Stabilization of Activity of Oxidoreductases by Their Immobilization onto Special Functionalized Glass and Novel Aminocellulose Film Using Different Coupling Reagents

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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 ($K_{M,app}$). 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 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.[1–7] 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.[8] 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,[9,10] while most studies investigate the influence of the support.[11,12]

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),[13] and a novel NH_2-functionalized coating on glass (NH_2-glass)[14] were developed as transparent NH_2-functional support matrixes. 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

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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 SiO_2 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 SiO_2 coating forms silanols which are condensed to a vitreous polymer film ("polymerized SiO_2").

This polymerized SiO_2 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.[13] 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′-biphenyldis-

ulfonyl 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-diacetylbenzene 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 $NaNO_2$ 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[15] containing 4-aminoantipyrine, 2,4,6-tribromo-3-hydroxybenzoic acid, and enzyme substrate dissolved in 100 mM phosphate buffer, pH 7.0 ($\lambda_{max} = 514$ nm, $\epsilon = 27.2$ 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 H_2O_2) described by Gallati[16] ($\lambda_{max} = 414$ nm, $\epsilon = 36.8$ cm²/μmol). While the glass rod was stirred in the solution, the increase of absorbance was measured at λ_{max} of the test system used.

The above-mentioned test systems and procedures were used to determine the $K_{M,app}$ 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 H_2O_2 (HRP), and 0.05−10 mM L-lactate (LOD). The $K_{M,app}$ values were calculated by mathematical hyperbola curve fitting (standard error range 2−13%) of the well-known Michaelis−Menten algorithm ($v = v_{max}S/(K_M + S)$) 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-
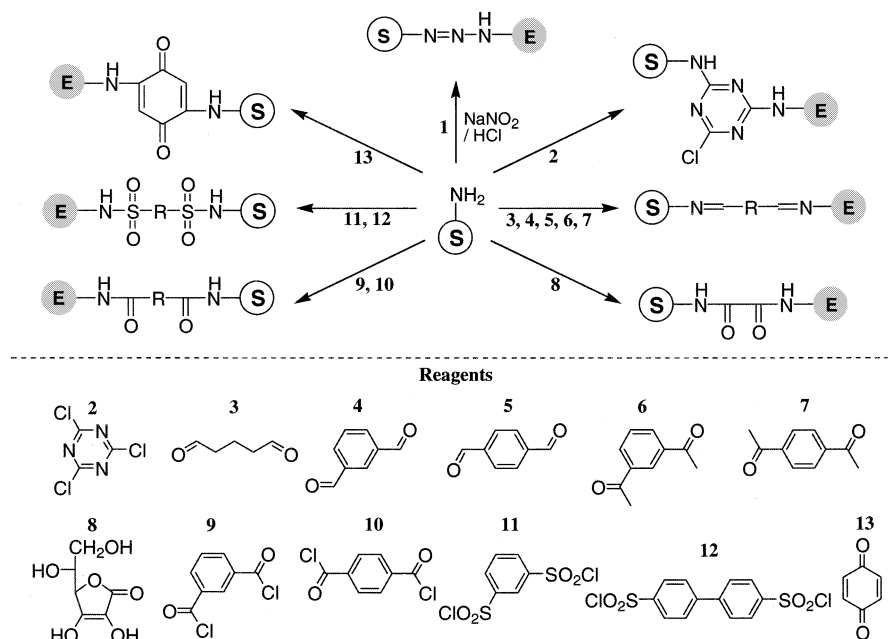
Figure 1. Structural formulas: coupling reagents and coupling structures between NH_2 support (S) and enzyme protein (E).

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 $\tau_{1/2} = \ln(0.5)/k$.[17] 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 μL 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.[18] 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/cm² 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/cm²).[19] HRP was immobilized up to 400 mU via benzoquinone onto NH_2-glass and up to 200 mU/cm² 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/cm²). 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

Table 1. Activities of Oxidoreductase Enzymes GOD, HRP, and LOD Immobilized on NH_2-Glass and NH_2-Cellulose Films

| | | immobilized enzyme activitiy[a] (mU/cm²) | | | | | |
| | | GOD | | HRP | | LOD | |
no.	coupling[b]	NH_2-glass	NH_2-cellulose	NH_2-glass	NH_2-cellulose	NH_2-glass	NH_2-cellulose
1	NaNO₂/HCl	c	194	c	69	c	220
2	cyanuric chloride	168	78	24	59	265	119
3	glutaraldehyde	160	187	70	206	175	100
4	isophthalaldehyde	26	94	40	58	27	61
5	terephthalaldehyde	24	56	23	48	32	21
6	1,3-diacetylbenzene	38	51	34	76	85	123
7	1,4-diacetylbenzene	57	70	14	104	88	52
8	L-ascorbic acid	140	165	111	200	84	192
9	isophthaloyl chloride	15	34	18	121	201	286
10	terephthaloyl chloride	116	60	70	27	205	131
11	1,3-benzenedisulfonyl chloride	23	168	11	48	333	c
12	4,4′-biphenyldisulfoyl chloride	82	27	12	35	339	c
13	benzoquinone	219	7	408	12	65	4

[a] Samples used for stability and kinetics investigations were in a 10% range of the given values. [b] For structural formulas of the coupling reagents see Figure 1. [c] Not available.

films, possibly because it appears to be a multilayer consisting of unordered NH_2-cellulose chains (about 100 nm size) in all space directions, which diminishes any spacer effects as observed earlier.[20] An influence of the coupling-molecule geometry was not observed for the immobilized activity of HRP and LOD on either of the NH_2-functionalized support surfaces.

A possible explanation for the lack of the geometry effect is that reactive amino acid residues, especially lysine (Lys) residues, are, in contrast to GOD, present at peripheral positions of the HRP and LOD enzyme molecules serving as binding sites for enzyme immobilization without deforming the active enzyme protein conformation.

Storage stability and thermostability are two important factors for the applicability of immobilized enzymes, because native enzymes usually quickly lose their activity.[21] The greatest stabilizing effect for GOD activity regarding storage was achieved when the enzyme was immobilized via L-ascorbic acid and benzoquinone on NH_2-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 NH_2-cellulose and the NH_2-glass surface, except for the 1,3-bifunctional aromatic coupling reagents (cf. Figure 2a). In the case of NH_2-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.

Apparently, 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 NH_2-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 immobiliza-

tion 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.[22] 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 NH_2-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 NH_2-functionalized support materials exhibits lower thermal inactivation compared to the native enzyme. The cellulosic NH_2-support stabilizes the enzyme to a greater extent than NH_2-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 NH_2-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 NH_2-glass, even as much as 100% (see Figure 2b). Similar to the results obtained for the storage stability, GOD coupled via 1,3-
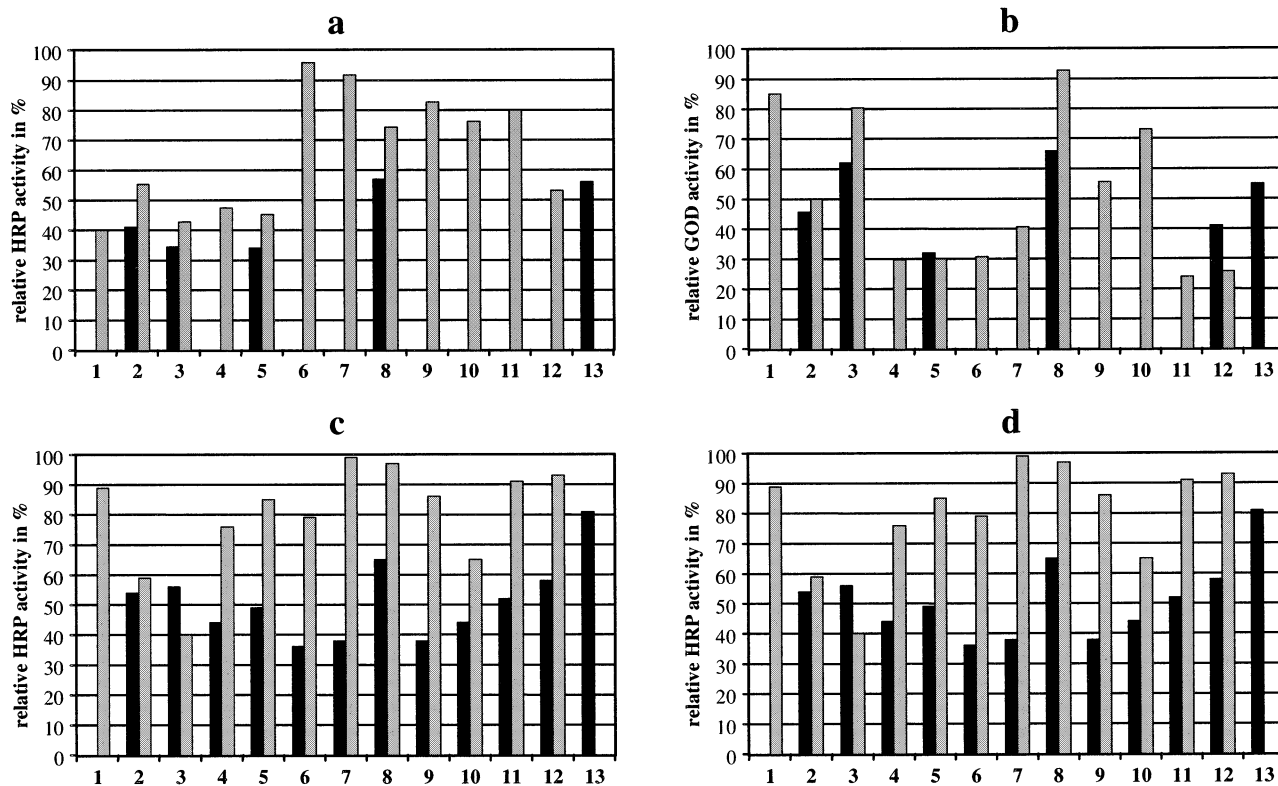
Figure 2. Storage stability and thermostability of GOD and HRP immobilized on NH_2-glass (dark columns) and NH_2-cellulose films (pale columns) using different coupling reagents (structural formulas see Figure 1): immobilized GOD (a) storage stability and (b) thermostability; immobilized HRP (c) storage stability and (d) thermostability. (To investigate the thermostability, the enzyme-functionalized support surfaces were treated in distilled water at 65 °C for 30 min.)

Table 2. Operational Enzyme Stability of HRP Immobilized on NH_2-Glass and NH_2-Cellulose Films[a]

NH_2 support	operational enzyme stability $\tau_{1/2}$ (min)[b,c]												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
NH_2-glass	*d*	*d*	77	35	*d*	42	*d*	105	23	60	*d*	*d*	60
NH_2-cellulose	80	168	110	75	71	131	118	148	354	282	473	683	*d*

[a] Reaction conditions: 25 °C, pH 5.0, 10 mM H_2O_2, 100 mM ABTS. [b] The half-life time $\tau_{1/2}$ 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.

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.[22] Further, it is described that a smaller number of binding points results in less enzyme stability.[23] 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.,[24] 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[25]). 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
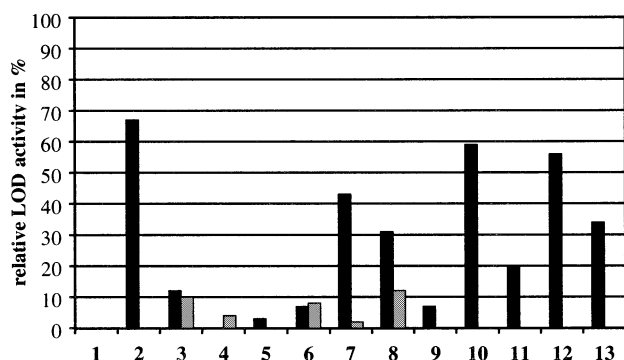
Figure 3. Storage stability of LOD immobilized onto NH_2-glass (dark columns) and NH_2-cellulose (pale columns) using different coupling reagents (structural formulas see Figure 1).

enzyme was also reached by coupling via amide-bond-forming bifunctional reagents, such as L-ascorbic acid (cf. ref 1) and aromatic dicarbonyl and disulfonyl dichlorides, on both NH_2-functionalized supports, with the exception of coupling the enzyme by means of the aromatic disulfonyl dichlorides onto NH_2-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 NH_2-functionalized supports (Table 1), but only the immobilization onto the NH_2-glass surface could stabilize the enzyme activity (Figure 3). For example, while LOD coupled by terephthaloyl chloride onto NH_2-glass retained 60% of its initial activity after 30 days storage at 4 °C, the enzyme immobilized onto NH_2-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 NH_2-glass and is comparable with literature values.[26]

The enzyme could not be stabilized against thermal degradation. LOD immobilized on both NH_2-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 NH_2-glass and NH_2-cellulose films, respectively, by the coupling reagents shown in Figure 1 was

continuously measured in a test solution containing 10 mM H_2O_2 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 H_2O_2 follows a pseudo-first-order kinetic in a good approximation ($P < 0.05$).[17] The HRP activity decreases under the influence of H_2O_2 (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 H_2O_2/HRP−enzyme reaction. As seen in Table 2, in all cases, immobilized HRP was stabilized against H_2O_2-induced inactivation compared to the native enzyme. Nevertheless, the cellulosic NH_2-functionalized support stabilizes the immobilized enzyme to a greater extent than NH_2-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 NH_2-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 NH_2-glass and NH_2-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 NH_2-functionalized support surface (see Table 1), the apparent Michaelis constant of immobilized GOD seems to be influenced drastically by the coupling reagent and the NH_2-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 NH_2-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%.

Table 3. $K_{M,app}$ Values of Oxidoreductase Enzymes GOD, HRP, and LOD Immobilized on NH_2-Glass and NH_2-Cellulose Films

no.	coupling[b]	K_M values (mM) of the immobilized enzymes[a]					
		GOD		HRP		LOD	
		NH_2-glass	NH_2-cellulose	NH_2-glass	NH_2-cellulose	NH_2-glass	NH_2-cellulose
1	NaNO$_2$/HCl		2.3		0.25		0.37
2	cyanuric chloride	25	17.8	0.63	0.25	0.38	0.50
3	glutaraldehyde	18	7.1	0.52	0.30	0.40	0.35
4	isophthalaldehyde	26	15.4	0.74	0.24	0.57	0.37
5	terephthalaldehyde	19	16.2	0.70	0.22	0.50	0.29
6	1,3-diacetylbenzene	46	14.7	0.52	0.20	0.40	0.33
7	1,4-diacetylbenzene	36	7.0	0.80	0.21	0.37	0.34
8	L-ascorbic acid	11	1.4	0.67	0.23	0.35	0.28
9	isophthaloyl chloride	65	9.2	0.86	0.32	0.51	0.35
10	terephthaloyl chloride	32	18.8	0.35	0.19	0.45	0.40
11	1,3-benzenedisulfonyl chloride	33	5.0	0.80	0.25	0.37	
12	4,4′-biphenyldisulfoyl chloride	13	25.6	0.55	0.25	0.37	
13	benzoquinone	15		0.33		0.50	

[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.
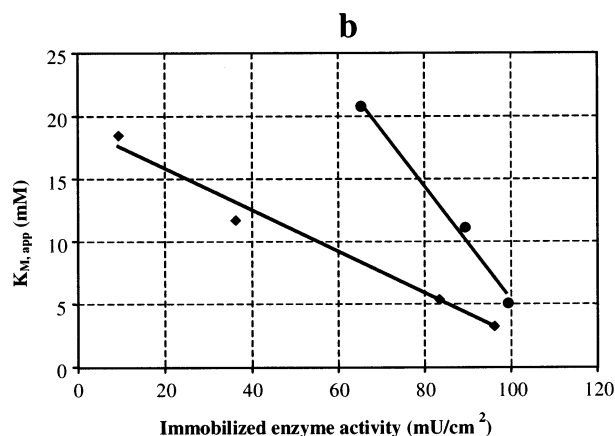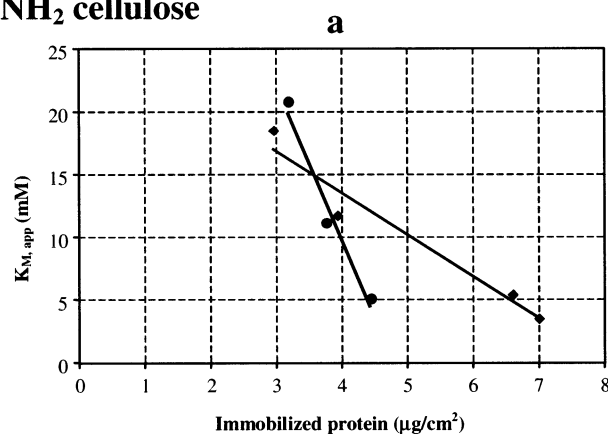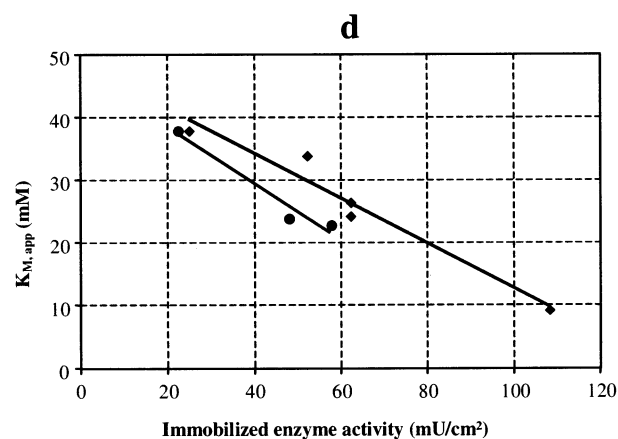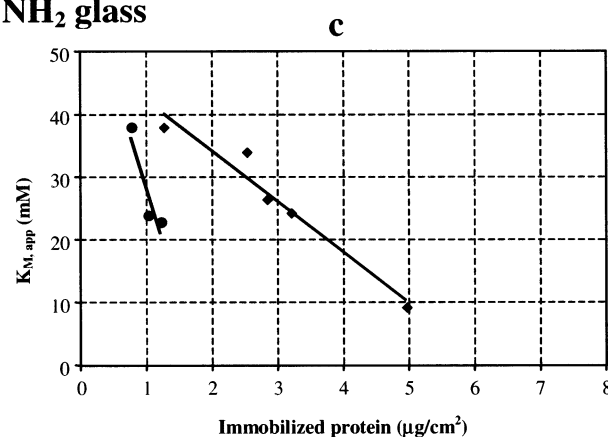[b] For structural formulas of the coupling reagents, see Figure 1.

NH$_2$ cellulose

NH$_2$ glass

Figure 4. Plot "K_M values vs immobilized GOD activities and protein quantities", using the support surfaces NH_2-glass and NH_2-cellulose films as well as the coupling reagents glutaraldehyde (●) and L-ascorbic acid (◆): (a) NH_2-cellulose films, K_M values vs protein quantities; (b) NH_2-cellulose films, K_M values vs GOD activities; (c) NH_2-glass, K_M values vs protein quantities; (d) NH_2-glass, K_M values vs GOD activities. Note that lines in the graphs a to d are drawn by hand to illustrate the general trend of the data. They do not indicate a linear slope.

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 $K_{M,app}$ value decreases with increasing immobilized enzyme activity per surface as well as with greater density of coupled enzyme protein.

L-Ascorbic acid, a bifunctional coupling reagent, is capable of fixing high amounts of enzyme protein per NH_2-functionalized support surface 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

specific activities (up to 40% for NH$_2$-cellulose film, up to 80% for NH$_2$ glass), but binds less protein. The immobilized GOD enzyme quantity per surface influences the $K_{M,app}$ values to a greater extent than the coupling reagent.

The reason for altering the $K_{M,app}$ values of immobilized enzymes compared to native ones is not fully understood.[30] 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 $K_{M,app}$ values with greater protein density.[31] They explained this effect as a hindrance of the electron flow, caused by a diffusion-controlled reaction, which is typical of entrapped enzymes. Klei et al., who found the same effect for GOD immobilized on an ion-exchanger resin from solutions with different concentrations, suggested that a lower distortion of the immobilized enzyme molecules with higher immobilized density would lead to a higher substrate affinity.[32]

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 there is also the possibility of falsification of K_M values if the enzyme substrate enriches on the NH$_2$-functionalized support surface, where the enzyme is immobilized. In the case of the GOD reaction no indication of glucose enrichment was found on the NH$_2$-cellulose film surface.

The results of the enzyme immobilization, especially in the case of GOD, lead to the conclusion that the NH$_2$-functionalized support structures exert an optimizing influence on the kinetic and stability, particularly of the immobilized enzyme activity.

Our conception is that the NH$_2$-functionalized support environment influences the "active" GOD–protein conformation probability measure. A working hypothesis is, for example, that NH$_2$ groups present in a positively charged form and less reactive with respect to coupling reagents, have a stabilizing effect on the active conformation of the 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 NH$_2$-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 $K_{M,app}$ 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.[33] 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.[34]

In conclusion, NH$_2$-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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