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Enzyme immobilization via microbial transglutaminase: a method for the generation of stable sensing surfaces

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Abstract

Transglutaminase from a variant of *Streptoverticillium mobaraense* (MTGase), which catalyzes the acyl-transfer between γ-carboxyamide groups and various primary amines was applied for the immobilization of biomolecules on electrode surfaces. Upon addition of suitable polypeptides (poly-L-lysine, poly-L-glutamine) or other proteins, which serve as substrates for MTGase, such as casein, gelatin or fibrinogen, networks can be generated by the entrapped and/or covalently attached sensing enzymes. These matrix proteins were used as cross-linkers and allow for the preparation of an enzyme layer with tailor made properties. The preparation of MTGase catalyzed enzyme layers on disposable screen printed electrodes using glucose oxidase (GOx) as a model enzyme is described. Best results were achieved using 2.3 U GOx mm^{-2} and 0.1 mg (poly-L-lysine/poly-L-glutamine) mm^{-2}, giving rise to a sensitivity of 0.15 nA l μmol^{-1} mm^{-2}. In a further approach the storage as well as the operational stability of lactate enzyme electrodes obtained upon immobilization of lactate oxidase (LOx) in a fibrinogen network was determined in comparison to biosensors prepared by classical chemical cross-linking. After storage of 20 weeks at 4°C in humid atmosphere, the MTGase based electrodes remained fully stable as judged by their sensitivity, whereas the electrodes obtained via glutaraldehyde (GDA) cross-linking lost 40% of their sensitivity. The lifetime (LT50) of the MTGase derived electrodes was determined at ambient temperature to be 133 ± 26 h ($n = 4$), whereas a lifetime of only 53 ± 9 h ($n = 3$) was found for the electrodes with GDA cross-linking. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Microbial transglutaminase; Immobilization; Stabilization; Protein cross-linking; Fibrinogen

1. Introduction

The most important features of biosensors are their specificity and sensitivity towards the target analyte(s). The specificity is principally governed by the properties of the biological component, the sensitivity of the integrated device, however, is dependent on both the biological component and the transducer. The relatively slow progress of biosensor technology from inception to fully functional commercial devices for these applications is a reflection of both technology-related and market factors. A wider acceptance of biosensors as reliable analytical tools is impaired by their limited storage and operational stability. Improvements in the stability and retention of biochemical activity in in vitro environments can be considered vital to the success of these devices.

Though a vast number of techniques for the immobilization and stabilization of biomolecules

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have been elaborated [1–5], only a few general conclusions can be drawn. In practice the individual biomolecule fixed on a certain transducer requires a tailor made solution. Thus, there is a growing interest in alternative methods for the gentle immobilization of proteins on transducer surfaces, preferably by simple adsorption. Moreover, the technique should be fully compatible to the application of soluble stabilizing additives.

Transglutaminase (*R*-glutaminyl-peptide: amine γ-glutaminyl transferase, EC 2.3.2.13), from a variant of *Streptoverticillium mobaraense* (MTGase), catalyzes the cross-linking of proteins, promoting the formation of isopeptide bonds between the γ-carboxyl group of a glutamine residue and the ε-amino group of a lysine [6]. MTGase is highly specific towards the acyl-donor, however, besides lysine residue as an acyl-acceptor, primary alkylamines may be used as substrates, which allows for the selective alkylation of proteins via accessible glutamine residues.

Up to now, MTGase has been used mainly for food biotechnological applications, e.g., for the alteration of the foaming and emulsifying properties of food proteins [7] or to improve the nutritive value of food by cross-linking soy-proteins or casein with lysine dipeptides [8] or other amino acids [9]. Another important application is the gelation of soluble edible proteins, e.g., casein [10,11] or the meat binding, which allows a restructured sliceable product from cut or molded beef to be obtained. Moreover, in enzyme technology MTGase was applied for the immobilization of enzymes in casein-gels [12,13] or on ion exchange resins [14].

In contrast to mammalian transglutaminases, which are found in many tissues and play an important role in the post translational modification of proteins or, as factor XIII, in blood coagulation [15], the physiological role of microbial transglutaminase is only poorly understood.

Compared to classical chemical methods the MTGase catalyzed cross-linking requires physiological conditions, which allow for an immobilization of labile enzymes. This makes MTGase a powerful tool in the field of analytical biotechnology, in particular in the development of biosensors. Recently, we reported the use of MTGase for the formation of immunoconjugates to be applied in ELISA or biosensors [16,17]. In this paper we describe the preparation of stable enzyme layers on transducer surfaces cross-linked by MTGase.

In the first part of the present study the MTGase mediated immobilization of glucose oxidase (GOx), as a model enzyme, on disposable screen printed electrodes in the presence of additional cross-linkers is described. The optimization of the immobilization procedure with respect to the content of cross-linker and the enzyme load is demonstrated. In the second part of this manuscript the MTGase derived immobilization procedure is transferred to the preparation of lactate enzyme sensors and the effect of different types of cross-linkers on the sensor performance is discussed. The storage stability of these enzyme electrodes is compared to those obtained by chemical cross-linking via glutaraldehyde (GDA). Moreover, the compatibility of this new method with the use of soluble additives is shown and data on the operational stability of the lactate enzyme sensors will be given, which demonstrate the benefits of this techniques compared to conventional chemical cross-linking.

2. Experimental

2.1. Materials

Microbial transglutaminase from a variant of *S. mobaraense* (500 U g^{-1}) was a kind gift from Ajinomoto (Aichi 481, Japan) [18]. The activity was measured by a colorimetric hydroxamate assay using *N*-carbobenzoxy-L-glutaminyl glycine [19]. One unit of MTGase activity is defined as the amount of enzyme which is necessary for the formation of one micromole of

hydroxamic acid per minute. GOx (*Aspergillus niger*, 300 U mg^{-1}) was obtained from Boehringer (Mannheim, Germany) and lactate oxidase (LOx, *Pediococcus* sp., 28.3 U mg^{-1}) from Fluka (Buchs, Switzerland). Poly-L-lysine (PLL, MW > 300,000), poly-L-glutamine (PLG, MW 3500), casein (sodium salt from bovine milk), fibrinogen (fraction I, type I-S from bovine plasma), gelatin (from cold water fish skin (teleostean gelatin), 50% aqueous solution) and 60 kDa dextran from *Leuconostoc mesenteroides* were purchased from Sigma (St. Louis, IL, USA). DEAE-dextran and T500 dextran were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Other chemicals were obtained from Aldrich (Steinheim, Germany), Fluka and Sigma in analytical reagent grade.

Buffers and aqueous solutions were prepared using double-distilled, 0.2 μm filtered water. The phosphate-buffered saline (PBS) contained 0.145 M sodium chloride, 8 mM sodium hydrogen phosphate and 2 mM potassium phosphate.

2.2. Preparation of the electrodes

The experiments with immobilized GOx were carried out using Pt-sputtered electrodes with a geometrical sensor area of 7 mm^2 as working electrodes as described elsewhere [20].

The screen printed electrodes, for the lactate measurements, were prepared using ED 965 SS from Acheson (Colloiden, Scheenda, the Netherlands) as graphite track and T 33 PT from MCA Services (Melbourn, S. Cambridgeshire, UK) as Pt–C track. The thickness of the tracks was 20 μm, the geometrical area for immobilization was 3.14 mm^2. The tracks were sealed with laminate foil (thickness 150 μm) at 130°C.

2.3. Preparation of the enzyme membranes

2.3.1. MTGase-catalyzed immobilization

The immobilization solution was obtained by dissolving enzyme, matrix proteins and MTGase in 5–10 μl PBS buffer, pH 7.4. The enzyme membranes were prepared by pipetting 1.5–5 μl of the immobilization mix directly onto the working electrode. The electrodes were stored on a water soaked filter in a receptacle in order to maintain a humid atmosphere. The cross-linking was allowed to proceed for approximately 2 h at room temperature, or overnight at 4°C. Finally, the electrodes were dried at air and were then ready for use. The additives were dissolved in the immobilization solution to give a final concentration of 1% (w/w). Electrodes prepared without additives served as control (see Fig. 5).

2.3.2. Chemical cross-linking with GDA

For the immobilization of LOx via GDA cross-linking, an enzyme solution was prepared containing 2.81 μl LOx (1 U μl^{-1} H_2O_{dest}), 2 μl BSA solution (50% BSA in H_2O_{dest}, w/w) and 1.19 μl H_2O_{dest}. Subsequently, 2 μl of this enzyme solution were mixed with 2 μl GDA (1% GDA in glycerol/H_2O, 1:4, v/v) and 1 μl H_2O_{dest} giving rise to an immobilization solution, which has to be applied directly to the electrodes. Two microliters of the immobilization solution is pipetted onto the screen printed electrode, which is ready for use upon 45-min incubation at room temperature.

For the determination of the storage stability at 4°C, the lactate enzyme electrodes were stored on a water soaked filter in a closed receptacle in order to prevent drying of the enzyme membrane. Upon storage of 4, 8, 12 and 20 weeks three electrodes from each immobilization batch were taken and the sensitivity was determined as described below. The storage of enzyme electrodes in presence of soluble additives was performed in the dry state in a closed receptacle in an air conditioned lab at 23°C. Enzyme electrodes without additives were stored under the same conditions and served as control. The initial sensitivity was determined with freshly prepared electrodes without prior storage ($t = 0$).

It is noteworthy that the enzyme membrane was dried after the incubation with MTGase and

prior to the measurements. The preconditioning comprised of reswelling of the enzyme membrane and a concomitant base line equilibration, which takes up to 30 min, depending on the matrix used. A further pretreatment at a higher potential was not necessary.

2.4. Sensor measurements in a batch arrangement

2.4.1. Determination of glucose

The measurements were carried out in a stirred acrylic glass cell (sample volume 6 ml) at ambient temperature. The experiments were carried out in the presence of PBS buffer, pH 7.4 at a stirrer speed of 600 rpm. A two electrode arrangement was used comprising of the GOx covered, screen printed, platinized working electrode polarized at +700 mV vs. an internal screen printed Ag/AgCl reference electrode on the same support [20]. After about 20–30 min the background current remained constant and the substrate was added. The voltage was adjusted and the current increase was monitored with an EP 30 electrochemical detector from Biometra (Göttingen, Germany).

2.4.2. Determination of lactate

The measurements were carried out as described for glucose, using LOx modified screen printed electrodes and an external Ag/AgCl reference electrode (3 M KCl) in the presence of PBS buffer, pH 6.5. All calibrations were performed upon successive addition of analyte.

2.5. Lactate determination in a flow system

The operational stability of lactate sensors, prepared by MTGase-catalyzed immobilization of LOx in a fibrinogen network, was determined in a simple flow-through set-up. For this application a system, consisting of a flow cell, a peristaltic pump (Meredos, Bovenden, Germany), a six-way-valve (Knauer, Berlin, Germany) and the EP 30 detector was used. The measurements were performed in PBS, pH 6.5 containing 0.01% sodium azide at a potential of +700 mV vs. an a screen printed Ag/AgCl reference electrode. Lactate (3 ml) and buffer (2 ml) were pumped alternately through the measuring cell. After the measurements the cell was washed with 11 ml of buffer. The software package FIABOLO (ICB, Münster, Germany) was used to control the valve and to monitor the electrochemical signal continuously.

3. Results and discussion

In initial experiments the formation of MTGase catalyzed protein networks was investigated using GOx as the model sensing enzyme. Since GOx is known as a poor substrate for MTGase [14], stable enzyme layers were obtained only in the presence of suitable matrix proteins, which display a number of cross-linkable glutamine and lysine residues on the surface.

3.1. Preparation of glucose enzyme sensors

The influence of the matrix proteins was investigated in detail, using PLL and PLG with a ratio of 1:2 (w/w) as cross-linkers (Fig. 1). According to preliminary studies the enzyme load was set to 2.3 U GOx mm^{-2}. At amounts of less than 0.04 mg (PLL/PLG) mm^{-2} the enzyme layer was mechanically not stable and became detached from the surface of the electrode. The optimum, with respect to signal intensity, was reached between 0.08 and 0.12 mg (PLL/PLG) mm^{-2}. The presence of higher amounts of PLL/PLG caused a strong decrease in the signal intensity probably due to the higher density of the enzyme layer causing limitations in substrate diffusion.

In further experiments the enzyme load was varied using 0.09 mg (PLL/PLG) mm^{-2} as cross-linker. Concomitantly, with a higher enzyme load, the signal increased up to an optimum between 2 and 3 U GOx mm^{-2} (Fig. 2).

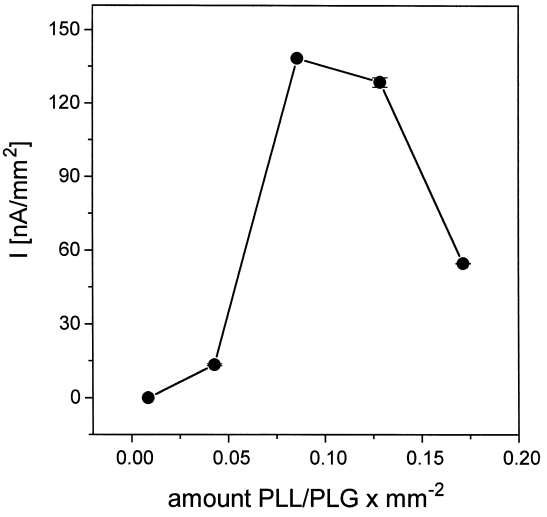

Fig. 1. Influence of the matrix protein (PLL/PLG) on the performance of glucose enzyme sensors. The enzyme load was set to 2.3 U GOx mm^{-2}. The sensor signal was determined at a concentration of 1 mmol l^{-1} glucose in PBS buffer, pH 7.4, at a potential of +700 mV vs. Ag/AgCl; the geometrical area for immobilization was 7 mm^2; mean values ± range ($n = 2$).

Best results were achieved using an enzyme load of 2.3 U GOx mm^{-2} and 0.1 mg (PLL/PLG) mm^{-2} as cross-linker, which corresponds to an 11-fold excess (w/w) of the matrix proteins over the sensing enzyme. The enzyme sensors obtained under these conditions were calibrated with glucose, in order to determine the sensitivity. The electrodes showed linearity up to 2 mmol l^{-1} glucose (data not shown). The sensitivity was 0.15 nA l μmol^{-1} mm^{-2} and the detection limit was determined to 1 μmol l^{-1} glucose.

Literature data revealed several proteins, which may serve as substrates for MTGase [13,14]. Following the process of blood coagulation, initial experiments were carried out in the presence of thrombin, in order to first degrade fibrinogen to fibrin, which was subsequently cross-linked by MTGase. The enzyme electrodes, however, showed a dramatic decay of the sensor signal within the first 15 min (data not shown), which might be explained by a proteolytic attack of GOx by thrombin. Thus, fibrinogen was used directly for cross-linking. A comparative study on the effect of casein, PLL/PLG or fibrinogen, as protein matrices on the sensor performance, exhibited the highest sensor response for the fibrinogen network (data not shown). The sensor response of the fibrinogen type electrodes at a concentration of 1 mmol glucose was 1.6 and 13 times higher in comparison to that obtained from enzyme electrodes based on the PLL/PLG network and the casein polymer, respectively. Due to the different affinity of the substrates to MTGase the content of cross-linker and MTGase was optimized individually. The content of MTGase had to be reduced by two orders of magnitude for the fibrinogen based immobilization in order to prevent a premature polymerization of the mixture. Thus, the sensor performance cannot be attributed to the kind of cross-linker alone. The incubation time of only 2 h (at room temperature) to prepare the network and the short response time for glucose (< 10 s) recommended fibrinogen, however, as cross-linker for further studies. Investigations with fish gelatin as a

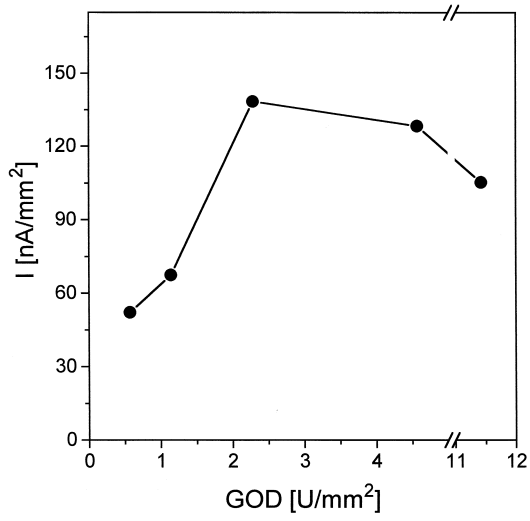

Fig. 2. Effect of the GOx load on the sensor response of MTGase derived enzyme electrodes. The amount of matrix protein was 0.1 mg (PLL/PLG) mm^{-2} at a ratio of 1:2 (w/w). The sensor signal was determined at a concentration of 1 mmol l^{-1} glucose in PBS buffer, pH 7.4, at a potential of +700 mV vs. Ag/AgCl; a representative single experiment is shown.

further matrix protein failed, due to problems arising from the unreproducible swelling of the enzyme membrane. For the gelatin based enzyme sensors, an increasing sensor response was observed over the time, which can be attributed to a sluggish swelling of the gelatin gel. First, the gelatin gel is packed very tightly. Concomitantly with the swelling of the enzyme membrane, the layer thickens and the pores of the gel widen, resulting in a less hindered diffusion of glucose in the gelatin gel.

3.2. Preparation of lactate sensors

The immobilization method established for glucose enzyme sensors was subsequently transferred to prepare lactate enzyme sensors using screen printed platinized carbon electrodes as transducer and fibrinogen as matrix protein. After optimization of the enzyme load and the fibrinogen content a calibration curve was obtained, which showed a linear range up to 1 mmol l^{-1} lactate and a detection limit of 3 μmol l^{-1} lactate (Fig. 3). The sensitivity in the range between 10–1000 μmol l^{-1} lactate was determined to 0.15 nA l μmol^{-1} mm^{-2}.

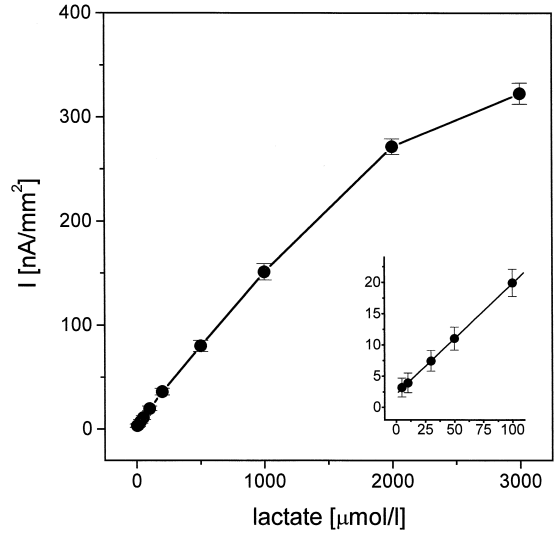

Fig. 3. Calibration curve of MTGase cross-linked lactate sensors. All data given relates to 1 mm^2 geometrical sensor surface: 0.12 U LOx, 0.05 mg fibrinogen, 6×10^{-5} U MTGase, the measurements were performed in PBS buffer, pH 6.5 at a potential of +700 mV vs. Ag/AgCl reference and Pt counter electrode; the geometrical area for immobilization was 3.14 mm^2; mean values ±S.D. ($n=8$, four electrodes from the same immobilization batch were determined in duplicate).

3.3. Effect of different matrix proteins on the sensor performance

As for the glucose sensors, the effect of different matrix proteins on the sensor performance was investigated using PLL/PLG, casein and fibrinogen. In contrast to the studies described above, the content of cross-linker was kept constant for all experiments and not optimized individually. The experimental conditions elaborated for the cross-linking of fibrinogen were transferred to the other matrix proteins. The MTGase content for the cross-linking of PLL/PLG and casein had to be increased by two orders of magnitudes in order to obtain a stable polymer. Compared to the other matrix proteins, fibrinogen is a much better substrate for MTGase. Under the elaborated experimental conditions all immobilization solutions polymerized within 1–2 h at room temperature.

The freshly prepared electrodes were calibrated and the sensitivity in the linear range between 10–1000 μmol l^{-1} lactate was determined (Table 1). In contrast to the studies performed with glucose enzyme sensors, the lactate sensors prepared with PLL/PLG as cross-linker gave no sensors response, probably due to the loss of LOx activity during immobilization. The sensors were sensitive to separately added hydrogen peroxide. Moreover, upon addition of free LOx and lactate to the measuring chamber, the sensors were able to detect hydrogen peroxide as well. Thus, the malfunctioning of the sensors could not be attributed to diffusion limitation by the protein network itself.

The sensitivities obtained for the sensors based on casein and fibrinogen cross-linking were quite similar, though the MTGase contents used for cross-linking were different (Table 1). It can be assumed that a lower content of MTG-

Table 1
Effect of different matrix proteins on the performance of lactate enzyme sensors

Cross-linker	Sensitivity (nA l μmol^{-1} mm^{-2})	Response time, t_{95}^{a} (s)
Casein	0.086 ± 0.003	46.0 ± 8.3
Fibrinogen	0.083 ± 0.013	27.9 ± 2.4
PLL/PLG	–	–

[a]Determined at a substrate concentration of 1 mmol l^{-1} lactate. All data given relates to 1 mm^2 geometrical sensor surface: 0.12 U LOx, 0.05 mg cross-linker, 6×10^{-5} U MTGase (fibrinogen), 6×10^{-3} U MTGase (casein and PLL/PLG); the measurements were performed in PBS buffer, pH 6.5 at a potential of +700 mV vs. Ag/AgCl (3 M KCl) reference and Pt counter electrode; the geometrical area for immobilization was 3.14 mm^2; mean values $\pm$S.D. ($n = 4$).

ase yields a less cross-linked enzyme layer with a less limited diffusion, which is confirmed by the lower response time found for these sensors (Table 1). It is noteworthy that for this application, the results obtained with casein as cross-linker were much better compared to those observed for the preparation of Pt-sputtered glucose enzyme sensors.

Finally, the MTGase derived immobilization method was successfully applied to prepare a complex multi-enzyme sensor for the determination of *ortho*-phosphate comprising maltose phosphorylase, mutarotase and GOx as sensing enzymes (data not shown). In this case the more rigid enzyme layer obtained from PLL/PLG as cross-linkers resulted in a better sensor performance as the enzyme sensors based on fibrinogen cross-linking.

It can be summarized so far that the conditions for the MTGase derived immobilization of sensing enzymes strongly depend on the structural properties of the target enzymes, the electrode surface and the cross-linker, which forms the protein matrix. Thus, the experimental conditions have to be optimized individually.

3.4. Storage stability of lactate enzyme sensors obtained via MTGase and GDA cross-linking

For a comparative study of the new procedure with conventional cross-linking techniques lactate sensors were prepared by co-immobilization of bovine serum albumin and LOx in presence of GDA. This approach, which follows the lines of established standard cross-linking procedures, was then compared to an immobilization via MTGase and fibrinogen as cross-linker. Both types of electrodes were stored at 4°C in a sealed receptacle. Upon determination of the electrodes sensitivities after 4, 8, 12 and 20 weeks of storage the data revealed 100% stability for the MTGase derived electrodes, whereas the electrodes based on GDA cross-linking display only about 60% of the initial sensitivity (Fig. 4).

In similar experiments lactate enzyme sensors prepared in presence of several dextrans as additives were stored under dry conditions at 23°C. Enzyme membranes based on hydrogels often suffer from unreproducible sensor signals

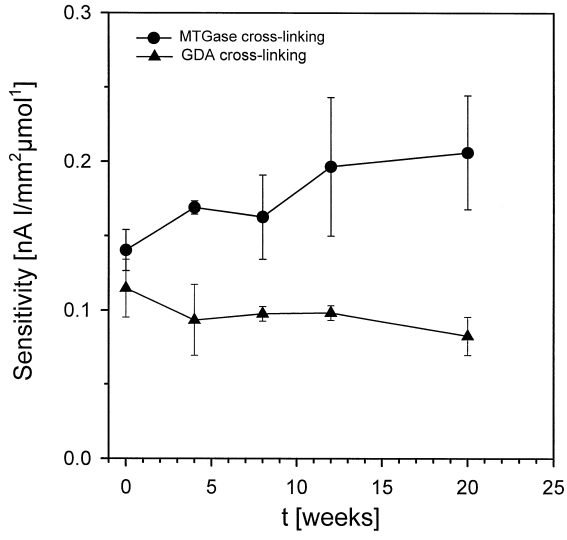

Fig. 4. Storage stability of lactate enzyme sensors prepared via MTGase and glutaraldehyde cross-linking. MTGase cross-linking, 0.12 U LOx mm^{-2}; 0.05 mg fibrinogen mm^{-2}, 6×10^{-5} U MTGase mm^{-2}; GDA cross-linking, 0.12 U LOx mm^{-2}; 0.085 μl BSA-solution mm^{-2} (50% BSA in H_2O, w/w); 0.25 μl GDA-solution mm^{-2} (1% GDA in glycerol/H_2O, 1:4, v/v); geometrical sensor area, 3.14 mm^2; buffer, PBS, pH 6.5; analyte, 0.005–3 mmol l^{-1} lactate; potential, +700 mV vs. Ag/AgCl (3 M KCl) reference and Pt counter electrode; mean values $\pm$S.D. ($n = 3$).

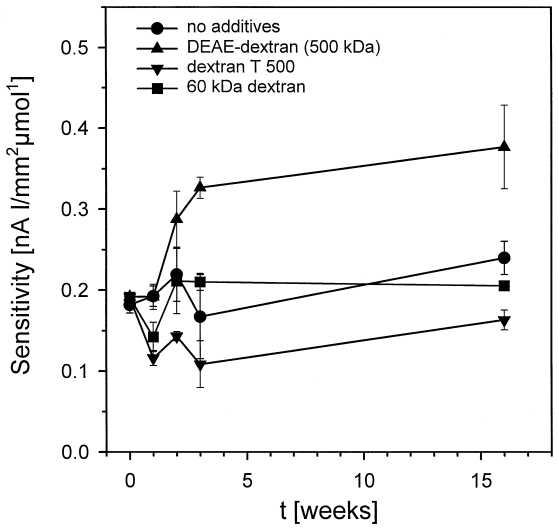

Fig. 5. Storage stability of lactate enzyme sensors in the presence of soluble additives. All data given relates to 1 mm^2 geometrical sensor surface: 0.12 U LOx, 0.05 mg fibrinogen, 6×10^{-5} U MTGase; additives, 1% (w/w) in the immobilization solution; the measurements were performed in PBS buffer, pH 6.5 at a potential of +700 mV vs. Ag/AgCl (3 M KCl) reference and Pt counter electrode; the geometrical area for immobilization was 3.14 mm^2; the electrodes were dried at air and stored at 23°C in an air-conditioned laboratory; mean values ± S.D. ($n = 3$).

due to cracks in the membranes observed after drying of the sensors (data not shown). In contrast, enzyme sensors obtained by MTGase cross-linking showed 100% stability even after 4 month storage (Fig. 5). Best results were obtained upon addition of DEAE-dextran as soluble additive. The sensitivity increased during the first month of storage and remained constant at a level 1.5-fold higher compared to the control. The presence of a low molecular weight dextran (60 kDa) had no improving effect on the storage stability. These results are in line with those published by Gibson et al. [21], and might be explained by the formation of an electrostatic cage by the DEAE-dextran. The additive is applied at a pH above the isoelectric point of LOx converting the enzyme into the anionic form. The positively charged diethylaminoethyl group counterbalances this charge and thus electrostatically stabilizes the target enzyme. The stabilizing effect of the charged groups was demonstrated by using dextran T500 as control. Dextran T500 corresponds to DEAE-dextran in molecular weight but lacks the ionic headgroups. In this case the sensitivity of the lactate sensors decreased over time (Fig. 5). The increase in sensitivity, however, observed in the presence of DEAE-dextran cannot be explained straightforward. It is often observed that enzymes tend to recover after immobilization however, no explanation can be given, why this phenomenon was observed in the presence of DEAE-dextran only.

The response time (t_{95}) of the lactate sensors containing DEAE- and T500 dextran was < 20 s, which supports the idea, that the presence of high molecular weight additives gives rise to larger pore sizes and thus reduced diffusion limitations.

3.5. Determination of the operational stability of LOx

In a further approach the operational stability of lactate sensors, prepared by MTGase-cata-

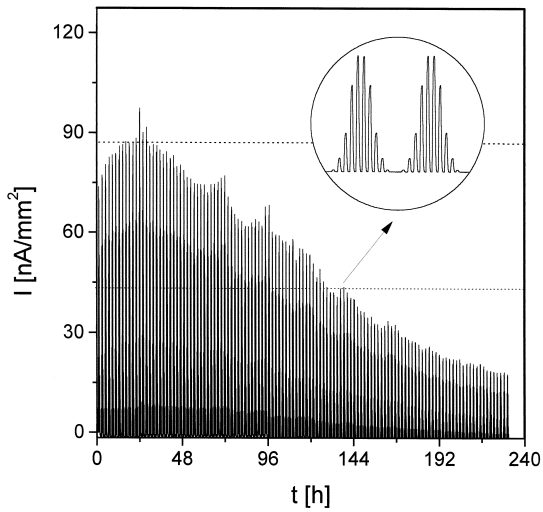

Fig. 6. Operational stability of lactate sensors prepared via MTGase cross-linking. All data given relates to 1 mm^2 geometrical sensor surface: 0.12 U LOx, 0.05 mg fibrinogen, 6×10^{-5} U MTGase, lactate was solved in PBS, pH 6.5 at concentration of 0.01, 0.1, 0.3, 0.7 and 1.0 mmol l^{-1}; flow, 0.5 ml min^{-1}; potential, +700 mV vs. screen printed Ag/AgCl reference electrode; the geometrical area for immobilization was 3.14 mm^2; a single representative experiment is shown.

lyzed immobilization of LOx in a fibrinogen network, was determined (Fig. 6). For this experiment a flow system was used, which allows for the application of different concentrations of lactate (10–1000 μmol l^{-1}) alternating with PBS buffer. According to the IUPAC recommended definitions for electrochemical biosensors the lifetime (LT50) is defined as the operational time, which is necessary to cause a 50% decrease in the sensitivity, determined within the linear range of the calibration curve. For the LOx electrodes a lifetime of 133 ± 26 h ($n = 4$) was determined at room temperature. For electrodes obtained by chemical cross-linking of LOx with GDA in the presence of BSA a lifetime of 53 ± 9 h ($n = 3$) was observed.

4. Conclusions

The use of MTGase catalyzed immobilization has been successfully applied for simple one-enzyme sensor systems (GOx and LOx), as well as for a more complex three-enzyme sequence, demonstrating the broad potential of this method. The main advantage of the MTGase-mediated cross-linking results from the mild reaction conditions, which especially recommends this procedure for the immobilization of labile enzymes. A chemical modification of the active center of the sensing enzyme, as observed with unspecific low molecular weight reagents, is not possible due to the size of MTGase (38 kDa). Upon addition of suitable polypeptides (poly-L-lysine, poly-L-glutamine) or other proteins, which serve as substrates for MTGase, such as casein, gelatin or fibrinogen, networks can be generated by the entrapped and/or covalently attached sensing enzymes.

PLL/PLG-generated networks are tighter, compared to fibrinogen-based layers but display the same self adhesive properties. As a result, the response times of the PLL/PLG-derived electrodes are longer, however, the leaching of the target enzyme is lower in PLL/PLG networks compared to fibrinogen cross-linking. PLL/PLG was successfully applied for the preparation of enzyme membranes containing up to three sensing enzymes on Pt-sputtered electrodes, but failed to immobilize LOx on Pt–C screen printed electrodes.

The cross-linking of fibrinogen by MTGase, described here for the first time, generates a brittle protein network with properties like a blood clot, which exhibited a high affinity to the surfaces of Pt-sputtered as well as Pt–C screen printed electrodes. A short response time of the enzyme sensors prepared in this way, indicated a fast diffusion of the reaction compounds through the fibrinogen network.

It was shown that lactate enzyme sensors obtained by MTGase mediated immobilization of LOx exhibited a higher storage and operational stability compared to sensors prepared by chemical cross-linking via GDA. Moreover, the storage stability can be further improved upon addition of soluble additives, e.g., DEAE-dextran. In contrast to other enzyme membranes, which are formed by entrapment of enzymes in hydrogels, the MTGase derived enzyme membranes remain homogeneous during drying at air, which has a beneficial effect on the reproducibility of the enzymes sensors.

The conditions for the MTGase mediated immobilization of biomolecules depend strongly on the structural properties of the target enzyme(s), the electrode surface and the matrix proteins, which serve as cross-linkers. By variation of the matrix protein the physical properties of the enzyme membrane, such as mechanical rigidity and the adhesive and diffusion properties, can easily be adapted to the different analytical problems. This versatility, which allows to tailor the enzyme membrane for various applications, makes MTGase a powerful tool not only for the immobilization of sensing enzymes.

Further investigations are now in progress to investigate the stability of the MTGase derived enzyme layers in comparison to those obtained from other gentle protein entrapping matrices, e.g., polyurethane hydrogels or photo-cross-linkable polyvinylalcohol.

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References

[1] S.S. Wong, L.J. Wong, Enzyme Microb. Technol. 14 (1992) 866–874.
[2] I. Moser, T. Schalkhammer, F. Pittner, G. Urban, Biosens. Bioelectron. 12 (1997) 729–737.
[3] S. Lofas, B. Johnsson, A. Edstrom, A. Hansson, G. Lindquist, R.M.M. Hillgren, L. Stigh, Biosens. Bioelectron. 10 (1995) 813–822.
[4] K. Lippert, E.A. Galinski, Appl. Microbiol. Biotechnol. 37 (1992) 61–65.
[5] W.J.J. van den Tweel, A. Harder, R.M. Buitelaar (Eds.), Stability and stabilization of enzymes, Proc. of an International Symposium held in Maastricht, the Netherlands, 22–25 November, Elsevier, 1992.
[6] M. Motoki, K. Seguro, N. Nio, K. Takinami, Agric. Biol. Chem. 50 (1986) 3025–3030.
[7] A. Kato, T. Wada, K. Kobayashi, K. Seguro, M. Motoki, Agric. Biol. Chem. 55 (1991) 1027–1031.
[8] K. Ikura, K. Okumura, M. Yoshikawa, R. Sasaki, H. Chiba, Agric. Biol. Chem. 49 (1985) 1877–1878.
[9] K. Ikura, M. Yoshikawa, R. Sasaki, H. Chiba, Agric. Biol. Chem. 45 (1981) 1877–1878.
[10] K. Ikura, T. Kometani, M. Yoshikawa, R. Sasaki, H. Chiba, Agric. Biol. Chem. 44 (1980) 1567–1573.
[11] M. Nonaka, H. Tanaka, A. Okiyama, M. Motoki, H. Ando, K. Umeda, A. Matsuura, Agric. Biol. Chem. 53 (1989) 2619–2623.
[12] M. Motoki, H. Aso, K. Seguro, N. Nio, Agric. Biol. Chem. 51 (1987) 993–996.
[13] M. Motoki, H. Aso, K. Seguro, N. Nio, Agric. Biol. Chem. 51 (1987) 997–1002.
[14] Y. Kamata, E. Ishikawa, M. Motoki, Biosci. Biotechnol. 56 (1992) 1223–1224.
[15] C.S. Greenberg, P.J. Birckbichler, R.H. Rice, FASEB J. 5 (1991) 3071–3077.
[16] A. Josten, M. Meusel, F. Spener, Anal. Biochem. 258 (1998) 202–208.
[17] P. Jülicher, L. Haalck, M. Meusel, K. Cammann, F. Spener, Anal. Chem. 70 (1998) 3362–3367.
[18] H. Ando, M. Adachi, K. Umeda, A. Matsuura, M. Nonaka, R. Uchio, H. Tanaka, M. Motoki, Agric. Biol. Chem. 53 (1989) 2613–2617.
[19] J.E. Folk, P.W. Cole, J. Biol. Chem. 240 (1965) 2951–2960.
[20] S. Hüwel, L. Haalck, M. Borchardt, F. Spener, in preparation.
[21] T.D. Gibson, J.N. Hulbert, B. Pierce, J.I. Webster, in: W.J.J. van den Tweel, A. Harder, R.M. Buitelaar (Eds.), Stability and Stabilization of Enzymes, Proc. of an International Symposium held in Maastricht, the Netherlands, 22–25 November, 1992, pp. 337–346.