumed silica nanoparticle (fsNP)–laccase conjugates produced with different applied laccase amounts

Applied laccase [U mg^{-1} fsNP]	Binding efficiency[a] [%]	Enzyme load[b] [U mg^{-1} fsNP]	Specific activity[c] [U mg^{-1} protein]
7.5	75.1±6.4	2.56±0.22	8.36±0.71
6.0	81.0±5.7	2.66±0.19	8.9±0.63
4.0	93.9±4.6	2.92±0.15	11.3±0.60
3.0	98.2±4.3	2.47±0.11	10.6±0.47

[a] Binding efficiency was defined as the laccase activity bound to fsNP in percent [%] after exhaustive washing relative to the initially applied laccase activity (100%)

[b] Laccase activity load was defined as the activity remaining associated to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) applied fsNP

[c] Specific activity was defined as the laccase bound to the fsNP after exhaustive washing in units per milligram (U mg^{-1}) protein

The beads of fsNP–laccase conjugates aggregates after modification with laccase and glutaraldehyde according to SAI (Fig. 2) as well as P-M (not shown) methods were clearly larger and did not display the cauliflower-like shapes. The aggregates were rather round shaped with a smooth surface.

Surface area measurements by Brunauer–Emmett–Teller analysis

Besides SEM, differently treated fsNP samples were analyzed for surface area by means of BET analysis. BET analyses of non-suspended fsNP (directly used for BET without previous suspension in buffer) revealed a surface of 320.3±16.1 m^2 g^{-1} fsNP (Fig. 3). When suspensions of fsNP in wastewater and PB with subsequent lyophilisation were analyzed the surface area decreased to 305.1±15.9 and 209.8±11.1 m^2 g^{-1} fsNP, respectively. FsNP with sorbed laccase showed a surface of 193.1±12.6 m^2 g^{-1} fsNP. The surface of fsNP decreased stepwise in the time course of the immobilization procedure. A surface of 158.3±8.3 m^2 g^{-1} fsNP was measured subsequently to the APTES modification step. The laccase–glutaraldehyde reaction led to a further decrease of the nanoparticle surface as a surface of 108.7± 4.8 m^2 (for SAI method) and 110.6±6.5 m^2 g^{-1} fsNP–laccase conjugates (for P-M method) was measured. As an element of comparison, spNP had a surface of only 17.4± 0.9 m^2 in suspension and 15.9 m^2 g^{-1} spNP (single measurement) after APTES modification.

Stability assay in PB

The activity of free laccase as a benchmark and immobilized laccase on fsNP was tested for its stability in PB (pH 7) over a 1 month period. Free laccase was rather unstable over time. After 29 days incubation in PB, only 20.1±1.7% of the initial laccase activity in an assay with ABTS as a substrate was observed (Fig. 4). On the contrary, the laccase immobilized on fsNP was much more stable. The activity of fsNP–laccase conjugates decreased to 63.3± 1.3% (for P-M method) and 57.0±2.2% (for SAI method) after 32 days incubation. As a result, the enzymatic stability of the conjugates obtained using the SAI method seemed to be slightly inferior (6.3% less) to those produced by the P-M method. Nevertheless, as the laccase activity load is higher with the novel developed SAI method than applying the P-M method (2.67±0.08 and 1.47±0.04 Umg^{-1} fsNP, respectively), the total laccase activity remained considerably higher in SAI-produced conjugates after 1 month of incubation. The pH of the free laccase samples as well as the samples with fsNP–laccase conjugates was constant (7.0) over incubation time (data not shown).

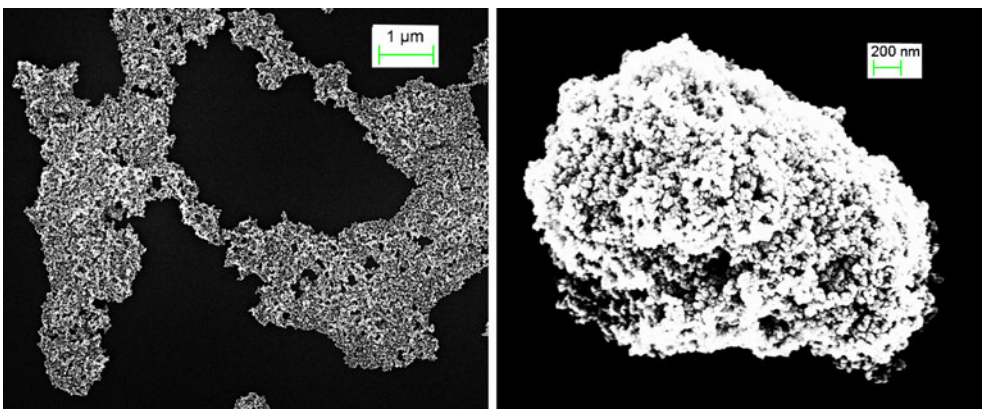

Fig. 1 SEM analyses. *Left*, fumed silica nanoparticles (fsNP) highly aggregated on mica surface, ×20,000 magnification, 20 kV; *right*, fsNP aggregated to a large clump, ×57,050 magnification, 15 kV. Samples were taken from a suspension in phosphate buffer (pH 7)

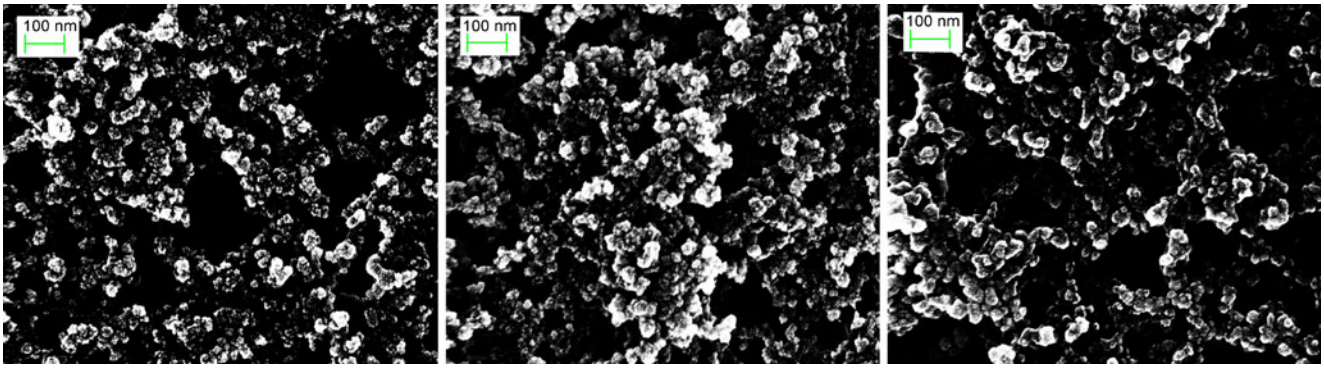

Fig. 2 SEM analyses. *Left*, close-up view of fumed silica nanoparticles (fsNP); *middle*, close-up view of fsNP after modification with APTES; *right*, close-up view of fsNP after modification with laccase and glutaraldehyde according to novel-developed sorption-assisted immobilization method. Samples were taken from a suspension in phosphate buffer (pH 7), ×150,000 magnification, 20 kV

Stability assay in wastewater

The stability of the enzymatic activity associated to the fsNP–laccase conjugates (according to SAI method) as well as for free and sorbed laccase was assessed under application-relevant conditions in real wastewater (pH 8.2) over 1 month (Fig. 5). The SAI-produced conjugates were satisfyingly stable as 77.4±1.7% of the initial laccase activity remained after 25 days incubation (81.0% after 32 days incubation, but not in triplicate). In contrast, the activity of free laccase in wastewater decreased to 2.5±1.2% within 29 days. The activity of laccase simply sorbed to fsNP decreased in a comparable fashion to 9.5±0.9% after 31 days. The original pH of wastewater (8.2) dropped instantly to 7.0 when the free laccase samples and the sorbed laccase samples were prepared at the beginning of the stability assay and remained constant thereafter (data not shown). In the samples with fsNP–laccase conjugates the pH dropped instantly to 7.3, decreasing to 7.0 during the stability assay (data not shown).

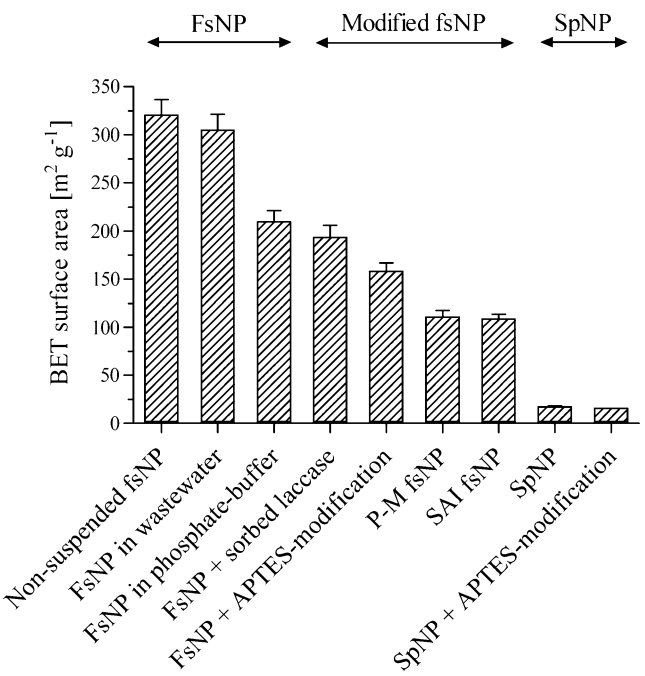

Fig. 3 Surface area determination by BET analysis of differently treated fumed silica (fsNP) and spherical nanoparticles (spNP). *P-M* optimized protocol method (Galliker et al. 2010). *SAI* novel-developed sorption-assisted immobilization method. *SpNP+ APTES-modification* not in triplicate but single measurement

Discussion

The immobilization of different laccases on solid surfaces becomes of increasing interest. Immobilization on magnetically separable mesoporous silica spheres (Zhu et al. 2007), on nanoporous gold particles (Qiu et al. 2009), and mesostructured cellular foams (Rekuć et al. 2009) have

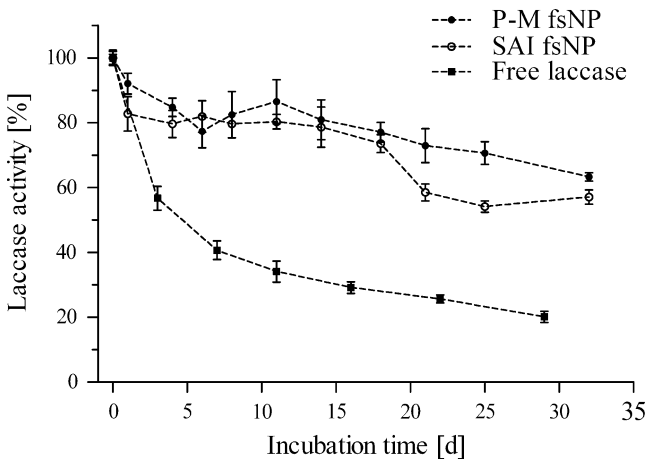

Fig. 4 Stability assay in phosphate buffer (pH 7) over 1 month: fumed silica nanoparticle (fsNP)–laccase conjugates obtained by various methods and free laccase as a reference were tested. *P-M* optimized protocol method (Galliker et al. 2010). *SAI* novel-developed sorption-assisted immobilization method

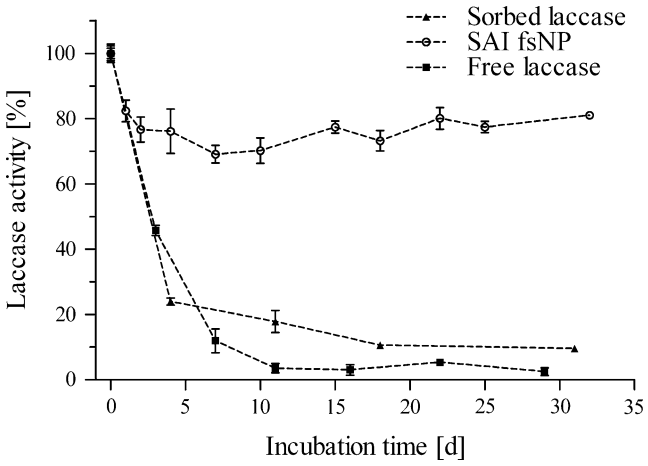

Fig. 5 Stability assay in wastewater (pH 8.2) over 1 month: fumed silica nanoparticle (fsNP)–laccase conjugates obtained by the novel-developed SAI method (sorption-assisted immobilization), fsNP with sorbed laccase and free laccase as a reference were tested. Data point of SAI fsNP at t=32 [d] not in triplicate but single measurement

successfully been carried out during the last few years. In this study, a further successful laccase immobilization strategy was developed.

With the novel SAI method, it was possible to bind 2.67±0.08 $U mg^{-1}$ nanoparticles of laccase activity to fsNPs. Due to the fact that the laccase load with SAI method is almost twice as high as the coupling procedure with the P-M (Galliker et al. 2010) (1.47±0.04 $U mg^{-1}$ fsNP), we assume that the immobilization of laccase does not only occur via classic cross-linking to the amino groups of APTES-modified particles. In addition, simple sorption of the laccase to the fsNP surface with subsequent immobilization through intermolecular cross-linking of the sorbed enzyme molecules by glutaraldehyde may occur, leading to the formation of so-called covalently linked enzyme aggregates (CLEAs) at the surface of the silica material. An improved immobilization through cross-linking with glutaraldehyde was demonstrated before for chloroperoxidase (Kadima and Pickard 1990), glutaryl 7-aminocephalosporanic acid acylase, D-amino acid oxidase, and glucose oxidase (López-Gallego et al. 2005), even though other support materials and slightly different procedures were applied. The validity of this hypothesis is reflected by the tendency of the laccase to sorb to fsNP as demonstrated by means of a sorption test (1.50±0.04 $U mg^{-1}$ fsNP).

Since fsNP have a strong tendency to aggregate to bigger particles forming structures with large surface and numerous interstices (as seen by SEM), laccase is sorbing to the fsNP surface/interstices (cross-linking with itself and APTES) and is afterwards immobilized with glutaraldehyde, which leads to a more random structure compared to the clearly structured covalent binding on P-M fsNP. The structure of the fsNP aggregates is consisting of small cauliflower-like beads. These tiny structures disappeared after the glutaraldehyde addition, which is "coating" the fsNP resulting in a smooth surface. The decrease of the fsNP surface area by glutaraldehyde was detected by BET surface area analysis. While the surface was 158.3±8.3 $m^2 g^{-1}$ for fsNP after the APTES modification, the final fsNP–laccase conjugates had a surface of approximately 110 $m^2 g^{-1}$ fsNP, independently of the method used for the last step of the immobilization procedure.

Our method led to different results as those obtained by another group (Cabana et al. 2009a) who reported that the simultaneous addition of laccase and glutaraldehyde to diatomaceous earth support Celite® R-633 results in less activity remaining associated to the solid surface than adding laccase after the removal of excess glutaraldehyde (P-M method). This effect was essentially attributed to cross-linking of laccase building CLEAs (Cabana et al. 2007a; Sangeetha and Abraham 2008). Further complementary experiments showed that CLEAs (without NP) were only marginally produced using the novel method: Applying laccase and glutaraldehyde into microtubes and centrifuging the possible CLEAs at 5,000×g for 5 min did not reveal any pellet (data not shown). Furthermore, centrifugation (5,000×g for 5 min) of fsNP–laccase conjugates on the one hand in PB and on the other hand through a 40% sucrose solution showed that maximally 1% of the total laccase activity remained in the top layer of the microtubes (data not shown).

The material used by Cabana et al. (2009b) had a surface of 1.3 $m^2 g^{-1}$ material, whereas our fsNP have a surface of 320.3±16.1 $m^2 g^{-1}$. This surface difference combined with the different surface structure of their material is most probably the reason why Cabana et al. (2009a) were able to bind maximally 0.0008 $U mg^{-1}$ material compared to 2.67±0.08 $U mg^{-1}$ obtained with the present procedure. Testing the spNP possessing a surface of 17.4±0.9 $m^2 g^{-1}$ (theoretical surface calculation for spNP, ~21 $m^2 g^{-1}$) and low porosity (Galliker et al. 2010) did as well result in less activity binding (0.23±0.01 $U mg^{-1}$ spNP).

The silanization of nanoparticles with APTES was tested as well in acetone but led to unsatisfying results compared to the modification in PB. A positive effect of water on the polymerization of the reagent during silanization of silica surface was described before (Krasnoslobodtsev and Smirnov 2002).

Small differences in specific activities in all experiments should be neglected since the Lowry test is slightly disturbed by interferences with a wide variety of chemicals (Rodríguez-Vico et al. 1989; Winters and Minchin 2005). In our experiments, fsNP silanized with APTES (without any protein) revealed a minor protein amount (data not shown) with the Lowry test. In fact the amino groups of the APTES can lead to a false-positive result (Lleu and Rebel 1991).

Due to the cross-linking on fsNP–laccase conjugates produced after SAI method, more amino groups of the APTES are saturated and do not contribute to the protein content any more. This might explain, why SAI-produced fsNP–laccase conjugates have a higher specific activity than P-M ones, as well as spNP–laccase conjugates have a lower specific activity than fsNP ones. Furthermore, diffusion limitations of the substrate ABTS and selective immobilization of laccase on nanoparticles cannot be excluded.

When looking at the SEM pictures, it was visible that most of the fsNP samples were aggregating to microparticles in the range of 1–10 μm. This is actually an advantage in perspective of possible future applications of immobilized laccases for the elimination of EDCs such as bisphenol A in wastewater treatment plants. It is much cheaper to retain bigger particles (e.g., fsNP) with a filter membrane (ultrafiltration) than particles in the nanometer range (e.g., spNP) by nanofiltration due to increasing energy costs with decreasing membrane pore size (Schäfer et al. 2001). However, further research is necessary concerning the rigidity of the formed aggregates.

The coupling procedure for fsNP–laccase conjugates was optimized to bind as much of the initially applied laccase as possible and to obtain the highest loads of laccase activity on the fsNP. With 4.0 $U mg^{-1}$ fsNP, the highest activity (2.92±0.15 $U mg^{-1}$ fsNP) was bound. Applying 3.0 U laccase, only 2.47±0.11 $U mg^{-1}$ fsNP was observed, but less laccase (only about 1.8%) was lost. Under these conditions, a good compromise between the cost of the enzyme and the immobilized activity was found.

The stability of immobilized enzyme such as the presently developed fsNP–laccase conjugates is a crucial parameter if the material is intended for wastewater treatment applications. Due to harsh environmental conditions such as non-optimal pH or non-ideal temperature (Brady and Jordaan 2009) as well as the presence of proteases leading to proteolytic reactions, free soluble enzymes are not suitable for applications in reality (Modaressi et al. 2005). The free laccase quickly lost about 80% of its activity in PB and 97% in wastewater over 1 month. The large amount of impurities present in wastewater is supposed to be the reason why free laccase is more stable in a synthetic buffer. The fsNP–laccase conjugates produced by P-M as well as SAI methods lost about 40% of the laccase activity over 1 month in PB, whereas in the application relevant wastewater, SAI fsNP–laccase conjugates lost only about 23% of its activity. This might be due to the slightly higher pH in wastewater (originally 8.2, instantly decreased to 7.3 when the conjugates were added) compared to PB (7.0) or due to the presence of non-identified components (organics, inorganics) and necessitates further investigation. The loss of activity in both cases is supposed to be due to a partial inactivation of the immobilized enzymes and additional release into the surrounding solution with subsequent fast inactivation of the free enzymes, since almost no activity was found in the corresponding supernatants. The stabilizing effect of laccase immobilization on solid surfaces due to reduced number of contact points for proteases, decreased possibility of disadvantageous enzyme folding and increased stability against unfavorable pH was also shown by other studies (Brady and Jordaan 2009; Rekuć et al. 2010; Wang et al. 2010; Zhao et al. 2011). Additionally, it is noteworthy that the shear forces under the applied stability assay conditions (shaking 230 rpm) were actually much stronger than in a wastewater treatment plant. Therefore, the stability test applied rather represents a "worst-case scenario". In conclusion, the presently developed "sorption-assisted immobilization" constitutes an efficient method to stabilize laccase for use under environmentally relevant conditions.

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