

Article

Approaching Immobilization of Enzymes onto Open Porous Basotect[®]

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Abstract: For the first time, commercial macroporous melamine formaldehyde foam Basotect[®] (BT) was used as a basic carrier material for both adsorptive and covalent enzyme immobilization. In order to access inherent amino groups, the Basotect[®] surface was pretreated with hydrochloric acid. The resulting material revealed 6 nmol of superficial amino groups per milligram Basotect[®]. Different optimized strategies for tethering the laccase from *Trametes versicolor* and the lipase from *Thermomyces lanuginosus* onto the pre-treated Basotect[®] surface were studied. Particularly, for covalent immobilization, two different strategies were pursued: lipase was tethered via a cross-linking method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and laccase was bound after functionalizing Basotect[®] with hydrophilic copolymer poly(ethylene-*alt*-maleic anhydride) (PEMA). Prior to laccase immobilization, the PEMA coating of Basotect[®] was verified by ATR-FTIR analysis. Subsequent quantification of available high-reactive PEMA anhydride moieties revealed an amount of 1028 ± 73 nmol per mg Basotect[®]. The surface-bound enzyme amounts were quantified as 4.1–5.8 μ g per mg Basotect[®]. A theoretical surface-covered enzyme mass for the ideal case that an enzyme monolayer was immobilized onto the Basotect[®] surface was calculated and compared to the amount of adsorptive and covalently bound enzymes before and after treatment with SDS. Furthermore, the enzyme activities were determined for the different immobilization approaches, and the stability during storage over time and against sodium dodecyl sulfate treatment was monitored. Additionally, PEMA-BT-bound laccase was tested for the elimination of anthropogenic micropollutant bisphenol A from contaminated water in a cost-effective and environmentally-friendly way and resulted in a degradation rate higher than 80%.

Keywords: Basotect[®]; enzyme immobilization; laccase; lipase; PEMA; EDC; bisphenol A

1. Introduction

Industrially-produced duroplastic Basotect[®] (BT) is a white slabstock foam with recent applications in lightweight constructions, as well as for acoustic and thermal insulation. BT polymer consists of highly cross-linked melamine and formaldehyde monomers, leading to high mechanical stability and hardness. In addition, BT reveals high chemical stability and is non-soluble in any organic solvent. Due to its chemical structure, amino and hydroxyl functionalities are buried within the framework, making this material a promising candidate for covalent enzyme immobilization approaches. Since enzyme immobilization is preliminary performed using films and beads [1,2],

BT's open porous morphology is favorable for enzyme immobilization and its application as a compact block under flow conditions, as well.

In general, the immobilization of enzymes strongly enhances the usability of biocatalysts for industrial or catalytic purposes [1,3,4]. Therefore, not only high loadings of enzymes on carrier materials are favorable, but also the carrier material characteristics play an important role in how effective the enzyme-modified material will be. It often depends on the carrier material whether the transport of substrates to and products from an enzyme is ensured. Key factors in enzyme immobilization, besides the reaction medium and diffusion limitations, are the precipitation of products, the viscosity of the mixture and reaction thermodynamics [5]. These requirements have to be considered when choosing the carrier material for any special application. In cases where low flow rates or highly viscous fluids have to be used, an open porous carrier with bigger pores is necessary. BT provides excellent preconditions as a carrier with respect to flow-through processes.

There are many different techniques available to immobilize enzymes, which are continuously enhanced [1,5–7], resulting in heterogeneous catalysts with advantageous properties over homogeneous catalyst. In particular, re-use or continuous operation of the biocatalyst, as well as enhancement of stability [8] against thermal influences or organic solvents [9] are of interest. Due to its simplicity, physical adsorption is a very common approach to immobilize enzymes, although issues with enzyme desorption over time exist [10]. Covalent immobilization, as well as cross-linking of enzymes are also widely-used alternatives for tethering enzymes on surfaces [7]. Enzymes can be linked directly to the carrier surface, in case it is providing suitable functional groups, like the herein studied Basotect®. The water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [11] is an extensively-used coupling agent, which increases the reactivity of carboxylic groups as an active ester. However, activating enzyme carboxylic groups can lead to enzyme-enzyme cross-linking since activated enzyme molecules may react with nucleophiles, e.g., amino groups, of other enzymes [12]. As an alternative, if the carrier surface does not provide enough or suitable functional groups, surface modification with highly functional polymers is often applied [13]. The latter is an easy possibility for changing the surface chemistry of carrier materials to get optimized conditions for enzymes and thus improve immobilization rates and increase enzyme activity. Poly(ethylene-*alt*-maleic anhydride) (PEMA) is one example for this purpose and has been used by our group and others for several years to bind enzymes [14,15]. Due to the high density of anhydride moieties in PEMA, there is an increased amount of binding sites for protein immobilization along the polymer chain. Additionally, the functional groups for covalent tethering are well accessible because of the atactic configuration of the anhydride moieties and the short sidechain of the comonomer. Furthermore, the good solubility of the polymer in aqueous solution results in a hydrogel-like 3D swelling behavior of surface-bound PEMA and thus provides an optimal environment for hydrophilic enzymes [16]. Lipase and laccase immobilization have been extensively studied, particularly either for applications in the dairy industry, oil and fat processing and for producing fine chemicals or even biofuels [11,17–19], or in case of laccase, in the pulp and paper industry, cosmetics or for dye synthesis and biosensors, as well as wastewater treatment [20–23]. Different carrier materials, e.g., zeolites, silica gel, polypropylene, polyethylene, octyl-agarose and cellulose ester, as well as silica-based mesoporous cements and fibers, ceramic membranes or polyacrylonitrile beads, and immobilization techniques were applied [24–32]. Immobilized laccase is also a promising candidate for wastewater treatment to remove anthropogenic pollutants [33–37].

In the present study, we primarily aim at the evaluation of the industrial melamine formaldehyde foam Basotect® as a new and suitable carrier for the immobilization of enzymes using common and established adsorptive and covalent immobilization techniques. To our knowledge, the use of a melamine formaldehyde foam for enzyme immobilization has not been studied before. A few working groups [38] applied melamine-based resins in pyrolysis to form templates for mostly non-enzymatic catalysts. Furthermore, BT was used as the moving bed for fungal lipase producers [39]. Using BT as a carrier material for catalytically-active enzymes, either adsorptively or covalently bound,

is a completely new approach. Based on BT's chemical structure, inherent amino groups might be favorable for covalent immobilization approaches. Thus, we intended to develop a method to expose amino groups, followed by suitable characterization of the resulting material. Utilizing EDC-mediated cross-linking of lipase and modified BT, as well as laccase tethering on PEMA-functionalized BT, two different covalent immobilization approaches were pursued. Aiming at an implementation of a particularly reactive BT surface for covalent immobilization, an intermediate PEMA layer providing highly reactive anhydride functional groups should be proven. Utilizing a theoretical model, the limits of loading the BT with an enzyme monolayer should be calculated. The comparison of these theoretical values with the actually measured degree of immobilization of the respective enzyme should make it possible to evaluate the utilization of the available BT surface for enzyme attachment. In addition, further evaluations of the enzyme activity after binding to BT and the stability of the adsorptively- and covalently-bound enzymes against SDS treatment, as well as enzyme stability during storage, should allow a distinct assessment of the suitability of BT as a new carrier material for enzyme immobilization. Finally, laccase-BT should be applied exemplarily to degrade bisphenol A in wastewater, to demonstrate the functionality of enzyme-loaded BT.

2. Results

2.1. Characterization of Basotect®

In order to evaluate the accessibility of BT's inner surface and to obtain information about flow-through conditions, pore diameter and fillet widths were determined by SEM. The analyzed BT was a white open porous foam. Its macro-porous morphology was characterized by a set of three to five slight fillets, connected in a coupling point. By these interconnections, a highly branched three-dimensional network was formed, as shown in Figure 1a. Furthermore, BET characterization of the surface area was performed, and additional micropore analysis (de Boer and Saito-Foley method) showed an absence of any micro-pores on the BT surface. Results are shown in Table 1.

Table 1. Characteristics of Basotect®.

| | |
|---------------|--------------------------------------|
| Pore diameter | 30–240 µm |
| Fillet width | 4–7 µm |
| BET surface | 5.91 m ² ·g ^{−1} |

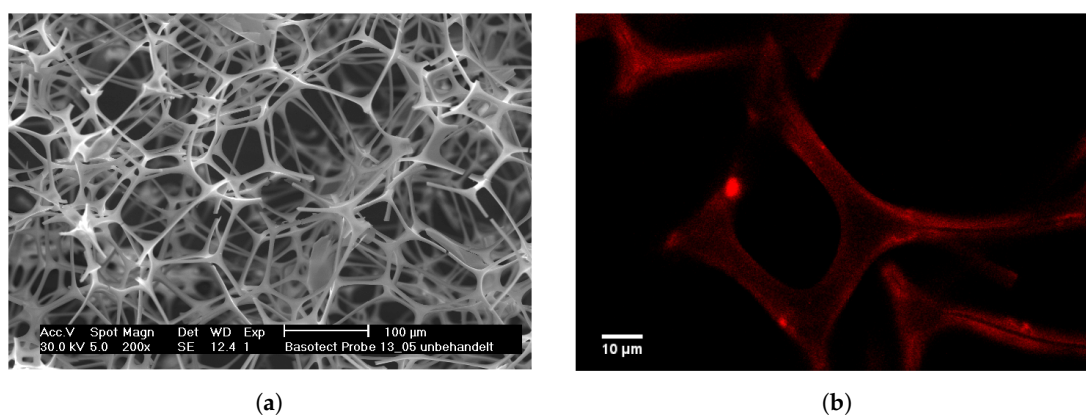


Figure 1. (a) Scanning electron microscopy view of pure Basotect®; (b) fluorescence microscopy of ATTO-TAG™ labeled amino functionalities on the Basotect® surface after partial hydrolysis with concentrated HCl. The labeled sample was excited at 488 nm, and the emission was detected at 590 nm.

Initial investigations of the BT solubility showed a distinct non-solubility in all organic solvents, as this is typical for duroplastics. In contrast, applying hydrolysis conditions using concentrated hydrochloric acid at 55 °C resulted in a cleavage of chemical bonds and a total dissolution after 4 h. A reduction of hydrolysis time made the decomposition incomplete, which resulted only in a slight shrinkage of the foam framework. This process led to an exposure of primary amino functionalities, suitable for covalent enzyme immobilization.

The potential exposition of primary amino groups by a slight HCl-induced etch of the BT surface was analyzed by the specific fluorescence marker ATTO-TAGTM FQ (3-2-(furoyl quinoline-2-carboxaldehyde). Its special performance is based on the non-fluorescent status of the non-reacted aldehyde form, whereas after reacting with a primary amino group, the resulting isoindole product reveals a distinct red fluorescent emission. After treatment of BT with concentrated HCl for 20 min, ATTO-TAGTM FQ indicated the availability of primary amino groups, as shown in Figure 1b, whereas for non-treated samples, no reaction with the dye was observed.

The amount of amino groups on the polymer surface was quantified by a photometric assay with the azo dye Orange II (β -naphthol orange). The assay was used as previously described by Noel et al. [40] with some modifications. It is based on ionic interactions between the sulfonate group of the dye and the protonated amino groups in an acidic milieu. If the pH value rises, the interactions get weaker, and the dye is released in solution where it is measured at a 484-nm wavelength. For acid pretreated BT, the amount of amino groups was characterized by the Orange II assay, and a degree of amination of $6.0 \pm 0.7 \text{ nmol} \cdot \text{mg}^{-1}$ was determined for pieces of the typical size of $10 \times 3 \times 3 \text{ mm}^3$.

2.2. PEMA Functionalization of Basotect[®] and Characterization

The coating of BT with PEMA was shown by ATR-FTIR. Acid pre-treated BT was treated with PEMA and compared to PEMA-free samples. As shown in Figure 2, the PEMA-BT samples show distinctive signals of carboxylic acid anhydride groups at 1775 cm^{-1} and 1850 cm^{-1} , which proves the tethering of PEMA to the BT surface. As PEMA provides cyclic anhydride moieties, the signal at 1775 cm^{-1} is larger than the other one at 1850 cm^{-1} .

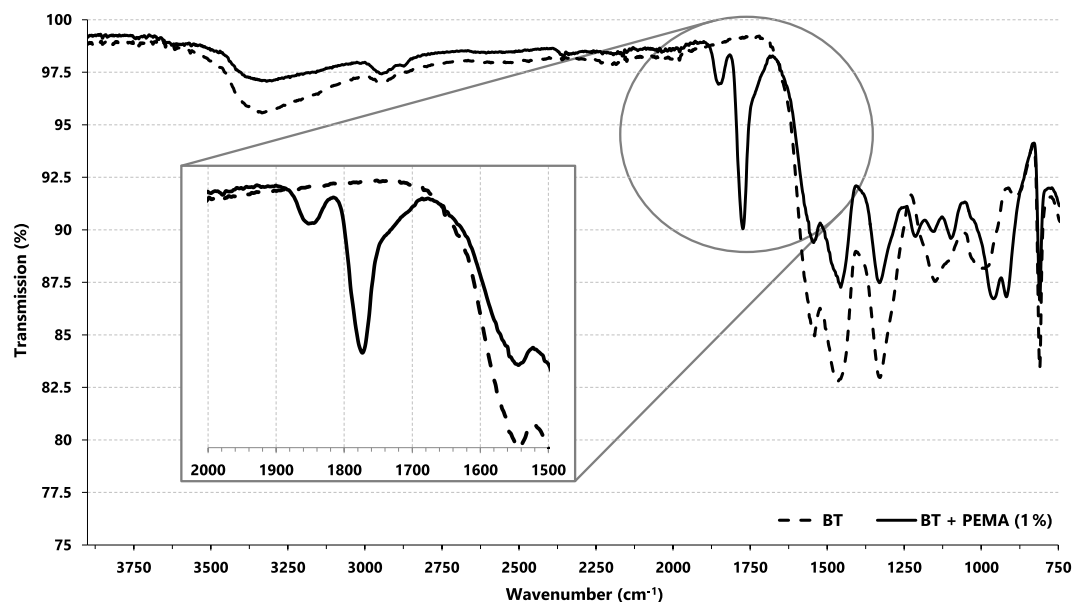


Figure 2. Comparison of ATR transmission spectra of plain Basotect[®] (BT) and PEMA functionalized BT (not normalized). The figure shows untreated and PEMA functionalized BT for comparison. The signals at 1775 cm^{-1} and 1850 cm^{-1} account for tethering of PEMA to the BT surface.

The amount of free surface bound carboxyl groups (degree of carboxylation, DoC) on the BT surface after functionalization with PEMA was additionally quantified via a photometric assay using toluidine blue O. On average, $1028 \pm 73 \text{ nmol} \cdot \text{mg}^{-1}$ carboxyl moieties on PEMA-coated BT surfaces were detected.

2.3. Enzyme Immobilization

2.3.1. Adsorptive and Covalent Immobilization

This study aimed at the evaluation of a melamine formaldehyde slabstock foam as a suitable carrier material for the immobilization of the enzymes lipase and laccase. Thus, the immobilized enzyme quantities and activities were analyzed after adsorptive immobilization on acid-treated BT samples, as well as after covalent immobilization using two different approaches. Lipase was bound by the EDC-induced linkage. Covalent laccase immobilization was performed after linkage of PEMA on the BT surface, creating multiple attachment points.

Immobilization methods varied in immobilization time, as well as in concentration and pH value of the enzyme solution. Details are described in Section 4.5. In initial experiments, the optimum lipase and laccase concentrations in solution were stated for achieving the highest amount of immobilized enzyme quantities on the BT surface. Furthermore, the composition of enzyme solution and its pH value was ascertained for the best maintenance of the enzyme stability. To evaluate the differences in the amount of adsorptively- and covalently-immobilized enzyme, the immobilization conditions were selected identically within a certain enzymes. As shown in Table 2, the adsorptive immobilization of laccase and lipase resulted in an immobilized mass of just under $6 \mu\text{g}$ protein per mg of BT, respectively. Covalently-bound enzymes were treated with SDS, additionally, to desorb non-bound enzymes. Thus, the residual lipase and laccase amount decreased slightly, in contrast to the adsorptive immobilization method.

Verification of enzyme activity after immobilization is essential for further applications of the enzyme-BT assemblies. Specific activity assays, as described in the literature [39,41], were utilized. For lipase activity measurements, the hydrolysis of the fatty acid derivative *p*-nitrophenyl palmitate was used, and laccase activity was detected by transformation of ABTS. In addition to the activity of immobilized enzymes, the activities of dissolved enzymes were determined. Compared to the free enzyme, a general decrease of activity after enzyme immobilization was determined and is shown in Table 2. After adsorptive immobilization, lipase activity decreased to eight-fold less than free lipase activity, whereas after EDC induced covalent immobilization, the activity was a quarter compared to free lipase. In contrast, laccase activity after adsorptive and covalent immobilization was decreased to about a quarter of the free laccase value, and no distinct difference in remaining activity due to the immobilization method was detected.

Table 2. Protein quantity and laccase- or lipase-specific activity, respectively, after adsorptive or covalent immobilization on BT. Covalent lipase immobilization was performed on acid pre-treated BT using EDC, whereas laccase was tethered on PEMA-BT. Further immobilization conditions were described in Table 4. $n = 6$.

| | Adsorptive | | Covalent | | |
|---------|---|--|---|--|---|
| | Quantity on BT ($\mu\text{g} \cdot \text{mg}^{-1}$) | Activity ($\text{U} \cdot \text{mg}^{-1}_{\text{enzyme}}$) | Quantity on BT ($\mu\text{g} \cdot \text{mg}^{-1}$) | Activity ($\text{U} \cdot \text{mg}^{-1}_{\text{enzyme}}$) | Activity of Free Enzyme ($\text{U} \cdot \text{mg}^{-1}_{\text{enzyme}}$) |
| Lipase | 5.8 ± 1.2 | 0.3 ± 0.1 | 4.3 ± 0.4 | 0.5 ± 0.1 | 2.2 ± 0.1 |
| Laccase | 5.8 ± 1.6 | 4.5 ± 0.8 | 4.1 ± 1.2 | 4.2 ± 0.7 | 18.5 ± 3.6 |

2.3.2. Surface Coverage with Enzyme

For further estimation of the ability of BT binding enzymes, the coverage of available BT surface with immobilized enzyme was analyzed. Aiming at this, the theoretical minimum and maximum

enzyme quantities that cover a specific BT surface were calculated (see Section 4.9) and compared to the actual immobilized enzyme quantity for lipase, as well as laccase.

For comparison, the calculated theoretical surface coverage and the experimental data for lipase and laccase immobilization according to the adsorptive or covalent immobilization technique, as well as the effect of SDS treatment are shown in Table 3. In particular, for covalent enzyme immobilization, SDS was used to remove non-covalently bound enzyme molecules.

Table 3. Determined surface coverage of lipase and laccase after adsorptive and covalent immobilization and calculated theoretical minimum and maximum coverages of the enzymes on the BT surface. Additionally, the effect of SDS treatment after enzyme immobilization for each immobilization technique on surface coverage is presented. Immobilization conditions were described in Table 4. $n = 6$.

| Surface Coverage | | Lipase | Laccase |
|---|-----------------------|--------------|--------------|
| Theoretical ($\text{ng}\cdot\text{cm}^{-2}$) | min | 25 | 114 |
| | max | 44 | 125 |
| After adsorptive immobilization ($\text{ng}\cdot\text{cm}^{-2}$) | without SDS treatment | 98 ± 20 | 98 ± 37 |
| | after SDS treatment | 17 ± 15 | 63 ± 19 |
| After covalent immobilization ($\text{ng}\cdot\text{cm}^{-2}$) | without SDS treatment | 113 ± 14 | 126 ± 36 |
| | after SDS treatment | 73 ± 7 | 69 ± 20 |

Analyzing lipase surface concentration, the measured value exceeded the theoretical maximum after adsorptive immobilization. SDS treatment after immobilization resulted in a distinct decrease of bound enzyme amount (83%) associated with the fall of the indicated value below the theoretical minimum surface coverage. In contrast, after covalent lipase immobilization and SDS treatment, the remaining lipase concentration decreased by 35%, but continued to exceed the theoretical maximum surface concentration. After adsorptive immobilization, laccase surface coverage was slightly below the theoretical minimum, indicating an almost complete coverage of the BT surface. A subsequent SDS treatment resulted in a slight decrease of surface concentration (36%). However, SDS treatment after adsorptive laccase immobilization did not lead to the same extent of laccase molecule detachment from the BT surface that was observed after adsorptive lipase immobilization. As a result, the same surface coverage was obtained after adsorptive and covalent laccase immobilization followed by SDS treatment. As detected with lipase, the laccase surface coverage after covalent immobilization and SDS treatment was lower, compared to covalent (45%) and adsorptive immobilized laccase without SDS treatment.

Since adsorptive immobilization of lipase and laccase was performed using uniformly acid-pretreated BT, a consideration of the molar surface concentration of both enzymes on BT after using the same molar enzyme solution concentrations allows a comparison of the ability of the BT to bind the respective enzyme. With the respective enzyme mass concentration shown in Table 4, a molar surface concentration of $1.8 \text{ pmol}\cdot\text{cm}^{-2}$ for laccase and $3.4 \text{ pmol}\cdot\text{cm}^{-2}$ for lipase on the BT surface was achieved. In contrast, the use of the same molar enzyme solution concentrations of approximately $9 \text{ nmol}\cdot\text{mL}^{-1}$ (corresponding to $0.25 \text{ mg}\cdot\text{mL}^{-1}$ of lipase solution and $0.5 \text{ mg}\cdot\text{mL}^{-1}$ of laccase solution, respectively) resulted in an almost similar molar surface coverage of $1.6 \text{ pmol}\cdot\text{cm}^{-2}$ for lipase and $1.8 \text{ pmol}\cdot\text{cm}^{-2}$ for laccase on the BT surface. Additionally, in the case of a lower lipase solution concentration, a lipase surface concentration of $47 \pm 3 \text{ ng}\cdot\text{cm}^{-2}$ was almost similar to the theoretical maximum concentration.

2.4. Storage Stability of Enzyme-Basotect® Interactions

The stability of immobilized enzymes was estimated after tethering on BT and subsequent storage for one or seven days at two different conditions. After adsorptive and covalent immobilization, the lipase-BT specimens were frozen at $-18\text{ }^{\circ}\text{C}$, whereas covalent fixed laccase on PEMA-BT was stored at $4\text{ }^{\circ}\text{C}$. Results in Figure 3 show a decrease of laccase activity to 60% relative to initial activity after one day. However, remaining activity was constant during the next six days. The activity of adsorptive immobilized lipase decreased stepwise after freezing. After one day, activity decreased by about one-fourth, and further storage led to a remaining activity of 12%. A similar trend of activity decrease was determined analyzing covalently-bound lipase on BT, but remaining activity after seven days freezing was at about 40%.

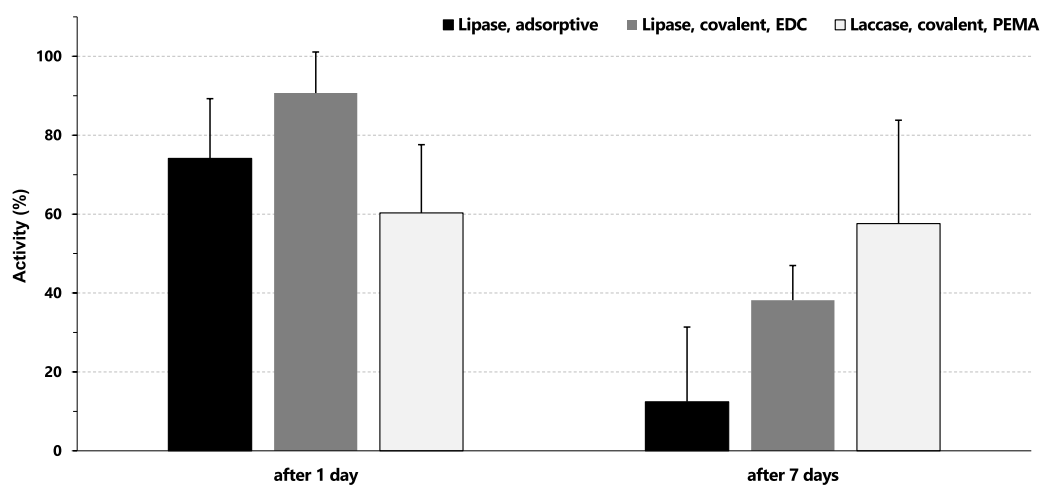


Figure 3. Remaining relative activity of lipase and laccase on BT after storage for one or seven days, respectively. Lipase-loaded BT was stored at $-18\text{ }^{\circ}\text{C}$ and laccase-loaded PEMA-BT at $4\text{ }^{\circ}\text{C}$.

2.5. Application of Immobilized Laccase for the Elimination of Bisphenol A

To demonstrate the function of immobilized laccase, we applied a laccase from *Trametes versicolor* for the degradation of several anthropogenic organic micropollutants in surface water and wastewater effluent. Among others, bisphenol A (BPA) was tested in degradation experiments. Results are shown exemplarily in Figure 4. It was possible to degrade the pollutant BPA almost completely within one week, or by nearly 90% within 48 h. After one week of experiments, the same functionalized BT pieces were placed in another mixture of the same micropollutant mix again, and the degradation was monitored for a second time (second curve in Figure 4).

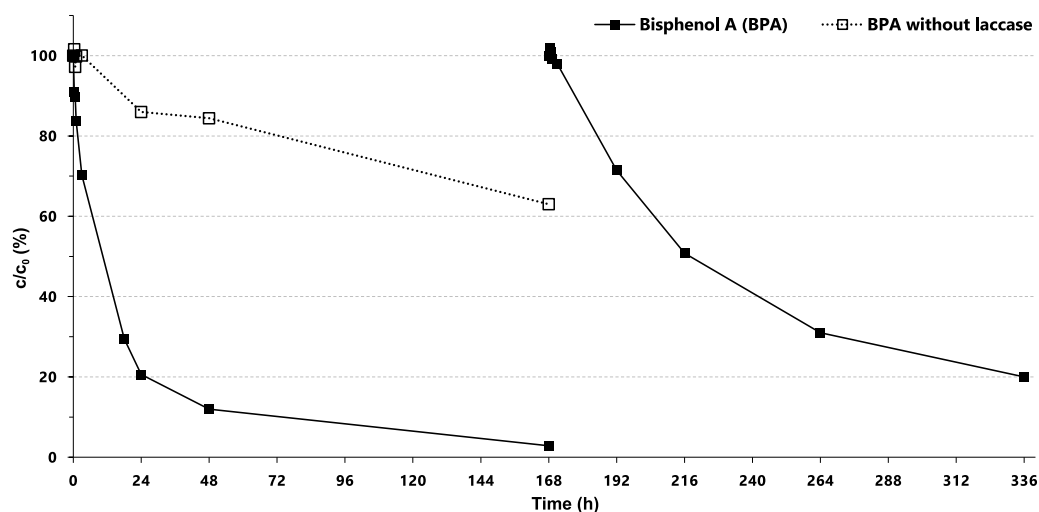


Figure 4. Oxidative elimination of BPA with BT-PEMA bound laccase from *Trametes versicolor* over two periods of one week. After the first week, the same amount of pollutants was added again (second cycle). Dotted curves represent the same setup with BT pieces without laccase. $n = 3$.

3. Discussion

3.1. Characterization and Functionalization of Basotect®

Due to its open porous structure, BT is characterized by its favorable flow performance and, consequently, its ability for easy mass transport through the pore network. Furthermore, a suitable specific surface and a useful surface chemistry are important for successful application as an enzyme carrier material. Despite large pores, BT offers a good mechanical stability and hence can be used for further functionalization. Enzymes and other proteins can be tethered to the inner surface either adsorptively or via covalent immobilization. The chemical structure of BT is very stable and resistant, but can also easily be functionalized by partly breaking chemical bonds between melamine and formaldehyde with strong acids. We used concentrated HCl to uncover amino moieties onto the surface of BT in a quick and easy way. These amino groups can be used for further functionalization. However, with increasing HCl treatment time, the BT becomes less stable. We optimized the exposure process of amino groups to balance out both mechanical stability and preferably the degree of amination (DoA).

DoA was measured with a photometric assay using the Orange II azo dye. A mean amount of $6.0 \text{ nmol} \cdot \text{mg}^{-1}$ for DoA was found. Since the application of BT for enzyme immobilization is a completely new approach, comparison with likewise findings or similar materials is limited. Leirião et al. [42] reported a value of $3.3 \pm 0.95 \mu\text{mol}$ of NH_2 groups per mg material for a polyacrylonitrile membrane enzyme support material. Due to different densities and unknown BET surface areas for these materials, a direct comparison is not possible. Others report NH_2 group amounts only per particle [43] or as the concentration of nitrogen [44]. Additionally, every quantification method provides deviating results and hence is inappropriate for exact comparability.

HCl-treated BT was further functionalized with the copolymer PEMA, to attach anhydride moieties to the carriers inner surface as an additional option for optimized covalent enzyme tethering. PEMA was successfully linked to BT, and remaining maleic carboxylic groups were identified by ATR-FTIR and quantified using the TBO assay. A mean amount of $1027 \text{ nmol} \cdot \text{mg}^{-1}$ of available carboxylic groups was found. This is more than 170-fold of the identified amount of amino groups, so it can be assumed that the introduction of a PEMA interlayer increases the capability of BT to bind enzymes. Although the inner surface of BT might not get fully covered with a PEMA coating

during the functionalization process, the long polymer chains can be tethered to a few amino groups. Thus, a significant increase of binding possibilities seems reasonable.

3.2. Enzyme Immobilization

For the selected enzymes lipase and laccase, adsorptive and covalent immobilization to the BT surface was the aim. Exclusively considering one enzyme, the results of achieved surface amounts were compared. The generation of an enzyme-carrier interaction depends significantly on the spatial presentation of suitable functional groups or binding domains of the enzyme, as well as the surface chemistry of the carrier material. Prior to its application for enzyme immobilization, the surveyed BT was treated with HCl, leading to superficial hydrolysis. Polymer inherent amino groups were thereby exposed, which were not available for the raw material. This generated BT surface served as a basis for adsorptive immobilization of the enzymes or was used either directly for covalent attachment or after implementation of a reactive PEMA layer on BT surface. Lipase was fixed directly on the BT surface by EDC activation of carboxyl functions of the enzyme, and covalent laccase tethering was performed onto PEMA-modified BT. The advantage of the additional PEMA hydrogel layer is based on the introduction of highly reactive anhydride moieties to the surface. These moieties ensure, on the one hand, the linkage of the interlayer to the amine-containing carrier, creating stable imide bonds. On the other hand, unused anhydride groups enable covalent fixation of enzymes. However, the enzyme might also adsorb additionally onto the surface and thus add to the final amount of immobilized enzymes. Compared to the use of EDC for the activation of carboxyl functions on the enzymes' surface, PEMA provides reactive groups located on the BT surface. The latter allows for specific covalent bonds to be formed only between enzyme and carrier and prevents the uncontrolled additional linkage of enzyme molecules to each other, thus preventing loss of enzyme activity.

After adsorptive lipase and laccase immobilization, a higher amount of immobilized enzymes was determined, as after covalent fixation, since SDS treatment was applied afterwards. It can therefore be assumed that the covalent type of immobilization includes also a portion of adsorptive immobilization, as well, because the covalent immobilization cannot occur independently of the adsorptive one. The hypothesis can be proposed that this portion of non-covalent-bound enzyme molecules has been desorbed, leading to a decline of immobilized enzyme amount. As SDS treatment is a very rough approach for detaching enzymes from surfaces, the remaining amounts can be seen as firmly attached to the BT surface. For enzyme-BT samples after adsorptive tethering, no SDS treatment was performed so that a high number of non-covalent interactions can persist. This interplay may occur between enzyme and carrier and, particularly, between enzyme molecules themselves, but does not allow for permanent anchoring of the enzyme molecules to the carrier surface.

Figure 5 shows a comparison of the used immobilization techniques for both chosen enzymes. Based on the theoretical assumptions made in section 2.3.2, immobilization results are compared to a theoretical ideal monolayer of immobilized enzymes. Therefore, the amount of immobilized enzymes, as well as the enzyme activity of the samples were considered. Adsorptively-bound lipase showed the highest amounts of immobilized enzymes. As it was significantly higher than for an expected monolayer, it can be assumed that multilayer immobilization occurred. However, it did not result in higher amounts of enzyme activity. For laccase, adsorptively-bound enzyme resulted in slightly better enzyme activity compared to the covalent binding method, which is also the case when comparing the immobilized enzyme amounts. The difference between the immobilization results of adsorptively-bound lipase and laccase can partly be explained by taking the concept of 'hard' and 'soft' proteins into account, which was introduced by Norde and Anusiem in 1992 [45]. While 'soft' proteins, with their lower internal structural stability, can be adsorbed better onto surfaces, 'hard' proteins, in contrast, have stronger inner connections and hence a higher stability of their native structure.

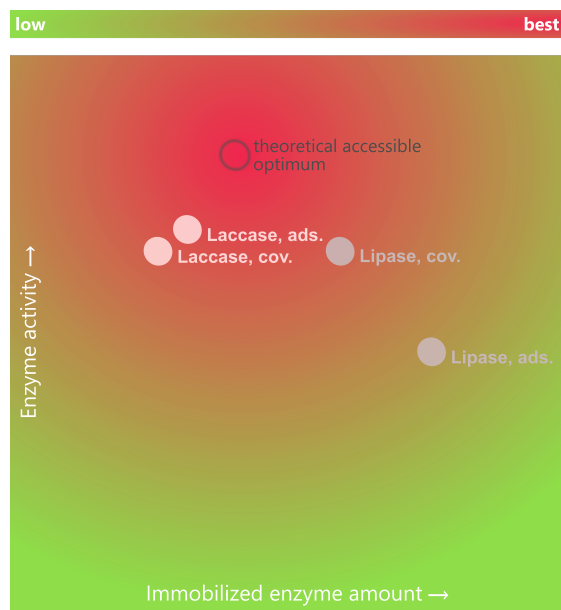


Figure 5. Classification of used immobilization strategies for laccase and lipase compared to a theoretically-accessible optimum of enzyme immobilization. Red shading represents better results, while green shading otherwise symbolizes lower immobilization rates and/or lower enzyme activity. The figure compares the respective optimal conditions of enzyme immobilization for adsorptive (ads.) immobilization without SDS treatment and for covalent (cov.) immobilization results after washing with SDS solution. Activity values were compared to the free enzymes as given in Table 2.

In comparison to the dissolved enzymes, the immobilization leads to an activity decrease for both biocatalysts. Nonetheless, the respective best activity results reached a comparable level for both enzymes. The used immobilization methods tether the enzyme molecules to the BT surface, but it cannot be excluded that access to the active side is hindered by the spatial orientation of the enzymes on the BT surface. Furthermore, there is a probability that during covalent immobilization, on the one hand, functional groups of the active site or some close to it are linked to the BT surface or to other functionalities of further lipase molecules during the lipase immobilization. On the other hand, besides the linkage of nucleophilic groups on the surface of the laccase molecule with the anhydride moieties on the BT-PEMA surface, a covalent bond between anhydride groups and amino acid sidechains of the active side of laccase can occur. Moreover, structural changes in the secondary and tertiary structure of the enzyme molecules caused by the immobilization processes are possible. All these factors would explain a decrease in enzyme activity.

3.3. Analysis of Basotect® Surface Coverage with Enzyme

Determination of the theoretical surface coverage and its comparison with the BT surface actually covered by enzyme molecules allows for the evaluation of the BT surface to be available for immobilization. It was assumed that the enzyme molecules' spheres are rectangular cuboid-shaped and the surface is coated with an enzyme monolayer. For its formation, a theoretical coverage area is edged by the two extreme states of enzyme molecule orientation on the BT surface. These boundaries represent the minimum or maximum surface-specific enzyme mass and frame a permitted surface coverage range (see Figure 6). For this purpose, it was assumed that all enzyme cuboids are bound exclusively to the BT with their smallest (maximum surface-specific enzyme mass) or exclusively with their largest contact surface (minimum surface-specific enzyme mass). All values below the minimum surface-specific enzyme mass indicate that the surface is not completely covered by enzyme molecules and free space is present. In contrast, measured surface-specific enzyme masses above the maximum limit allow the assumption that enzyme aggregates or multilayers were formed. For the range between

the two limits, it can be assumed that an enzyme monolayer was formed. However, local enzyme aggregations associated with uncovered regions cannot be excluded, although the surface-specific enzyme mass is within the permissible range.

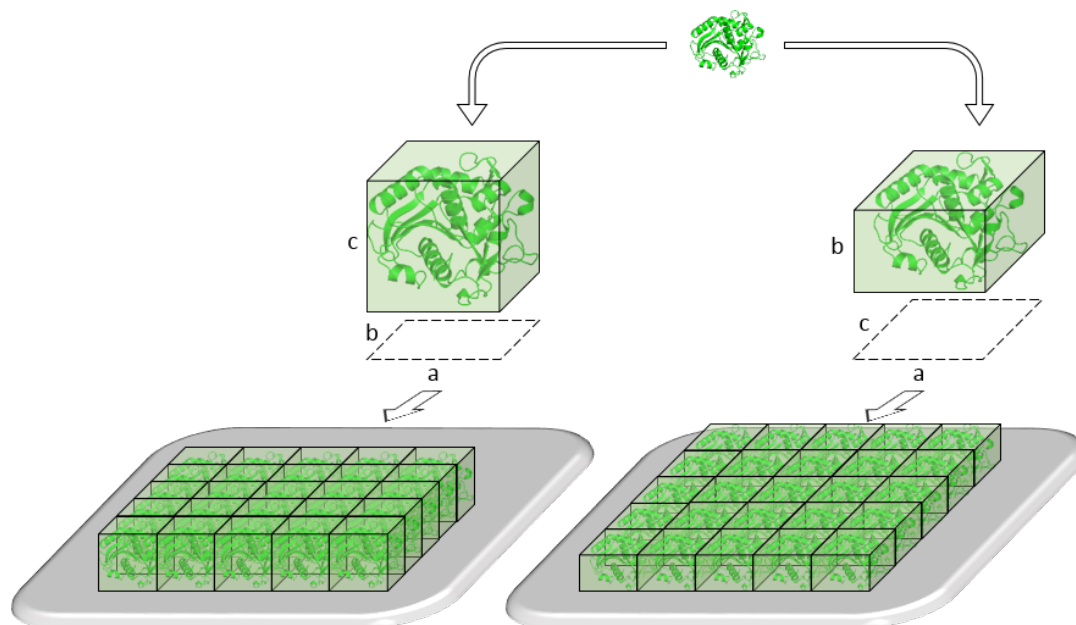


Figure 6. Visualization of the theoretical orientation and distribution of a lipase molecule on the BT surface. Assuming a cuboid morphology, lipase can theoretically attach to the BT surface with its smallest (area $a \times b$) or largest (area $a \times c$) cuboid area. After complete coverage of the carrier surface with enzyme, a minimum and maximum of lipase molecules per BT surface can be calculated and represent a range of theoretical surface-specific enzyme mass. In comparison, the real orientation of the lipase molecules on the BT surface is assumed as a random process. Furthermore, real coverage can take place in three states: (I) below the theoretical minimum, representing incomplete BT surface coverage, (II) above the theoretical maximum, representing an enzyme multilayer or enzyme aggregate formation, and (III) within the theoretical minimum and maximum, when immobilized enzyme mass is in this range, but enzyme aggregates exist with free space between. However, comparing the theoretical and measured states allows an assessment of utilizing the available BT surface for enzyme immobilization. The 3D model of lipase was taken from the RCSB Protein Data Base (ID code: 1DT3).

After adsorptive immobilization of lipase, the determined surface concentration indicates a multilayer composition or aggregation of enzyme molecules on the surface, since the maximum theoretical surface concentration has been exceeded. A subsequent SDS treatment also showed inadequate interactions between enzyme aggregates and the BT surface. The lipase molecules could be detached from the surface so much that an incompletely covered surface remained. For covalent lipase immobilization utilizing EDC, exceeding of the maximum theoretical surface coverage was found to be positive in regard to the amount of fixed lipase. It has been suggested that during this immobilization, two processes run in parallel: lipase molecules bind to the BT surface adsorptively and other lipase molecules aggregate to the existing enzyme layer. At the same time, EDC induces the covalent linkage of lipase and BT, as well as the cross-linking of lipase molecules among themselves. However, this non-directed immobilization must be regarded as critical, since the chemical bond can also be generated with amino acids of the active side and thus lead to enzyme inactivation. The fact that the lipase molecules in the aggregates are not completely interconnected with each other or with the BT surface is suggested by SDS treatment. Nevertheless, in comparison to adsorptive immobilization, a higher amount of lipase could be stably bound to the BT, which has not been detached from the surface by SDS. Furthermore, in comparison to adsorptive lipase immobilization,

the higher surface concentration of the enzyme automatically leads to a higher BT-specific activity after covalent immobilization.

Considering the range of theoretical surface coverage of the BT with laccase, the measured surface concentrations indicate that after adsorptive and covalent immobilization and without SDS treatment, almost the entire surface is covered. After covalent immobilization and SDS treatment, however, slightly more than half of the surface was occupied with laccase. This is a clear difference to lipase immobilization, in which the theoretically maximum surface concentration was consistently exceeded. Furthermore, the additional detachment of enzyme molecules by SDS treatment indicates that parts of the covalently-immobilized laccase have not formed a sufficiently strong bond to the BT surface. Furthermore, the almost equal surface concentrations after adsorptive immobilization at BT and covalent immobilization at PEMA-BT with subsequent SDS treatment indicate that the covalent technique does not lead to an enhancement of the binding stability between enzyme and BT in laccase immobilization. It can be assumed that adsorptive interactions between laccase and BT are beneficially developed such that no desorption from the BT surface can be initiated by SDS treatment. The lack of formation of the covalent bond between laccase and PEMA-BT is also conceivable.

Since BT was pretreated in the same way for the adsorptive immobilization without subsequent SDS treatment for both enzymes, a comparison of the immobilization ability of the BT can be carried out. For this purpose equal molar enzyme solution concentrations of about $9 \text{ nmol} \cdot \text{mL}^{-1}$ were used for the immobilization. If the obtained surface coverage of both enzymes is compared, regardless of the pH of immobilization solution (see Table 4) and the isoelectric point of the enzymes ($\text{pI}_{\text{lipase}} = 4.4$, $\text{pI}_{\text{laccase}} = 5.8$), almost the same molar surface concentration at the BT could be achieved after adsorptive lipase and laccase immobilization when using the same molar enzyme solution concentrations. This suggests that the immobilization process with the acid pretreated BT surface is primarily independent of the polarity of the binding partners. Despite having the same molar concentrations in the immobilization solution, the BT surface could not be fully occupied when using laccase compared to lipase. This is due to the smaller size of a laccase molecule in comparison with lipase (for sizes of the enzymes, see Section 4.9).

3.4. Stability of Immobilized Enzymes

Immobilization of enzymes on solid support materials can fix their geometrical structure and increase stability. This enables, i.e., an increase of operating time, the application in enzyme hostile reaction systems and the more economical application of these biocatalysts. Furthermore, the immobilization of enzymes enables the preservation of activity during storage periods, while the activity of free enzymes frequently decreases. According to the immobilization method and storage conditions, immobilization of the enzymes on BT preserve the activity. Lipase was bound adsorptively and covalently via EDC activation to the BT and frozen at -18°C . The results indicate that the freeze and thaw process changes the tertiary structure of the lipase and thus continuously reduces the activity after seven days. However, it was found that the decline of activity after adsorptive lipase tethering was significantly higher than after covalent linkage, possibly caused by additional desorption processes. This suggests that the generation of covalent cross-links between BT and lipase and between lipase molecules themselves better preserves the enzyme structure than adsorptive fixation. In contrast, the activity of immobilized laccase could be maintained at a constant level during storage at 4°C . Accordingly, it can be concluded that the cooled and non-frozen storage of immobilized enzymes on BT carrier is advantageous.

3.5. Prospect and Applications

Due to its porous structure, functionalized Basotect[®] can be easily applied as a catalyst for utilization in fluid media. A possible application for immobilized enzymes is the elimination of hazardous trace substances from wastewater or drinking water [46,47]. The oxidoreductase laccase was immobilized on PEMA-functionalized BT and tested in an application in order to demonstrate

an alternative possibility to reduce water pollution of anthropogenic contaminants. Often, many of these substances cannot be removed from urban wastewater by conventional wastewater treatment plants mainly due to their polarity and toxicity and thus become persistent in the environment over time [48]. In recent years, alternative methods were developed, to get rid of such pollutants mainly by oxidizing them with UV radiation or ozonation [49]. Other alternatives like the Fenton process and sonification were also considered [50]. All these new approaches can be summarized by the term advanced oxidation processes (AOPs) [51] and are mostly still under development for technical use. Using oxidizing enzymes for a similar approach is, therefore, also a promising option. The persistent water pollutant bisphenol A was chosen to show that immobilized laccase can be utilized to eliminate organic micropollutants. Immobilized laccase on BT carrier was applied to eliminate bisphenol A out of a mix of water pollutants. A reduction of initial BPA amount by 90% within 48 h was figured out. A higher result could be achieved by a longer reaction time. To examine multiple uses of immobilized laccase in a second attempt, the same pieces of BT were used again to reduce the amount of bisphenol A, and a reduction of the initial BPA content by 80% within the first two days was shown. Degradation efficiency and rate can be adjusted by the amount of applied laccase-functionalized BT pieces. As aging of the immobilized enzyme and maybe also desorption of adsorptively-bound laccase occur, the degradation rate becomes lesser when immobilized laccase was applied repeatedly. For a technical application of this first approach, a scale up is necessary. Furthermore, reaction times may also decrease, if immobilized oxidoreductases are applied in a flow-through reactor. Basotect[®] analyzed in this study provides an excellent carrier material for such an application, especially because it can be easily flowed through due to its wide open porous structure. Additionally, it is easy to functionalize with several biocatalysts and ensures their multiple and long-term usability. Particularly for such use cases, we can recommend this carrier material, which supplies a highly customizable surface and allows also an easy exchange between the biocatalysts and their substrates.

4. Materials and Methods

The melamine formaldehyde resin Basotect[®] was provided by BASF Schwarzheide GmbH as white slabstock foam. The copolymer PEMA with a molecular weight of 100 to 500 kg·mol⁻¹ was obtained from Sigma Aldrich (Taufkirchen, Germany). Lipase from *Thermomyces lanuginosus* was acquired as type “NOVAREKO” solution from NovaBiotech Dr. Fechter GmbH. Laccase was used from the white rot fungus *Trametes versicolor* and purchased from Sigma Aldrich (#51639 with 10 kU·g⁻¹).

4.1. Size and HCl Treatment of Basotect[®] Samples

BT was cut into pieces having a final size of 10 × 3 × 3 mm³ (each with a weight of about 500 µg), predominantly, to generate samples for the analyses.

For all enzyme immobilization experiments, BT was pretreated with hydrochloric acid. In detail, BT samples were immersed in concentrated HCl solution for 20 min at 24 °C without previous cleaning of the raw material. Afterwards, the samples were rinsed with ultrapure water and incubated in 1 mL 0.1 M phosphate buffer pH = 8.0 for 15 min. Finally, the water-rinsed samples were dried at 60 °C for 4 h.

4.2. Scanning Electron Microscopy of Basotect[®]

Morphological analysis of BT was performed using a Philips XL 30 scanning electron microscope. Samples were examined in native form and after incubation in concentrated hydrochloric acid at 24 °C for 90 min, respectively. Subsequently, acid-treated samples were rinsed with ultrapure water intensively and dried at 60 °C for 4 h. After mounting on an aluminum specimen holder, the samples were coated with gold utilizing an Emitech K550 sputter-coater to create a layer of approximately 20 nm.

4.3. Amino Group Detection

4.3.1. ATTO-TAG FQ Fluorescence Labeling

Primary amino groups on the BT surface were identified by derivatization with the ATTO-TAGTM FQ Amine-Derivatization Kit (Molecular Probes, Eugene, OR, USA) with subsequent fluorescence microscopy. For fluorescence labeling, the manufacturer's protocol was modified to perform the reaction with superficially available amino groups. In detail, 2.5 mg·mL^{−1} ATTO-TAGTM FQ solution in methanol, 0.01 M KCN solution and a 0.1 M borate buffer pH = 9.5 were mixed at a volume ratio of 1:2:2.5. BT samples were immersed in 0.3 mL of these solution and incubated at room temperature for 1 h. Subsequently, samples were rinsed intensively with ultrapure water and analyzed using a Leica TCP SP laser scanning microscope (excitation 488 nm, emission 590 nm).

For the positive control, glass slides were cleaned as described elsewhere [52], subsequently placed in a Petri dish (diameter 150 mm), and 100 µL of 3-aminopropyltriethoxysilane were added on a small watch-glass. The Petri dish was sealed with parafilm, and gas phase silanization was carried out at room temperature overnight. The resulting amino silanized glass slides were rinsed with toluene and dried with nitrogen. ATTO-TAGTM FQ treatment of the glass slides was carried out as described for BT.

4.3.2. Orange II Assay

The assay, which had been previously described by Noel et al. [40], is based on the pH-driven ionic interaction between a negatively-charged dye and the positively-charged surface and was modified for use with BT. A piece of foam was incubated in 1.5 mL Orange II solution (0.5 mM, in water at pH = 3 adjusted with hydrochloric acid; Orange II was obtained from Sigma Aldrich, Taufkirchen, Germany) at 40 °C for two hours and shaken vigorously at 750 rpm. Afterwards, the pieces were washed with at least 4 mL of HCl (pH = 3) four times. Desorption of bound dye was carried out by incubation of washed and dried BT pieces in 1 mL NaOH (pH = 12) for ten minutes. Finally, one-hundredth fuming HCl (37%) was added to the solution and its absorption in a 10-mm cuvette was measured at 484 nm. DoA was determined from absorption ΔE by the following formula using the Orange II molar attenuation coefficient ϵ of 18.1 mL·µmol^{−1}·cm^{−1}, the volume V of the assay solution, the mass of BT (m_{BT}) and the layer thickness d (length of the light path in the cuvette):

$$\text{DoA} = \frac{\Delta E \cdot V}{m_{BT} \cdot d \cdot \epsilon}.$$

4.4. PEMA Functionalization

To obtain a homogeneous coating of the inner surface of BT with the copolymer PEMA, HCl-pretreated pieces were submerged in a solution of 1 wt% PEMA in acetone and THF (volume ratio 1:2) and shaken firmly at 150 rpm for 24 h. The small pieces were removed from solution, and the remaining solvent was withdrawn by using a pipette until dry. Afterwards, the BT foam was tempered at 120 °C for at least two hours. In a final step, the cooled functionalized pieces were flushed with acetone at 120 rpm twice for 15 min and subsequently tempered again at 120 °C for two hours. The functionalized carrier was used immediately or stored dry and in the dark.

4.4.1. ATR-FTIR Analysis

The surface modification of PEMA-functionalized Basotect[®] was analyzed with a NicoletTM iSTM10 FTIR spectrometer (Thermo Scientific, Dreieich, Germany) equipped with a diamond crystal.

4.4.2. TBO Assay

Toluidine blue O (TBO), a blue cationic dye, was purchased from Sigma Aldrich and used as an easy photometric assay to quantify free carboxylic moieties onto PEMA-functionalized BT surfaces.

This application was earlier specified by Sano et al. [53] and is based on pH shifting. At basic conditions carboxylic groups exist as carboxylate anion, so cationic TBO is able to bind via ionic interactions [43]. At first, all carboxylic groups were hydrolyzed by immersing BT pieces in water twice for 30 min at 40 °C and shaken at 750 rpm. Dyeing of BT was performed afterwards by incubation of one BT piece in 1 mL 0.25 M TBO solution in 0.1 M NaOH for two hours at 40 °C and 1000 rpm. The unbound dye was subsequently removed by washing BT pieces repeatedly with 10 mL 0.1 M NaOH until the washing solution appeared clear, or at least five times for 10 min each. Attached TBO dye was then dissolved by washing in 1 mL 50 vol% acetic acid for 30 min at 40 °C and 1000 rpm. Absorption (ΔE) of this solution was measured at 633 nm, and the degree of carboxylation (DoC) in nmol·mg^{−1} was calculated with a molar attenuation coefficient (ϵ) of 54.8 mL·μmol^{−1}·cm^{−1} and by assuming that 3.4 mole carboxylic groups bind one TBO molecule according to Henning et al. [54].

$$\text{DoC} = 3.4 \cdot \frac{\Delta E \cdot V}{m_{\text{BT}} \cdot d \cdot \epsilon},$$

where V is the volume of the assay solution, m_{BT} is the mass of applied BT and d the layer thickness (length of the light path in the cuvette).

4.5. Enzyme Immobilization

Detailed conditions for adsorptive and covalent enzyme immobilization are summarized in Table 4.

Table 4. Conditions for adsorptive and covalent immobilization of laccase and lipase on acid-pretreated BT or PEMA-BT, respectively.

| | Enzyme Concentration in Solution (mg·mL ^{−1}) | Immobilization Time (h) | Composition of Immobilization Solution | Wash Procedure |
|---------|---|------------------------------------|--|--|
| Lipase | 3.0 | 0.5 (adsorptive) 2.0 (covalent) | 1% Propylene glycol, 1.6% PEG400, 0.01% CaCl ₂ , pH = 6.1 | Five-times dipping into 30 mL ultrapure water, three wash cycles |
| Laccase | 0.5 | 4.0 | 0.2 M sodium citrate buffer, pH = 4.5 | Incubation in 5 mL 0.2 M sodium citrate buffer (pH = 4.5) on orbital shaker at 110 rpm for 15 min, three wash cycles |

4.5.1. Adsorptive Immobilization

The acid-pretreated slabstocks were incubated on a horizontal shaker at 100 rpm and at room temperature in 1 mL laccase or lipase solution, respectively, and washed subsequently.

4.5.2. Covalent Immobilization

For lipase immobilization, one piece of HCl-treated (see Section 4.1) and non-dried BT was immersed in 1 mL lipase solution containing 0.1 M EDC (Sigma Aldrich, Taufkirchen, Germany) at room temperature. For laccase immobilization, 1 mL of laccase solution was added to three PEMA functionalized BT pieces (see Section 4.4) and incubated on an orbital shaker at 90 rpm and room temperature. Afterwards, all samples were washed (see Table 4) and treated with SDS solution (see Section 4.5.3).

4.5.3. SDS Treatment

After covalent immobilization, enzyme-functionalized BT or PEMA-BT pieces, respectively, were treated with SDS solution. Lipase-BT samples were treated with 2 wt% SDS solution at 25 °C for 30 min. Laccase-PEMA-BT samples were treated with 1 wt% SDS solution at 25 °C while vigorously shaking at 800 rpm for 24 h and, subsequently, washed in Millipore water again for 24 h under the same conditions.

4.6. Storage Conditions

4.6.1. Laccase on PEMA-BT

Laccase functionalized PEMA-BT pieces were stored in citrate buffer solution (pH = 4.5) at 4 °C until the next measurement of laccase activity, as described in Section 4.8.

4.6.2. Lipase on BT

Lipase-loaded BT samples were stored in 0.1 M phosphate buffer (pH = 7.4) at −18 °C, and lipase activity was determined (see Section 4.8) after several periods.

4.7. Protein Quantification

The amount of surface-bound protein was analyzed using a micro-bicinchoninic acid (BCA) protein quantification assay (Pierce, Thermo Scientific, Waltham, MA, USA). BCA working reagent was prepared following the manufacturer's protocol. To one moist protein-loaded BT or PEMA-BT sample, ultrapure water was added to a final liquid volume of 500 µL. Subsequently, the sample solution was mixed with 500 µL of BCA working reagent and incubated on a Thermomixer (Eppendorf AG, Hamburg, Germany) at 60 °C and 750 rpm for 1 h. After cooling to room temperature, the resulting solution was transferred to polystyrene cuvettes, and absorption at 562 nm was analyzed. For the generation of blanks, immobilization procedures onto acid-pretreated BT and PEMA-BT were performed without enzyme, and BCA assay was applied.

4.8. Enzyme Activity

4.8.1. Lipase

For activity measurement, the lipase-catalyzed hydrolysis of *p*-nitrophenyl palmitate to palmitate and the chromatic *p*-nitrophenolate was followed spectrophotometrically. In detail, 9 mL of 0.1 M TRIS buffer pH = 8.0, containing 1.0 mg·mL^{−1} gum arabic (Sigma Aldrich, Taufkirchen, Germany) and 8 mg·mL^{−1} Triton X-100 (AppliChem GmbH, Darmstadt, Germany), was blended with 1.0 mL of 3.0 mg·mL^{−1} *p*-nitrophenyl palmitate (Sigma Aldrich, Taufkirchen, Germany) in 2-propanol in a beaker equipped with a magnetic stir bar. The temperature of the resulting solution was adjusted to 45 °C. Subsequently, one piece of lipase-loaded BT was added and stirred at 500 rpm. After 1 min and 5 min, respectively, 1 mL of solution was removed, and absorption was measured at 405 nm.

4.8.2. Laccase

Laccase activity was determined via the ABTS assay (ABTS = 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma Aldrich, Taufkirchen, Germany). A sodium citrate buffer (0.5 M, pH = 5.3) was used, and a 207 µM ABTS solution was prepared and tempered at 37 °C. Eight hundred fifty two microliters of ABTS solution and 648 µL buffer were mixed together with one piece of laccase-functionalized BT in a cuvette, and absorption at 420 nm was recorded for 30 min to 120 min every 30 s, while tempered at 37 °C. Every ten minutes, the porous BT was squeezed in order to get a homogeneous mixture. The slope of the linear front part of the curve (absorption vs. time) was used to calculate laccase enzyme units assuming that 1 µmol ABTS was oxidized per minute

To calculate the enzyme activity (EA) for the respective assay, the difference in absorption (ΔE) corrected by the blank was inserted in the equation:

$$EA = \frac{\Delta E}{\Delta t} \cdot \frac{V}{\varepsilon \cdot d},$$

where V is the total volume of the assay solution, Δt is the time difference, ε is the molar attenuation coefficient ($18.3 \text{ mL} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$ (for *p*-nitrophenolate) and $36.0 \text{ mL} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$ (for ABTS)) and d is the path length of the beam of light through the solution.

4.9. Calculation of Theoretical and Actual Enzyme Surface Concentration

The sizes of a laccase molecule (RCSB PDB ID code: 1GYC) is $9.2 \times 8.5 \times 8.4 \text{ nm}^3$ and of a lipase molecule (RCSB PDB ID code: 1DT3) is $13.9 \times 13.9 \times 8.1 \text{ nm}^3$. Values were taken from the RCSB protein database. Assuming a rectangular cuboid morphology of the enzymes as shown in Figure 6, the minimum and maximum rectangular planar surface area of a laccase molecule are 71.4 nm^2 and 78.2 nm^2 , respectively, and for lipase are 112.6 nm^2 and 193.2 nm^2 , respectively. The theoretical minimum and maximum of enzyme surface coverage was calculated, assuming the creation of enzyme monolayers in a compact side-on configuration. The quantity of enzyme molecules was identified, when a specific BT surface is fully covered either with all molecules in contact with the smallest or the largest contact surface of the enzyme cuboid, respectively. The theoretical surface coverage Γ is calculated using the equation:

$$\Gamma = \frac{M}{A_1 \cdot N_A},$$

where M is the molar mass of the enzyme, N_A is the Avogadro constant and A_1 is the respective smallest or largest contact area of one enzyme molecule cuboid.

For the calculation of actual enzyme surface concentration, the detected enzyme mass per mass of BT (see Table 2) was divided by the specific surface of BT.

4.10. Degradation of Bisphenol A Utilizing Laccase on PEMA-BT

4.10.1. Experimental Setup

Degradation, more precisely an oxidative removal, of anthropogenic micropollutants was done by treating a solution of organic micropollutants with laccase-functionalized pieces of PEMA-BT. The design of the experiment was established as follows: PEMA-coated BT foam pieces were functionalized with laccase as described above (see Section 4.5.2). Around twenty of them (which together carried $\sim 1 \text{ U}$ of laccase activity) were placed in a reaction tube, and a 10-mL mix of anthropogenic micropollutants was added (the final concentration of each pollutant was 1.4 nM). The mixture was then shaken vigorously for several days. The amount of micropollutants was monitored by LC-MS/MS at reasonable periods.

4.10.2. LC-MS/MS Detection of BPA

For quantification of bisphenol A (BPA; Sigma Aldrich, Taufkirchen, Germany), an Agilent Triple Quadrupole LC-MS/MS 6400 system was used. BPA was separated with a Phenomenex reversed phase column (Gemini-NX $3 \mu\text{m}$; C_{18} ; 110 \AA ; $100 \times 2 \text{ mm}$) and a mobile phase mixture of acetonitrile (A) and 0.05 vol\% NH_3 (B) from $67\% \text{ A}$ to $97\% \text{ B}$ within two minutes and back to $33\% \text{ B}$ (stop time: 4 min) with a retention time of 2.4 min and identified after ESI ionization in negative ion mode by its specific fragments at m/z 212 (quantifier) and m/z 195, 133 and 117 as qualifiers (see Tables 5 and 6 for detailed LC and MS parameters). BPA-16d was used as the internal standard for quantification. Calibration was established from 0.1 and $75 \mu\text{M}$ with 12 levels and a coefficient of determination of 99.8% .

Table 5. MS parameters for quantification of BPA.

| | Precursor Ion | Product Ion | Frag-Mentor (V) | Collision Energy (V) | CAV (V) |
|---------|---------------|-------------|-----------------|----------------------|---------|
| BPA-16d | 241 | 223 | 140 | 17 | 4 |
| | 241 | 142 | 140 | 23 | 3 |
| BPA | 227 | 212 | 140 | 17 | 4 |
| | 227 | 195 | 140 | 42 | 5 |
| | 227 | 133 | 140 | 23 | 3 |
| | 227 | 117 | 140 | 57 | 4 |

Table 6. LC parameters for quantification of BPA.

| Parameter | Value |
|----------------------------|--------------------------|
| Temperature | 35 °C |
| Flow | 0.4 mL·min ^{−1} |
| Injection volume | 15 µL |
| Source | 300 °C |
| Gas flow (N ₂) | 11 L·min ^{−1} |
| Capillary | 4000 V |

5. Conclusions

This study has found that the applied macroporous BT is generally suitable for enzyme immobilization. The developed enzyme-BT assemblies were active in reactions with model substrates, but the immobilization reduces the enzyme activity. Due to the uncovering of amino groups on its surface, BT represents a favorable framework for covalent enzyme immobilization. Existing amino groups on the BT surface can be presumably used to achieve a stable covalent attachment of lipase molecules by an EDC-mediated reaction. Furthermore, PEMA modification of the BT surface leads to high-reactive anhydride moieties that increase the number of functionalities for covalent tethering compared to amino moieties and are more suitable for enzyme immobilization than EDC. The assessment of BT surface coverage with enzyme molecules suggests that lipase not only interacts with the BT surface, but also forms appropriate interactions within lipase molecules. It is assumed that aggregations or multilayers are formed on the BT surface. This intermolecular interaction probably does not exist between laccase molecules. Consequently, interactions between BT and laccase molecules lead to the almost complete coverage of the BT surface by the formation of an enzyme monolayer. Although the immobilized enzyme amount is low, compared to other carrier materials, due to its open porous structure and resultant favorable flow conditions, as well as its chemical resistance, the potential as an enzyme carrier for technical and industrial application is ascribed to BT.

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