Glucose oxidase (GOD), horseradish peroxidase (HRP), and lactate oxidase (LOD) were covalently immobilized on special NH$_2$-functionalized glass and on a novel NH$_2$-cellulose film via 13 different coupling reagents. The properties of these immobilized enzymes, such as activity, storage stability, and thermostability, are strongly dependent on the coupling reagent. For example, GOD immobilized by cyanuric chloride on the NH$_2$-cellulose film loses approximately half of its immobilized activity after 30 days of storage at 4 °C or after treatment at 65 °C for 30 min. In contrast, GOD immobilized by L-ascorbic acid onto the same NH$_2$-cellulose film retains 90% of its initial activity after 1 year of storage at 4 °C and 92% after heat treatment at 65 °C for 30 min. Unlike GOD, in the case of LOD only immobilization on special NH$_2$-functionalized glass, e.g., via cyanuric chloride, led to a stabilization of the enzyme activity in comparison to the native enzyme. The operational stability of immobilized HRP was up to 40 times higher than that of the native enzyme if coupling to the new NH$_2$-cellulose film led to an amide or sulfonamide bond. Regarding the kinetics of the immobilized enzymes, the coupling reagent plays a minor role for the enzyme substrate affinity, which is characterized by the apparent Michaelis constant ($K_{M,app}$) ($K_{M,app}$). The NH$_2$-functionalized support material as well as the immobilized density of the protein and/or immobilized activity has a strong influence on the $K_{M,app}$ value. In all cases, $K_{M,app}$ decreases with increasing immobilized enzyme protein density and particularly drastically for GOD.

1. Introduction

Biomolecule-functionalized support surfaces are of fundamental importance for analyte recognition in biosensor technology or as biocatalysts in biotechnology. To develop enzyme-functionalized support surfaces for biosensors, numerous support materials have been applied with varying degrees of success. The limited functional stability of enzyme-functionalized supports still restricts their practical application, e.g., for biosensors in medical and in vivo diagnostics.

Oxidoreductase enzymes, e.g., glucose oxidase (GOD) and lactate oxidase (LOD), are still of great importance as analyte recognition systems in biosensors. Various immobilization approaches, such as ionic and covalent immobilization, cross-linking, the bioaffinity-based method, graft copolymerization, and entrapment, have been described. However, in most cases, the immobilization techniques could not fulfill all requirements for targeted application, like high enzyme stability and reactivity under the given process conditions. Of the immobilization methods, covalent binding often exhibits the highest stabilization of enzyme activities because the active conformation of the immobilized enzyme is stabilized. The support material and the coupling reagent thus provide the microenvironment, including the hydrophilic–lipophilic balance, pH value, and ionic strength. The coupling reagent additionally controls the protein bonding (how many and which amino acid residues are chemically changed). Both factors strongly influence the activity and the kinetic properties of the immobilized enzymes.

Surprisingly, despite the long history of enzyme immobilization, there are, especially in the case of oxidoreductase enzymes, only a few examples of systematic investigations of the influence of coupling reagents on the properties of the immobilized enzymes, while most studies investigated the influence of the support. The goal of our recent investigations is the development of new ultrathin and transparent solid phases with an incorporated oxidoreductase enzyme for fiberoptical biosensors. To this end, a novel film-forming NH$_2$-cellulose derivative, 6-deoxy-6-(4-aminophenyl)-amino)cellulosetosylate (NH$_2$-cellulose film), and a novel NH$_2$-functionalized coating on glass (NH$_2$-glass) were developed as transparent NH$_2$-functional support matrices. In this study, we present the 13 in part new NH$_2$-reactive coupling reagents for the immobilization of the oxidoreductase enzymes glucose oxidase, horseradish peroxidase, and lactate oxidase on the NH$_2$-functionalized supports. It is shown that the immobilization method can have a strong influence on the...
activity of the immobilized enzymes, their storage stability, thermostability, and their operational stability. The kinetic properties of the immobilized enzymes were also investigated.

2. Experimental Section

Materials. Glucose oxidase (GOD) EC 1.1.3.4 from Aspergillus niger Grade II (60 U/mg), horseradish peroxidase (HRP) EC 1.11.1.7 Grade II (200 U/mg), and lactate oxidase (LOD) from Pediococcus species (20 U/mg) were purchased from Sigma, and azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from Boehringer (Mannheim, Germany). All other chemicals (analytical grade or purer) were from Aldrich or Fluka and were used without further purification.

Preparation of the NH₂-Glass Surfaces. The NH₂-glass surfaces were prepared on the tips of glass rods (diameter 1 mm, length 10 cm). These rods were coated with an ultrathin SiO₂ layer (some hundred nanometers in thickness). A commercial procedure—the so-called Pyrosil technique (Sura Chemicals GmbH, Jena, Germany)—is especially well suited for this purpose. Within 1—2 h in air, due to the influence of the atmospheric humidity, the SiO₂ coating forms silanols which are condensed to a vitreous polymer film (“polymerization of SiO₂”).

This polymerized SiO₂ was then treated with (3-aminopropyl)triethoxysilane in toluene. The procedure is described elsewhere in more detail (see ref 14).

Preparation of the NH₂-Cellulose Surfaces. A glass rod (as used above) was dipped into a 5% solution of 6-deoxy-6-(4-aminophenyl)amino cellulose tosylate (NH₂-cellulose) in N,N-dimethylacetamide (DMA). The NH₂-cellulose synthesis is described elsewhere. The transparent film formed at room temperature was air-dried for at least 2 h.

Enzyme Immobilization Procedures. (i) Glutaraldehyde Activation. The NH₂-cellulose film on the glass rod was swollen in DMA for about 10 s and then immersed in 20% aqueous glutaraldehyde solution and incubated at room temperature for 10 min. The NH₂-glass rod was immersed in the same solution for 15 min. The activated support surfaces were then washed with bidistilled water and placed in an enzyme solution in bidistilled water.

(ii) Ascorbic Acid Activation. The NH₂-functionalized supports were placed in a saturated solution of ascorbic acid in DMA (400 mg/mL) and incubated at room temperature for 15 min. The samples were then thoroughly rinsed with bidistilled water and immediately added to an enzyme solution in bidistilled water.

(iii) Benzoquinone Activation. The NH₂ supports were placed in a DMSO/benzoquinone solution (200 mg/mL). After incubation for 15 min at room temperature, the samples were rinsed until the rinsing solution was no longer yellowish and then added to an enzyme solution in bidistilled water.

(iv) Activation by Means of Aromatic Diacid Dichlorides, Aromatic Disulfonic Acid Dichlorides, and Cyanuric Chloride. The NH₂-functionalized supports were added to a solution of 100 mg of iso- and terephthaloyl chloride, 1,3-benzenedisulfonyl chloride, 4,4′-biphenyldisulfonyl chloride, or cyanuric chloride dissolved in 10 mL of DMA and incubated at room temperature for 15 min. In the case of NH₂-glass activation with cyanuric chloride, the incubation time was 5 min. The activated surfaces were then thoroughly rinsed with ethanol until they became clear, quickly washed with 100 mM phosphate buffer, pH 7.0, and immediately placed into an enzyme solution in the same buffer, which had previously been cooled to 4 °C.

(v) Activation by Means of Aromatic Dialdehydes and Diketones. The NH₂-functionalized supports were placed into a solution of 100 mg of iso- and terephthalaldehyde, 1,3- or 1,4-diacylbenezene dissolved in DMA, incubated at room temperature for 15 min, rinsed with ethanol and bidistilled water, and added to an enzyme solution in bidistilled water.

(vi) Diazo Coupling. A NH₂-cellulose film was swollen in DMA for 10 s and then placed into 10 mL of ice-cooled 0.5 M HCl. One milliliter of a 1 M solution of NaNO₂ in bidistilled water was added dropwise turning the polymer film yellow. After incubation for another 10 min the film was rinsed with ice-cooled water and placed into an enzyme solution in 100 mM phosphate buffer, pH 7.0.

Enzyme Immobilization. An activated NH₂-functionalized support was immersed in 0.1 mL of an enzyme solution: 1 mg of enzyme per mL of bidistilled water (activation procedures i, ii, iii, and v) or 100 mM phosphate buffer, pH 7.0 (activation procedures iv and vi), in an Eppendorf tube. After incubation at 4 °C for 16 h the nonattached enzyme was removed by rinsing with a stream of bidistilled water for at least 1 min and the sample was stored at 4 °C in bidistilled water.

Determination of Enzyme Loading and of the Michaelis Constant. (i) Immobilized Enzyme Activity. The immobilized enzyme activity was determined photometrically using a UV—vis spectrophotometer (Beckman DU-64). The enzyme-functionalized glass rod was placed in a cuvette containing a solution of the respective enzyme activity test system. In the cases of GOD and LOD a test system modified after Trinder and Webster was used containing 4-aminopyrine, 2,4,6-tribromo-3-hydroxybenzoic acid, and enzyme substrate dissolved in 100 mM phosphate buffer, pH 7.0 (λₘₐₓ = 514 nm, ε = 27.2 cm²/μmol). The determination of the immobilized HRP was carried out according to the ABTS method (1 mg of ABTS per mL of 100 mM acetate buffer, pH 5.0, 10 mM H₂O₂) described by Galtti (λₘₐₓ = 414 nm, ε = 36.8 cm²/μmol). While the glass rod was stirred in the solution, the increase of absorbance was measured at λₘₐₓ of the test system used.

The above-mentioned test systems and procedures were used to determine the Kₘₐпп values of the immobilized enzymes by measuring the initial rate of substrate conversion in the concentration ranges of 1—200 mM glucose (GOD), 0.01—10 mM H₂O₂ (HRP), and 0.05—10 mM L-lactate (LOD). The Kₘₐпп values were calculated by mathematical hyperbola curve fitting (standard error range 2—13%) of the well-known Michaelis—Menten algorithm (v = vₘₐₓS/(Kₘ + S)) by means of Sigma Plot 5.0 scientific graphic software (Jandel Scientific).

(ii) Operational Stability. The activity of an HRP-functionalized support surface was determined by continu-
ously measuring the enzyme activity using the test system described above for 30–40 min with a hydrogen peroxide concentration of 10 mM. The half-life of the enzyme was calculated following a procedure suggested by Adediran and Lambeir whereby the half-life time ($\tau_{1/2}$) was calculated by $\tau_{1/2} = \ln(0.5)/k$. The pseudo-first-order rate constant $k$ was determined by mathematical exponential curve fitting of the first-order kinetic of HRP activity decrease ($V/V_0 e^{-kt}$) by means of Sigma Plot 5.0.

(iii) Protein Determination. An activated NH$_2$-functionalized support surface was treated with 1 mL of a GOD solution in water (1 mg/mL) and incubated under humidified conditions at 4 °C for 1–16 h. The nonattached enzyme protein was then washed off with 0.1 mL of bidistilled water. The protein content in the collected solution was determined by measuring the fluorescence of the FAD coenzyme group (excitation at 460 nm, emission at 520 nm) using a fluorescence spectrophotometer (Perkin-Elmer). Measurements with GOD solutions in the concentration range from 1 to 11 μg/mL gave a linear relation between emission intensity at 520 nm and GOD enzyme protein concentration. The immobilized activity was then calculated from the difference between the GOD concentration of the immobilization solution before and after immobilization.

3. Results and Discussion

The immobilization of the oxidoreductase enzymes glucose oxidase (GOD), lactate oxidase (LOD), and horseradish peroxidase (HRP) was performed on the tips of glass rods coated with the NH$_2$-functionalized support. The rods allow easy handling of the attached reagent solid phases. To create the special NH$_2$-glass, a glass rod was coated with a polymerized SiO$_2$ layer and subsequently treated with (3-aminopropyl)triethoxysilane (see ref 14). The cellulosic support was prepared by coating a glass rod with a novel NH$_2$-cellulose film. The NH$_2$-cellulose film is only some 200 nm in thickness as determined using atomic force microscopy and ellipsometry. The above-mentioned enzymes were covalently immobilized via 13 different coupling reagents, shown in Figure 1, onto both NH$_2$-functionalized support surfaces using the various reaction possibilities of the NH$_2$-groups. In all cases, the bifunctional reagents were used in great excess for activation of the matrix to avoid cross-linking, particularly, of the treated NH$_2$-cellulose film. This is necessary because each reagent, if applied in low concentration, may lead to a different extent of cross-linking resulting in a different extent of swelling, which can affect the immobilization of enzymes.

As can be seen in Table 1, the amount of immobilized enzyme activity varies depending on the oxidoreductase enzyme, the nature of the support material, and the coupling reagent. GOD could be immobilized up to approximately 200 mU/cm$^2$ on both NH$_2$-cellulose and NH$_2$-glass (see Table 1), which is close to the highest value of immobilized GOD activity described ($340$ mU/cm$^2$). HRP was immobilized up to 400 mU via benzoquinone onto NH$_2$-glass and up to 200 mU/cm$^2$ onto NH$_2$-cellulose films by means of various methods (see Table 1). The most suitable method for achieving high-immobilized LOD activity was coupling via 1,3-benzenedisulfonyl chloride and 4,4′-biphenyldisulfonyl chloride onto NH$_2$-glass (330 and 339 mU/cm$^2$). In the case of NH$_2$-glass, there seems to be a relation between the molecule geometry of the bifunctional reacting aromatic coupling reagents and the immobilized GOD activity (cf. Table 1). Greatly varying values of immobilized enzyme activities were obtained by applying a series of 1,3- and 1,4-bifunctional aromatic coupling reagents. The 1,3 coupling, for example, usually leads to a considerably lower enzyme immobilization efficiency than that obtained with the corresponding 1,4 coupling reactions. (Note that the aromatic 1,3 and 1,4 dialdehydes are an exception, where the values of the immobilized GOD enzyme activities hardly differ at all.) In contrast, this effect does not occur for NH$_2$-cellulose.
Native enzymes usually quickly lose their activity. This is due to various spacer lengths in enzyme immobilization on polyethylene membranes. Wang et al. discuss a relation between the spacer effect and the immobilized enzyme concentration or activity of GOD immobilization on polyethylene membranes. On the basis of the their data, the authors conclude that there is considerable deformation of the GOD protein if the enzyme is immobilized on polyethylene membranes without an alkylene diamino spacer group.

Storage stability and thermostability are two important factors for the applicability of immobilized enzymes, because native enzymes usually quickly lose their activity. The greatest stabilizing effect for GOD activity regarding storage was achieved when the enzyme was immobilized via L-ascorbic acid and benzoquinone on NH2-glass where the enzyme showed 99% and 96%, respectively, of its initial activity after 30 days at 4 °C. The storage stability of immobilized GOD activity strongly depends on the coupling method. It does not significantly differ between the NH2-cellulose and the NH2-glass surface, except for the 1,3-bifunctional aromatic coupling reagents (cf. Figure 2a). In the case of NH2-glass, GOD immobilized by means of 1,3-bifunctional aromatic coupling reagents exhibits drastically lower storage stability than the enzyme immobilized by the respective 1,4-bifunctional aromatic reagents.

Apparent, there is a relation between the storage stability of the GOD-functionalized support surfaces and the low immobilized GOD activity or protein quantity in the 1,3-coupling reaction (cf. Tables 1 and 2). Our conception is that, on one hand, the NH2-functionalized support structures and, on the other hand, the increasing immobilized enzyme activity or protein quantity will form the enzyme’s own efficient microenvironment leading to enzyme activity stabilization (cf. below and ref 18).

The so-called geometry effect seems to be a spacer effect. It results from various spacer lengths in enzyme immobilization by means of 1,3- and 1,4-bifunctional aromatic coupling bonds since in the 1,3 coupling the spacer effect is in principle about 1 Å shorter in comparison to the 1,4 coupling—as indicated by computer model estimates. The coupling with a 1,3-bifunctional aromatic reagent, which is sterically hindered compared to the respective 1,4-bifunctional aromatic reagent, results in a stronger deformation of the active enzyme conformation and/or a loss of (multipoint) bindings with the enzyme protein. The consequence is a higher conformational flexibility associated with a lower enzyme stability. This is also evident in the thermostability results (cf. below).

Wang et al. discuss a relation between the spacer effect and the immobilized enzyme concentration or activity of GOD immobilization on polyethylene membranes. On the basis of the their data, the authors conclude that there is considerable deformation of the GOD protein if the enzyme is immobilized on polyethylene membranes without an alkylene diamino spacer group.

The rate of inactivation of immobilized GOD decreases with greater storage time, e.g., GOD coupled via L-ascorbic acid to NH2-cellulose films loses 5% of its initial activity after 30 days, but retains 90% after storage for 12 months.

To investigate the thermostability, the GOD-functionalized films were treated in bidistilled water at 65 °C for 30 min. Native GOD lost more than 70% of its initial activity under these conditions. GOD immobilized on both NH2-functionalized support materials exhibits lower thermal inactivation compared to the native enzyme. The cellulosic NH2-support stabilizes the enzyme to a greater extent than NH2-glass as seen in Figure 2b. The highest thermostability was achieved for GOD immobilized by means of L-ascorbic acid, diazo coupling, or glutaraldehyde onto NH2-cellulose film, where the enzyme retained 93%, 85%, and 81%, respectively, of the initial activity. After the heat treatment all other GOD-functionalized films lost more than 25% of their initial activity and, in the cases of GOD immobilized by means of isophthalaldehyde and 1,3-diacetylbenzene on NH2-glass, even as much as 100% (see Figure 2b). Similar to the results obtained for the storage stability, GOD coupled via 1,3-
bifunctional aromatic coupling reagents to NH$_2$-glass exhibits a significantly lower thermostability than GOD immobilized by the respective 1,4-bifunctional aromatic reagents.

HRP was immobilized and then explored in the same manner as GOD. As seen in parts c and d of Figure 2, immobilized HRP is more stable when coupled to the NH$_2$-cellulose support compared to NH$_2$-glass. The best storage stability was obtained for HRP coupled via 1,4-diacetylbenzene or L-ascorbic acid, where it retained 99% and 97%, respectively, of its initial activity after 30 days of storage. The most stabilizing immobilization of HRP onto NH$_2$-glass was coupling by means of benzoquinone where the enzyme retained 81% of the initial activity after 30 days of storage. As seen in Figure 2c, there is no significant effect of the coupling molecule geometry for HRP immobilization via 1,3- and the respective 1,4-bifunctional aromatic coupling reagents.

An accepted reason for the stabilization effect of immobilized enzymes is that the active protein conformation is fixed by multipoint bonding formation between the NH$_2$ support and enzyme molecule. Further, it is described that a smaller number of binding points results in less enzyme stability. HRP (MW = 44 000 g/mol) is a small molecule in comparison to GOD (MW = 185 000 g/mol). It could be possible that for this reason a lower number of bonds between the HRP molecule and support surface are sufficient to stabilize the active enzyme conformation by immobilization. This supposition is supported by Garcia et al., who proved by means of $^1$H NMR that only approximately 1.7 Lys residues of the protein are accessible when it is immobilized by means of cyanuric chloride (cf. X-ray structure data quantity of Lys residues of HRP protein in Welinder). This leads to the conclusion that only a few Lys residues of the HRP protein might react with the 1,3-bifunctional aromatic coupling reagents—similar to cyanuric chloride.

This would diminish the geometry effect because the less favored 1,3-bifunctional aromatic coupling reagents lead to sufficient bond formation between the NH$_2$-functionalized support surface and HRP protein without deforming the active conformation of the enzyme protein.

Regarding the thermostability, coupling of HRP with 1,3- or 1,4-diacetylbenzene onto NH$_2$-cellulose film led to the highest stabilization against thermal inactivation at 65 °C. The activity remained 96% and 92%, respectively, of the initial activity of immobilized HRP after 30 min of thermal treatment at 65 °C. A high thermostability of the HRP

Table 2. Operational Enzyme Stability of HRP Immobilized on NH$_2$-Glass and NH$_2$-Cellulose Films

<table>
<thead>
<tr>
<th>operational enzyme stability $t_{1/2}$ (min)$^{a,b,c}$</th>
<th>NH$_2$ support</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_2$-glass</td>
</tr>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
</tr>
<tr>
<td></td>
<td>d  d  77  35  d  d  42  d  105  23  60  d  d  60</td>
</tr>
<tr>
<td></td>
<td>NH$_2$-cellulose</td>
</tr>
<tr>
<td></td>
<td>65  168  110  75  71  131  118  148  354  282  473  683  d</td>
</tr>
</tbody>
</table>

$^a$ Reaction conditions: 25 °C, pH 5.0, 10 mM H$_2$O$_2$, 100 mM ABTS. $^b$ The half-life time $t_{1/2}$ of HRP in solution was 17 min under the given conditions. The standard error of the values is 5–12%. $^c$ For structural formulas of the coupling reagents see Figure 1. $^d$ Not available.
enzyme was also reached by coupling via amide-bond-forming bifunctional reagents, such as L-ascorbic acid (cf. ref 1) and aromatic dicarboxyl and disulfonyl dichlorides, on both NH₂-functionalized supports, with the exception of coupling the enzyme by means of the aromatic disulfonyl dichlorides onto NH₂-glass (see Figure 2d). Because of this observation, it seems that stabilization of the active enzyme conformation has a bigger influence on the thermostability than the microenvironment.

In the case of LOD, comparable activity values of immobilized enzyme were measured on both NH₂-functionalized supports (Table 1), but only the immobilization onto the NH₂-glass surface could stabilize the enzyme activity (Figure 3). For example, while LOD coupled by terephthaloyl chloride onto NH₂-glass retained 60% of its initial activity after 30 days storage at 4°C, the enzyme immobilized onto NH₂-cellulose films using the same coupling reagent lost 99% of its initial activity after only 24 h at 4°C. The best storage stability for LOD (preservation of 69% of its initial activity after 30 days of storage) was achieved by coupling via cyanuric chloride onto NH₂-glass and is comparable with literature values.26

The enzyme could not be stabilized against thermal degradation. LOD immobilized on both NH₂-functionalized supports by means of all the coupling reagents used lost more than 60% of its initial activity after 30 min at only 45°C. As in case of GOD an influence of the molecule geometry of the 1,3- and 1,4-bifunctional aromatic coupling bonds was found for the LOD immobilization. As seen in Figure 3, in most cases, storage stability was found when the enzyme was coupled by the 1,4-bifunctional aromatic reagent compared to coupling by the respective 1,3-bifunctional aromatic reagent. Comparison of the results with GOD, HRP, and LOD leads to the conclusion that the influence of molecule geometry in the 1,3- and 1,4-bifunctional aromatic coupling reagents becomes greater in the order HRP < LOD < GOD, i.e., with increasing enzyme size.

Operational stability, a very important factor for the practical use of enzymes in biosensors or as biocatalysts,20 was tested for HRP because in the native state this enzyme quickly loses its activity in the presence of its substrate hydrogen peroxide.17 To investigate the decrease of HRP activity with operating time, the activity of the enzyme immobilized on NH₂-glass and NH₂-cellulose films, respectively, by the coupling reagents shown in Figure 1 was continuously measured in a test solution containing 10 mM H₂O₂ at pH 5.0 for a time period of 30–40 min. As described in the literature and confirmed by measuring the time-dependent activity decrease of HRP dissolved in the above-mentioned test solution, the inactivation of HRP in the presence of H₂O₂ follows a pseudo-first-order kinetic in a good approximation (P < 0.05).17 The HRP activity decreases under the influence of H₂O₂ (substrate) in solution. The formation of the inactive HRP intermediate “compound III” (see Dunford et al., e.g., ref 27) is the major reason for this effect. (Compound I and compound II are HRP intermediates containing two or one oxidizing equivalents in the HRP enzymatic cycle.)

The half-life of immobilized HRP was determined by fitting a pseudo-first-order function to the data of the H₂O₂/HRP−enzyme reaction. As seen in Table 2, in all cases, immobilized HRP was stabilized against H₂O₂-induced inactivation compared to the native enzyme. Nevertheless, the cellulose NH₂-functionalized support stabilizes the immobilized enzyme to a greater extent than NH₂-glass. With respect to the concept of enzyme stabilization by multipoint attachment,22 the stability of the immobilizing bonds against hydrolysis must influence the operational stability of HRP, because every cleaved immobilizing bond would lead to a more flexible enzyme conformation resulting in lower stability. The half-lives of HRP immobilized on NH₂-cellulose film (Table 2, column 2) show the presumed effect. While HRP immobilized via amide or sulfonamide bonds (coupling reagents 9–12, see Figure 1) is up to 40 times more stable than the native HRP, immobilization via Schiff’s base coupling structures (coupling reagents 3–6, see Figure 1) are less stable against hydrolysis than amide bonds and only stabilize HRP by a factor of 4–8.

The kinetic properties of the immobilized enzymes are characterized by the apparent Michaelis constant K_M,app, which is a measure of the enzyme−substrate affinity and additionally provides microenvironmental influences for the enzyme-catalyzed substrate conversion. The K_M,app Values of the reaction of oxidoreductase enzymes immobilized onto NH₂-glass and NH₂-cellulose films were determined as described in the Experimental Section. The immobilized enzyme activities of the investigated samples are given in Table 1. The K_M,app values of the immobilized enzymes were in most cases lower than those of the native ones in solution (see Table 3).

While the K_M,app values of immobilized HRP and LOD do not strongly depend on the coupling reagent but on the nature of the NH₂-functionalized support surface (see Table 1), the apparent Michaelis constant of immobilized GOD seems to be influenced drastically by the coupling reagent and the NH₂-functionalized support structure (K_M,app Values range from 1.4 to 65 mM). The K_M,app Values found in this study almost cover the wide range of 0.9–66 mM given in the literature.28,29 We found that the K_M,app values of GOD using the same NH₂-functionalized support and coupling reagent decrease with greater immobilized GOD activity. A similar effect was observed for immobilized HRP and LOD, with the difference that the K_M,app values did not vary by more than 25%.
To explore how the glucose (substrate)–GOD affinity depends on the amount of immobilized enzyme protein as well as on the immobilized enzyme activity, the GOD was coupled by glutaraldehyde and L-ascorbic acid onto both NH$_2$-functionalized supports. By variation of the time of enzyme treatment of the activated support surfaces, GOD could be immobilized with different amounts of enzyme activity and protein quantity per surface. As seen in Figure 4, the $K_{M,\text{app}}$ value decreases with increasing immobilized enzyme activity per surface as well as with greater density of coupled enzyme protein.

1,3-Diacetylbenzene, a bifunctional coupling reagent, is capable of fixing high amounts of enzyme protein per NH$_2$-functionalized support and exhibits a low specific enzyme activity (up to 23% for NH$_2$-cellulose films, up to 37% for NH$_2$-glass). Coupling with glutaraldehyde leads to higher

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**Table 3.** $K_{M,\text{app}}$ Values of Oxidoreductase Enzymes GOD, HRP, and LOD Immobilized on NH$_2$-Glass and NH$_2$-Cellulose Films

<table>
<thead>
<tr>
<th>no.</th>
<th>coupling$^b$</th>
<th>GOD NH$_2$-glass</th>
<th>NH$_2$-cellulose</th>
<th>HRP NH$_2$-glass</th>
<th>NH$_2$-cellulose</th>
<th>LOD NH$_2$-glass</th>
<th>NH$_2$-cellulose</th>
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<tr>
<td>1</td>
<td>NaNO$_2$/HCl</td>
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<td>0.25</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>13</td>
<td>benzoquinone</td>
<td>15</td>
<td></td>
<td>0.33</td>
<td></td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The immobilized enzyme activities are given in Table 1. The $K_{M}$ values of the free enzymes are 37 mM for GOD, 0.70 mM for HRP, and 0.57 for LOD.

$^b$ For structural formulas of the coupling reagents, see Figure 1.
specific activities (up to 40% for NH₂-cellulose film, up to 80% for NH₂ glass), but binds less protein. The immobilized GOD enzyme quantity per surface influences the $K_{M,\text{app}}$ values to a greater extent than the coupling reagent.

The reason for altering the $K_{M,\text{app}}$ values of immobilized enzymes compared to native ones is not fully understood. The immobilized enzyme substrate affinity can be affected by real effects, like improving the active conformation of the enzyme molecule, or by apparent effects, such as coupling-induced diffusion phenomena or microenvironmental influences. The effect of increasing substrate affinity of GOD with greater enzyme loading is still under discussion as well. For instance, Gregg et al., who entrapped GOD into a polymer on the surface of an enzyme electrode and measured the enzyme activity as an electrical signal, found decreasing $K_{M,\text{app}}$ values with greater protein density. They explained this effect as a hindrance of the electron flow, caused by a diffusion-controlled reaction, which is typical for GOD immobilized on an ion-exchanger resin from functionalized support structures exerting an optimizing influence.

In the present study where GOD was in fact immobilized onto the surface of ultrathin support layers, no diffusion phenomena could be observed in the kinetic behavior of the enzyme-functionalized support surfaces, and the concentration of the enzyme in solution was the same for all immobilization procedures. The kinetics of the immobilized enzymes, described in this study, follow the Michaelis–Menten mechanism. On principle, in the enzyme reaction environment influences the “active” GOD–enzyme.

The degree of deformation of the GOD–protein conformation is affected by the spacer and microenvironmental effects during enzyme immobilization. An “active/inactive” conformational transition ratio (conformational flexibility) is established at the NH₂-functionalized support surface, which is fixed to a greater or lesser degree depending on the type of coupling reaction. With increased GOD loading per support surface, the probability measure of the active conformation increases due to the enzyme’s own defining microenvironment, which is consequently associated with a decrease in the $K_{M,\text{app}}$ values of immobilized GOD. Our conception is consistent with the following literature data on GOD immobilization.

For instance, Barmin et al. found a significant decrease in the Michaelis constants of the immobilized GOD in comparison to the native enzyme when the GOD is coupled to a positively charged monolayer of polyethylene imine. Studies by Li et al. indicated that GOD is strongly adsorbed on positively charged glycolipid monolayers based on akyl-2-amino-2-deoxy-β-D-glucopyranoside, which is associated with a protein conformation change involving an increase of β-sheet formation.

In conclusion, NH₂-functionalized support surfaces and covalent enzyme immobilization with a large variety of coupling reagents described in this study are a powerful tool for optimizing the properties of oxidoreductase-functionalized support surfaces specifically for each enzyme and support material.

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References and Notes


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