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Surface immobilization strategies for tyrosinase as biocatalyst applicable to polymer network synthesis

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ABSTRACT

Enzymes have recently attracted increasing attention in material research based on their capacity to catalyze the conversion of polymer-bound moieties for synthesizing polymer networks, particularly bulk hydrogels. In this study, the surface immobilization of a relevant enzyme, mushroom tyrosinase, should be explored using glass as model surface. In a first step, the glass support was functionalized with silanes to introduce either amine or carboxyl groups, as confirmed e.g. by X-ray photoelectron spectroscopy. By applying glutaraldehyde and EDC/NHS chemistry, respectively, surfaces have been activated for subsequent successful coupling of tyrosinase. Via protein hydrolysis and amino acid characterization by HPLC, the quantity of bound tyrosinase was shown to correspond to a full surface coverage. Based on the visualized enzymatic conversion of a test substrate at the glass support, the functionalized surfaces may be explored for surface-associated material synthesis in the future.

Introduction

Enzymes as biocatalysts have been utilized for a long time to synthesize small molecule substances in biotechnological processes. More recently, the enzymatic conversion of substrates has attracted increasing attention in material research based on their capacity to convert polymer-bound moieties leading to polymer networks, specifically bulk hydrogels [1].

In this context, particularly those enzymes are of interest, which enable the coupling of functional moieties into netpoints, as it is the case for tyrosinase [2]. Its specificity to catalyze phenol oxidation has been suggested, at least for highly pure mushroom tyrosinase (MTyr), to potentially allow hydrogel synthesis also in the presence of tyrosine-free therapeutic peptides for drug release purposes [3].

While previous studies on MTyr-catalyzed hydrogels focus on bulk materials, transferring this principle of hydrogel formation to surfaces would be of high interest to create functional interfaces. However, a stable immobilization of MTyr to the surface is required to realize such concepts. Although various techniques for enzyme binding are known [4], the goal of a high surface coverage and preservation of catalytic activity demands a detailed evaluation for each specific enzyme. Here, a three-step process should be investigated for MTyr using glass as model surfaces comprising salinization to introduce amine or carboxyl anchor groups, reaction with glutaraldehyde and EDC/NHS chemistry, respectively, and eventually enzyme immobilization (Fig. 1). The success of the critical steps should be thoroughly characterized.

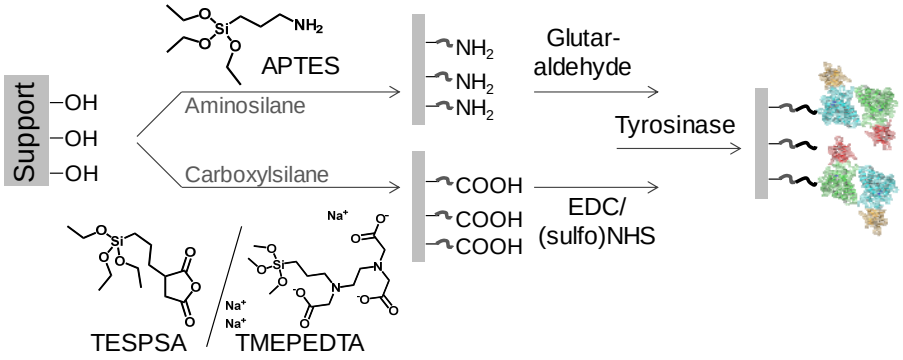

Fig. 1: *Immobilization strategy for MTyr comprising salinization, reaction with coupling reagents, and eventually protein binding.*

Experimental

Cover slips (Menzel glass, Germany) were cleaned with a 7.5:1 mixture of 96 wt.% H_2SO_4 and 30 wt.% H_2O_2, followed by extensive washing with water (always Millipore water used). Silanization was performed at room temperature with 5 vol.% *N*-[3-(trimethoxysilyl)propyl]-ethylenediamine triacetic acid (TMEPEDTA) in water, 2.5 vol.% 3-(Triethoxysilyl)-propylsuccinic anhydride (TESPSA) in a 1:1 mixture of ethanol and water (both silanes from ABCR GmbH, Karlsruhe, Germany; used at pH 4-5), or 5 vol.% (3-Aminopropyl)triethoxysilane (APTES) in ethanol. This step was followed by washing with water and ethanol, as well as drying at 70 °C for 15 min. Contact angles were determined after sample equilibration in water. A repeated dynamic analysis by the captive bubble method was conducted at two different sample positions with a DSA 100 (Krüss, Hamburg, Germany). The zeta potential was determined with a Delsa Nano C equipped with a Flat Surface Cell (Beckmann Coulter, Krefeld, Germany) by aid of reference particles according to the manufacturers protocol (Otsuka Electronics, Japan; 1:100 dilution). Silanized glass slips were glued to larger glass slides for proper fixation in the instrument cell. Confocal Raman microscopy was performed on a Senterra instrument (Bruker, Ettlingen, Germany) with a 532 nm laser at a power of 20 mW and a resolution of 2 cm^{-1}. For X-ray photoelectron spectroscopy studies, an Axis Ultra system (Kratos Analytical, Manchester, UK) with a monochromatic Kα beam source (1486.6

eV) was employed. The scans were conducted at 300 W with a pass energy of 160 eV (survey spectra) and 20 eV (regional spectra; C 1s, N 1s).

For enzyme coupling, APTES treated glass was incubated for 1 h in 2 vol.% glutaraldehyde, followed by washing in phosphate buffered saline (PBS, 50 mM, pH 7.4) and incubation with 2 $mg \cdot ml^{-1}$ MTyr (Sigma Aldrich, Steinheim, Germany) in PBS for 16 h at 4 °C followed by washing. TESPSA treated glass was first exposed at 4 °C for 1 h to ~0.3 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 0.1 M MES buffer pH 5.1 substituted with either 0.3 mM N-hydroxysuccinimide (NHS; all from Sigma Aldrich) or 0.3 mM N-Hydroxysulfosuccinimide (sulfo-NHS; G-Biosciences, USA). After washing with MES buffer, the samples were incubated with 2 $mg \cdot ml^{-1}$ MTyr in 0.1 M MES pH 8 at 4 °C for 16 h, again followed by washing with 0.8 M NaCl. All samples were stored in PBS. The MTyr quantification by amino acid analysis was conducted using HPLC as reported before [5]. Up to ten glass slides of 21 x 26 mm were incubated in 2 ml 6N HCl for enzyme hydrolysis at 120 °C for 24 h. For the MBTH assay, an oxygen saturated reagent solution with final concentrations of 6 mM 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH), 0.181 mM 3-(4-Hydroxyphenyl)-propionic acid (DAT, Sigma Aldrich) and 2 vol.% DMF in 50 mM PBS was used.

Results and discussion

In order to systematically explore the MTyr binding to the support surface, two approaches have been compared: (i) The introduction of amine moieties via APTES, which ideally react with one aldehyde group of glutaraldehyde to a Schiff base, while the second aldehyde function would be available to couple with primary amines of MTyr. (ii) The reaction with either TESPSA or TMEPEDTA as carboxylsilanes, activation via EDC/(sulfo)NHS chemistry, and binding of MTyr by forming an amid bond (Fig. 1).

Introducing anchor groups to glass surfaces by salinization

The effect of the carboxylsilane treatment on surface properties depending on reaction time was studied by analyzing the contact angle θ in water by the captive bubble method. Despite some alterations, the surfaces remained hydrophilic with $\theta < 40°$ (Tab. 1), which is well below the so-called Berg limit that often is used to describe hydrophobic surfaces that massively adsorb proteins by physical interactions [6]. Starting from a typical slightly negative zeta potential of pure glass, the treatment with carboxylsilanes did not show systematic shifts towards a more negatively charged surface with increasing reaction time. Therefore, an analysis of surface composition via X-ray photoelectron spectroscopy (XPS) was applied. By fitting the characteristic asymmetric carbon signal (C1s) with 3 peaks of Gaussian type, which can be attributed to C-C at 285 eV, -O-C=O, COOH at 289.1 eV or C-O-C, C-OH at 286.5 eV, the relative peak area corresponding to COOH functions was shown to clearly increase from 6 ± 1% for cleaned glass to 19 ± 6% or 12 ± 2% for 19 h of TESPSA and TMEPEDTA treatment, respectively (Tab. 1). In order to verify the introduction of COOH moieties by a second independent method, Raman microscopy with long accumulation times (4.5 h) was applied for selected samples (Fig. 2). As may be exemplified by the bands observed at 1731 cm^{-1} for 17 h TESPSA incubation, which can be assigned to the stretching vibration of carbonyl groups [7], successful introduction of COOH moieties to the glass surface was confirmed.

Table 1: Silanization of glass surfaces with the carboxyl-functionalized silanes.

Sample	Contact angle		Zeta potential	XPS surface analysis			
					Fitting of C1S signal		
	Advancing [°]	Receding [°]	[mV]	Total C1s signal [at %]	C-C [%]	C-O-C; C-OH [%]	-O-C=O; COOH [%]
Glass untreated	26 ± 4	25 ± 3	-11 ± 8	21 ± 2	78 ± 5	18 ± 5	4 ± 1
Glass cleaned	25 ± 6	21 ± 2	-5 ± 3	13 ± 3	75 ± 6	20 ± 5	6 ± 1
TESPSA 5 min	25 ±1	22 ± 2	-8 ± 2	7 ± 1	68 ± 3	23 ± 3	8 ± 0.5
TESPSA 3 h	35 ± 12	28 ± ±	-5 ± 2	17 ± 11	52 ± 25	28 ± 16	20 ± 9
TESPSA 19 h	39 ± 3	29 ± 4	-18 ± 13	11 ± 3	52 ± 17	29 ± 11	19 ± 6
TMEPEDA 5 min	24 ± 3	22 ± 2	-16 ± 5	7 ± 1	63 ± 7	28 ± 6	9 ± 1
TMEPEDA 3 h	30 ± 15	26 ± 9	-11 ± 4	9 ± 2	56 ± 7	31 ± 5	13 ± 3
TMEPEDA 19 h	23 ± 5	21 ± 2	-9 ± 7	14 ± 6	59 ± 5	29 ± 4	12 ± 2

n= 3-6, mean ± S.D.

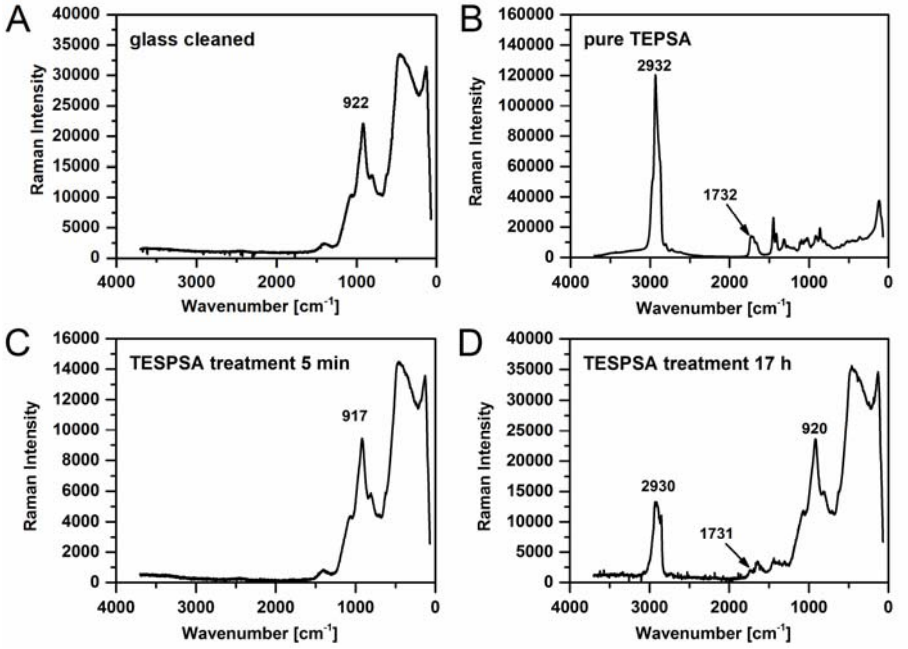

Fig. 2: Raman microscopy analysis of introduced anchor groups by salinization with TESPSA (2930 cm^{-1} C–H stretching vibration; 1731 cm^{-1} C=O stretching vibration, 920 cm^{-1} Si-O stretching vibration). Note: In this experiment, the longest TESPSA treatment was 17 h compared to 19 h in other experiments.

For surface treatment with the aminosilane APTES, the analysis of the zeta potential clearly demonstrated the anticipated shift towards less negative values, which can be assigned to the introduced amino groups. A clear evidence of amino moieties being exposed at the functionalized surface was provided by XPS analysis, where the nitrogen content increased from 0.5 at% for cleaned glass to 6 at% after 3 h of APTES treatment.

In summary, XPS supported by a second independent technique allowed concluding on a successful introduction for the carboxylsilanes TESPSA and TEMPEDA as well as the aminosilane APTES. For further experiments, the incubation with TESPSA and APTES for 3 h was chosen.

Table 2: Silanization of glass surfaces with the amino-functionalized silane.

Sample	Zeta potential [mV]	XPS surface analysis N 1s [at %]
Glass cleaned	-13 ± 5	0.5 ± 0.3
APTES 5 min	-2 ± 2	n.d.
APTES 3 h	1 ± 0.4	6 ± 3
APTES 19 h	-1 ± 1	n.d.

n.d. = not determined; n= 3-5, mean ± S.D.

Table 3: Quantification of surface bound MTyr by amino acid analysis.

Coupling reaction	Amount of bound tyrosinase [µg·cm^{-2}]	Estimated surface coverage * [%]
TESPSA + EDC/NHS	0.2 ± 0.02	65
TESPSA + EDC/sulfo-NHS	0.57 ± 0.09	230
APTES + Glutaraldehyde	0.71 ± 0.13	280

* Surface coverage of 100% corresponds to monolayer (0.31 µg·cm^{-2}); MW of MTyr 120 kDa; R_H 4.3 nm [8]; Close-packing of spheres P_{2D} 0.91, P_{3D} 0.74; Calculation of theoretical weight per mono/multilayer: MW [kDa] · 1.66·10^{-15} [µg·kDa^{-1}] · $P_{2D/3D}$ · π·(R_H [nm])$^{-2}$ · 10^{14} [nm^2·cm^{-2}]. Data are mean and SD of 3 analyses; experimental error of method ~ 20%.

Enzyme immobilization, quantification and proof of catalytic activity

As introduced above, the silanized surfaces were exposed to glutaraldehyde (APTES) as well as EDC/NHS or EDC sulfo-NHS (TESPSA), followed by washing steps and eventually MTyr treatment. The indirect quantification of bound protein from the supernatant of the MTyr coupling step by the BCA assay did not provide reasonable results, which was assigned to an interference of NHS with the BCA assay. Instead, the surface bound MTyr was quantified by a direct method. A high number of MTyr functionalized glasses were crushed and subjected to acidic conditions for MTyr hydrolysis to amino acids followed by pre-column functionalization with *ortho*-phthaldialdehyde and HPLC analysis [5]. This method could prove the binding of reasonable quantities of MTyr, which was clearly highest for silanization with APTES and coupling with glutaraldehyde (Table 3). For TESPSA treatment, sulfo-NHS was leading to higher MTyr binding compared to NHS, possibly due to the better solubility of the charged activating reagent and the formed active ester intermediates in aqueous environment [9]. As a rough estimate, the surface coverage has been calculated using the spherical projection area corresponding to the MTyr hydrodynamic radius R_H, also considering the close-packing P of spheres in 2D or 3D assemblies. This analysis suggests that a dense packing of MTyr at the surface can be achieved in some cases, even though one should consider presumably relevant systematic experimental errors of this approach due to the very low protein quantities being exposed.

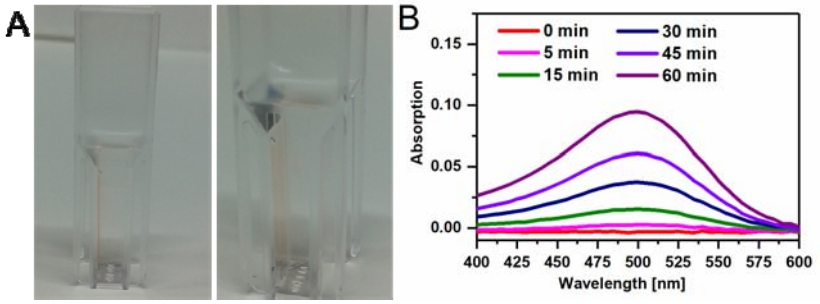

Fig. 3: Analysis of enzymatic activity of immobilized MTyr (TESPSA + EDC-sulfo-NHS) by the MBTH assay. (A) Photographs of cuvettes with functionalized glass slides mounted on the left wall of the cuvette, where a red color reaction was confirmed. (B) UV-Vis spectra, demonstrating the time-dependent absorption increase at 505 nm, λ_{max} of MBTH-quinone adduct.

The preservation of enzymatic activity is, besides the quantity of bound enzyme, a relevant property to evaluate the success of the immobilization method. A tyrosine derivative, desaminotyrosine, was used as substrate for enzymatic conversion to a quinone, the formation of which was visualized by reaction with Besthorn's hydrazone (MBTH) to a pink product. As exemplarily illustrated for MTyr immobilized via the TESPSA and EDC/sulfo-NHS approach, a local red coloration of the glass slide standing upright at the left wall of a cuvette very clearly showed a surface associated conversion of the substrate (Fig. 3A). Monitoring this reaction in a UV/Vis photometer by measuring the absorption of the fluid in the center of the cuvette suggested that this reaction is

exclusively taking place at the surface with a slow diffusion driven exchange kinetics of the enzymatic product with the bulk medium.

Conclusions

This study could demonstrate that functionally intact MTyr can be bound to support surfaces via glass silanization and a coupling reaction. Considering the need of oxygen as a cofactor for biocatalysis by MTyr, the enzyme may be switched on when changing a medium-filled container from anaerobic to aerobic conditions. Such surfaces will be of interest for the enzyme-catalyzed synthesis of surface-associated polymer networks. Due to the generally slow and thus well controllable reaction of MTyr, the kinetics of the enzymatic conversion of polymeric precursors may be a relevant subject for future studies.

Acknowledgments

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