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Investigations with respect to stabilization of screen-printed enzyme electrodes

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Abstract

Immobilization of enzymes on screen-printed electrode surfaces was performed by entrapment in UV-polymerizable, screen-printable pastes. The use of glucose oxidase, lactate oxidase, xanthine oxidase and horseradish peroxidase gave corresponding sensors in flow injection analysis (FIA) systems. The influence of various additives on different enzymes in the immobilization matrix was investigated. Activation as well as stabilization was achieved in some cases. An FIA system including dialysis-modules and stabilized glucose and lactate electrodes was successfully used to monitor animal cell cultivations.

Keywords: Animal cell cultivations; Enzyme electrode; Flow injection analysis; Glucose oxidase; Lactate oxidase; Screen-printing; Stabilization; Xanthine oxidase

1. Introduction

Analytical duties often require sensitive and selective tools. Modern instruments like HPLC or GC can often help to analyse complex mixtures but their use is expensive. Furthermore, they are of limited use in on-line analysis [1]. In contrast the use of enzymes as biochemical recognition elements combined with a transducer offers fast and sensitive determination of distinct analytes at low costs. The principle of the detection is the selective turnover of the substrates of the enzymes which is determined by the transducer. Enzyme electrodes containing oxidases like glucose oxidase (GOD) and lactate oxidase (LOD) are well known and applied to bioprocess control [2] or used in clinical analysis [3]. Xanthine oxidase (XOD) is used for estimating fish freshness [4,5] and could also be used for monitoring of microbial fermentations [6]. The common product of these enzyme reactions is hydrogen peroxide (1) which can be oxidized at screen-printed platinum working electrodes with an applied potential of +600 mV vs. a platinum reference electrode (2). The resulting current is related to the concentration of the substrate:

$$\text{Substrate} + O_2 \xrightarrow{\text{Oxidase}} \text{Product} + H_2O_2 \qquad (1)$$

$$H_2O_2 \xrightarrow{+600\,\text{mV vs. Pt}} O_2 + 2H^+ + 2e^- \qquad (2)$$

Horseradish peroxidase is often used as a label in the ELISA technique [7]. It catalyses the reduction of hydrogen peroxide (3) and various

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peroxides. A mediator like hydroquinone can serve as an electron donor to the HRP to reactivate the enzyme (4) which was oxidized in the first reaction step. The *p*-benzoquinone which is formed can be rereduced at the working electrode with an applied potential of −310 mV vs. platinum. The resulting current is related to the concentration of the *p*-benzoquinone respectively to the activity of the enzyme (5):

$$H_2O_2 + HRP_{red} \rightarrow 2\,H_2O + HRP_{ox} \quad (3)$$

$$HRP_{ox} + \text{hydroquinone} \rightarrow HRP_{red} + p\text{-benzoquinone} \quad (4)$$

$$p\text{-benzoquinone} + 2H^+ + 2e^- \xrightarrow{-310\,\text{mV vs. Pt}} \text{hydroquinone} \quad (5)$$

The main limitation in the use of enzymatic sensors is the decrease of the catalytic activity of the enzymes through thermal and chemical inactivation or just denaturation which are inherent qualities of many enzymes. Thus, there is a need to protect the enzymes from such destabilizing influences to extent the operational stability and the shelf life of enzymes in solution, freeze-thawed and immobilized in different matrices. Various substances were added to increase the stability and activity of different enzymes to achieve reliable and stable preparations. Good results were obtained with lactitol and diethylaminoethyl (DEAE)-dextran [8–10], Gafquat 755N [11] and various sugars [12]. But still it is not possible to foresee the effects of any given additive on a specific enzyme because they show a great variety in molecular weight, assembly of amino acids, conformation, charge distribution on the protein surface and isoelectric points. In addition additives can have different effects on enzymes in solution and in immobilizing matrices.

We immobilized enzymes on screen-printed electrodes to combine the specificity of the biological system with the sensitivity of electrochemical transduction. This technology enables the mass production of enzyme sensors at relative low costs. Reference electrode, auxiliary electrode and working electrodes consisting of conventional platinum thick film materials are screen-printed in a special layout onto Al_2O_3-substrates [13] and fired in a special temperature profile [14]. Mixtures of a commercially available UV-polymerizable paste, based on polymethylacrylates, with aqueous enzyme solutions and the various additives are printed onto the working electrodes. The resulting enzyme membranes showed good adhesion after the polymerization process. Placed in a special mounting the electrodes gave selective amperometric detectors which can be easily inserted in FIA systems. These devices enable an automation of on-line monitoring and control of bioprocesses [15–18].

We investigated the influence of various additives, which were reported to have stabilizing effects, on the performance of the sensors. Due to their importance in ELISA techniques we chose HRP, where we compared the behaviour of the immobilized to the dissolved enzyme. In addition, we chose GOD, LOD and XOD as oxidases and used the glucose and lactate electrodes for long term monitoring of animal cell cultivations.

2. Materials and methods

2.1. Reagents

The used enzymes were glucose oxidase from *Aspergillus niger* (Grade II, Boehringer, Mannheim, Germany), lactate oxidase from *Pediococcus* species (Lyophilisate, Boehringer, Mannheim, Germany), xanthine oxidase from cow milk (Lyophilisate, Boehringer) and horseradish peroxidase (HRP, Grade II, Boehringer). Bovine serum albumine (BSA) was obtained from Boehringer (Lyophilisate, Fraction V).

As substrates in the FIA devices we used glucose (D-glucose, monohydrate, Riedel de Haën, Seelze, Germany), lactate (L-lactate, Li-salt; Sigma, Deisenhofen, Germany), xanthine

(Na-salt, Sigma), hydrogen peroxide (H_2O_2, 30% p.a., Merck, Darmstadt, Germany) and hydroquinone (Merck). To prove the activity of the dissolved HRP 3,3′,5,5′-Tetramethylbenzidine (TMB, Boehringer) and H_2O_2 were used as substrates in microtiter plates (MaxiSorp, Nunc, Denmark).

The enzyme membranes were made with a UV-polymerizable, screen-printable paste based on polymethylacrylates. DEAE-dextran (Sigma), Gafquat 755N (ISP, Guildford, UK), lactitol (Sigma), polyethylenimine (PEI, high and low molecular weight, Aldrich, Gillingham-Dorset, UK) and polylysine (Poly-L-lysine hydrochloride; Aldrich, Milwaukee, WI, USA) were used as additives.

2.2. Solutions

Potassium phosphate buffer was made by mixing different volumes of 0.1 M solutions of K_2HPO_4 and KH_2PO_4 (both of p.a., Merck) to adjust the desired pH. Clark and Lubs buffer was made by adding an individual volume of 0.1 M NaOH to 500 ml of a 0.1 M KCl/boric acid solution to reach pH 8.0 and adding distilled water until 1000 ml (all from Riedel de Haën). Tris buffer and HEPES buffer were made by the use of the reagents of analytical grade (respectively Sigma, USA, ICN Biomedicals, USA). Acetate buffer was made by using a 0.1 M solution of Na-acetate-3-hydrate adjusting the desired pH with a 1% solution of citric acid (both of p.a., Riedel de Haën). H_2O_2/TMB solution contained 100 μl of 1% H_2O_2 and 400 μl TMB stock solution, which was made by dissolving of 6 mg TMB in 1 ml DMSO (dimethyl sulfoxide; Merck-Schuchardt, Hohenbrunn, Germany), in 25 ml acetate buffer pH 5.5. Na-azide at a concentration of 0.05% w/v was added to the calibration solutions during the cultivations to avoid microbial contamination (Merck-Schuchardt). All solutions were made by using distilled water and were degassed before use in the FIA systems.

2.3. Stabilization tests with horseradish peroxidase in solution

To prove the loss of activity of horseradish peroxidase in solution, 0.5 μg HRP/ml were dissolved in different buffers of various pH and stored in glass tubes (Duran, Germany) at 4°C. Tested were potassium phosphate buffer and Tris buffer pH 6.5, 7.5 and 8.5, HEPES buffer and Clark and Lubs buffer pH 7.5, acetate buffer pH 5.5 and distilled water. Different additives in various concentrations were added. The activity of the HRP was measured by adding 100 μl of an H_2O_2/TMB solution (in acetate buffer pH 5.5) to 50 μl of 1:100 diluted HRP solution in microtiter plates. The catalytical reaction of HRP with H_2O_2 and TMB led to a blue intermediate complex. The reaction was stopped after 10 min by adding 50 μl 1 M sulfuric acid. Under these conditions the blue complex turned into a yellow dye which could be measured spectrophotometrically at 450 nm vs. 650 nm [19]. Each solution was measured 3-fold and the mean value was taken as measuring point. The activities of the dissolved HRP after storage were set in relationship to a fresh solution.

2.4. Enzyme electrodes

The GOD, LOD, XOD and HRP electrodes were normally manufactured by mixing 100 mg of the UV-polymerizable paste with a mixture of 25 μl of the enzyme solution and 25 μl of the additive solution. The enzymes were dissolved in Clark and Lubs buffer pH 8.0 with a concentration of 100 mg/ml in the case of the GOD and LOD electrodes and 200 mg/ml in the case of the XOD electrodes. The concentration of the HRP was 0.3 mg/ml in potassium phosphate buffer pH 6.5. The additives were soluted in Clark and Lubs buffer pH 8.0 with a concentration of 20% w/v in the case of polylysine, DEAE-dextran and Gafquat 755N. In the case of lactitol the concentration was 50% w/v. BSA was soluted in Clark and Lubs

buffer pH 8.0 with a concentration of 100 mg/ml. The mixtures were screen-printed onto platinum working electrodes on planar aluminium oxide substrates (Hoechst CeramTech, Marktredwitz, Germany) by using screens in a special layout placed in a manual printing device (HAT 10, Fleischle, Brackenheim, Germany). Each electrode contained two working electrodes and one reference electrode made from platinum paste (Ferro 3804). In the case of the HRP electrodes the working electrodes were made by graphite paste (Ercon C458). After the screen-printing process the enzyme pastes were fixed by light induced polymerization with a UV-lamp (UN 50011 Light system Type 14012, Aktiprint mini 12, Technigraf, Grävenwiestrach, Germany).

2.5. FIA devices for stability tests

The tests of the enzyme electrodes were carried out in a simple FIA manifold. The sample solutions were injected into the carrier stream using the injection valve FIAstar (V-100, Perstorp, Rodgau, Germany), the sample volume in all cases was 40 μl. The flow rate of the carrier stream was 0.7 ml/min by use of a peristaltic pump (Meredos, Nörten-Hardenberg, Germany). All connector tubes were PTFE-tubes with an inner diameter of 0.8 mm. The detection unit consisted of the screen-printed enzyme electrode placed in a wall-jet cell, a special plexiglass mounting which was developed at the GBF (Braunschweig, Germany). The electrode was connected with a potentiostat (VA Detector 641, Metrohm, Herisau, Switzerland), a stainless steel capillary was used as inlet as well as auxiliary electrode. A potential of +600 mV vs. the platinum reference electrode was applied at the working electrodes in the case of the GOD and LOD electrodes, +300 mV in the case of the XOD electrodes and −310 mV in the case of the HRP electrodes. The whole FIA device was controlled by a personal computer using the program fiafox [20] which allowed also the collection of the data. Tests with GOD, LOD and XOD electrodes were normally carried out by using Clark and Lubs buffer (pH 8.0). In case of the HRP electrodes potassium phosphate buffer (pH 6.5) was used.

2.6. Animal cell cultivations

Cultivations were carried out with murine T-lymphocytes (EL-4 6.1) and with murine hy-

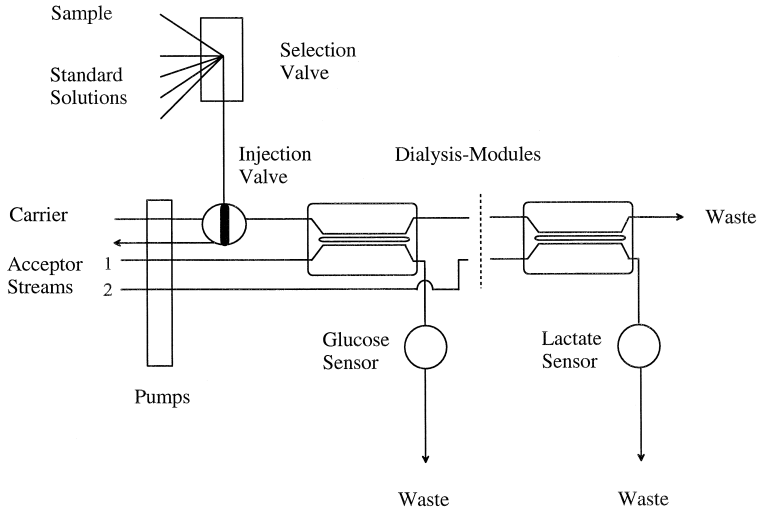

Fig. 1. Scheme of the flow injection analysis (FIA) device for monitoring animal cell cultivations; Clark and Lubs buffer pH 8.0 used; injection volume 40 μl; flow rates 0.7 ml/min; standard solutions containing 0.5, 1.0, 2.0 and 4.0 g/l glucose and lactate; complete cycle: 2 h 15 min.

bridoma cells (K4E7) in a 2.5-l fermenter with stirred perfusion membranes. These perfusion membranes allowed the harvest of the desired product under sterile conditions via a bypass and the retainment of the cells. A PTFE tube connected this bypass with the selection valve of the FIA device.

2.7. FIA system for monitoring animal cell cultivations

The animal cell cultivations were monitored using an automated FIA system (Fig. 1) controlled by a personal computer using the program fiafox [20] which also collected the data [21]. The system contained peristaltic pumps (Meredos, Bovenden, Germany), an injection valve (V-100, Perstorp, Rodgau, Germany), a selection valve (Besta, Wilhelmsfeld, Germany), dialysis modules (GBF), electrochemical wall-jet cells (GBF; similar to Ref. [22]) with integrated screen-printed GOD and LOD-electrodes and two channel potentiostats (Meredos, Bovenden, Germany) [13]. PTFE-tubes (inner diameter 0.5 mm, Omnifit, Cambridge, UK) connected the components according to the flow scheme (Fig. 1). More dialysis modules with following wall-jet cells could be integrated in order to detect additional analytes (dotted line in Fig. 1).

To calibrate the system four solutions with certain concentrations of the analytes glucose and lactate (0.5, 1.0, 2.0 and 4.0 g/l) were injected in sequence into the donor carrier stream. After 20 s, the donor carrier stream was stopped for 2 min and the analytes were allowed to diffuse into the acceptor carrier streams via the dialysis membranes (Spectra/Por, MWCO 12-14000, Spectrum Laboratories, USA) within the dialysis modules. Afterwards the acceptor streams were transported to the enzyme electrodes. Each of the four solutions was injected three times, followed by four injections of a sample taken from the perfusion line of the fermenter [23]. Clark and Lubs buffer pH 8.0 was used for carrier and sample streams. A complete measuring cycle took about 2 h and 15 min.

2.8. Off-line analysis

The samples taken during the cultivations were evaluated with regard to the concentrations of glucose and lactate by the use of Yellow Springs analyzers (model 2000, Yellow Springs Instrument, Yellow Springs, OH, USA), which represent the current method of evaluation.

3. Results

3.1. Stabilization of dissolved horseradish peroxidase

An activating and stabilizing effect of the additives DEAE-dextran and Gafquat 755N on dissolved HRP was observed in all used buffer systems. Fig. 2 shows the results for potassium phosphate buffer pH 6.5 with the activity of a freshly prepared HRP-solution being set at 100%. All other buffers showed similar results. First of all the activity of HRP was increased approximately 1.5-fold by adding DEAE-dextran or Gafquat 755N to the dissolved HRP. Moreover, there was a stabilizing effect ob-

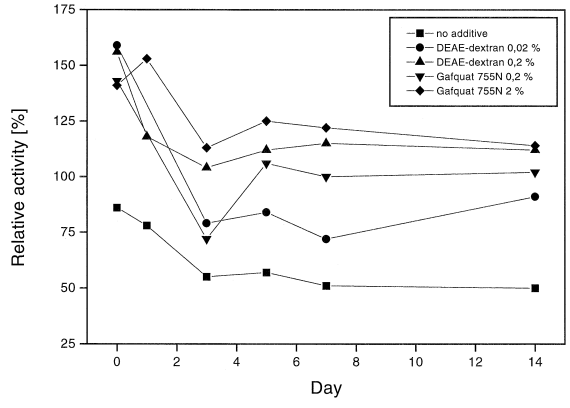

Fig. 2. Stability of dissolved HRP with DEAE-dextran, respectively Gafquat 755 N as additives; 0.5 μg/ml HRP stored in glass tubes at 4°C.

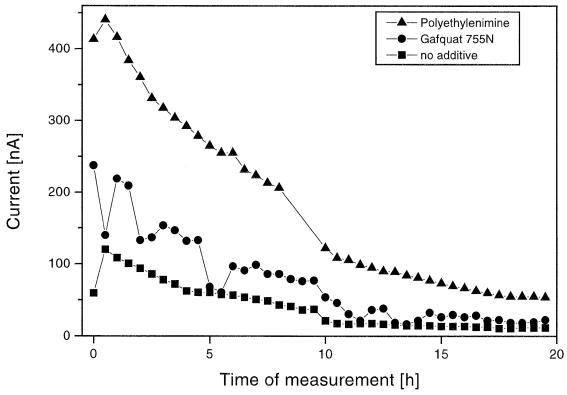

Fig. 3. Stability of immobilized HRP with polyethylenimine respectively Gafquat 755N as additives; immobilization matrix contains 100 mg UV-polymerizable paste, 25 μl HRP solution (0.3 mg/ml) and 25 μl of polyethylenimine, respectively Gafquat 755N (20% w/v each) added.

tained in the case of the additives. The activity decreased rapidly in all cases during the first 3 days. Later the loss of activity was almost negligible. Without additive the remaining activity was about 50% after 14 days. In the case of DEAE-dextran and Gafquat 755N the initial activity decreased about more or less 30%. The stabilizing effect increased in the case of higher concentration of the additives. Gafquat 755N had obviously a greater stabilizing influence than DEAE-dextran.

3.2. Effect on immobilized horseradish peroxidase

The activity of the HRP electrodes was investigated by injecting a solution of hydroquinone and hydrogen peroxide into the carrier stream. Both chemicals had a concentration of 4.3 mM. As seen in Fig. 3, the addition of Gafquat 755N and polyethylenimine to HRP in the immobilization matrix increased the signal significantly, each value representing the average of five measuring results. The response was approximately doubled in the case of Gafquat 755N respectively 4-fold in the case of polyethylenimine. A stabilizing effect could not be observed because the signals of all sensors decreased in the same degree.

3.3. Activation of the xanthine electrodes

The immobilized XOD showed a rapid loss of catalytic activity while measuring samples of 1 mM xanthine within 2.5 h (not shown). Every 1.5 min, one measurement was carried out. The activity of a xanthine electrode without additive and one with lactitol as additive increased up to 115% of the initial value after a short time. Then the activity decreased in the same degree in both cases. After 2.5 h the loss of activity related to the highest activity was about 45%. The use of BSA respectively polylysine led to a decrease of the activity right from the beginning of measuring. In the case of polylysine the loss of activity was stronger, the remaining activity was about 60% after 30 measurements or 45 min. However, in case of BSA the remaining activity was about 80% at this point. After 2.5 h the relative loss of activity was about 30% by adding BSA and 55% in the case of polylysine.

Combinations of these substances showed no positive influence with regard to stability (not shown), whereas the starting values depended on the use of additives (Fig. 4). The addition of BSA or lactitol did not show a significant effect. On the other hand, the addition of polylysine led to a 2.5-fold increased initial response. Combinations of lactitol/dextran, lactitol/

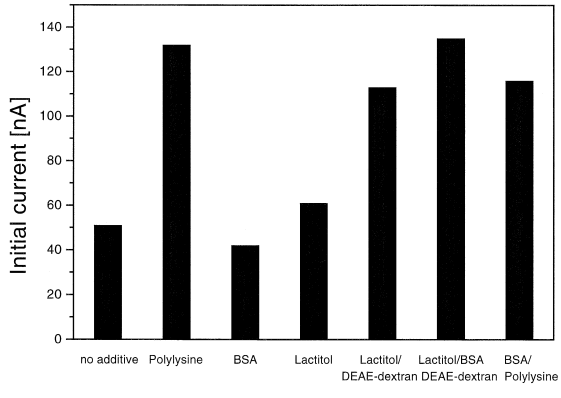

Fig. 4. Initial current of screen-printed xanthine electrodes with different additives; immobilization matrix contains 100 mg UV-polymerizable paste, 25 μl XOD solution (200 mg/ml) and 25 μl of the solutions (20% w/v) of each mentioned additive (BSA 100 mg/ml).

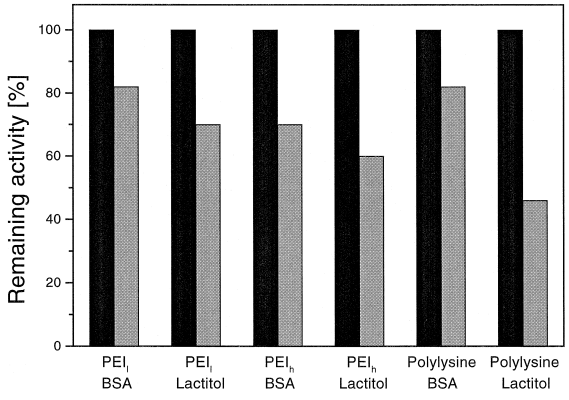

Fig. 5. Remaining activity of screen-printed glucose electrodes with different additives after storage at 4°C for 8 days; immobilization matrix contains 100 mg UV-polymerizable paste, 25 μl GOD solution (100 mg/ml) and 25 μl of the solutions (20% w/v) of each mentioned additive (BSA 100 mg/ml).

BSA/dextran and BSA/polylysine acted in the same manner.

3.4. Stabilization and activation of the lactate and glucose electrodes

As shown previously [13], the addition of DEAE-dextran/lactitol led to an increased activity of the lactate sensor. The highest signal was obtained by using 1% dextran and 5% lactitol in the immobilization matrix. An electrode with these additives showed a much higher sensitivity for lactate and a shortened linear range in comparison with an electrode without additives.

The influence of lactitol, lactitol/BSA and lactitol/BSA/DEAE-dextran as additives on the operation stability of GOD electrodes was investigated by the continuous measurement of 10 mM glucose (not shown). By adding only lactitol the sensor kept 59% of its activity after 2.5 h, while addition of lactitol/BSA and lactitol/BSA/DEAE-dextran resulted in a remaining activity of 93% after the same time. Replacing lactitol by polylysine led in the combination with BSA to a further extension of the stability. The corresponding GOD electrode gave constant signals for at least 18 h. Storing this electrode in Clark and Lubs buffer pH 8.0 at 4°C for 8 days reduced the activity only about 14%. During measurement it was stable again for at least 14 h.

To investigate the stability during storage additives in different combinations were tested. The electrodes were printed and the initial value of the current obtained by measurement of 10 mM glucose was set as 100%. After 8 days of storage at 4°C they were tested again and the value was compared to the initial value (Fig. 5). The best results were obtained with polyethylenimine (low molecular weight)/BSA and also polylysine/BSA with a remaining activity of 82%. Considering these results for further investigations we added polylysine/BSA to the GOD paste to get glucose electrodes with a good stability when stored or in use.

3.5. Monitoring of animal cell cultivations

The lactate electrode with 1% DEAE-dextran and 5% lactitol showed a good stability indicated by the results of the standard solutions during an animal cell cultivation of murine hybridoma cells (Fig. 6). There was almost no loss of enzymatic activity for about 96 h. The standard deviation of three subsequent measure-

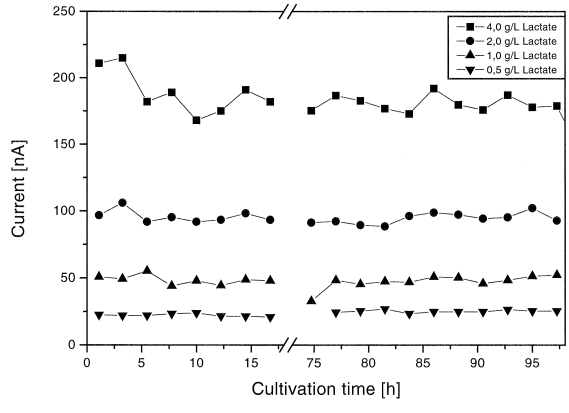

Fig. 6. Stability of screen-printed lactate electrode during animal cell cultivation; immobilization matrix contains 100 mg UV-polymerizable paste, 25 μl LOD solution (100 mg/ml), 1% DEAE-dextran and 5% lactitol.

ments was in most cases in the range from 0.5% up to 6.0%. The correlation coefficient for this calibration was typically better than 0.99. After a cultivation time of 200 h the lactate electrode was exchanged against a new one printed in the same batch as the first and stored in dry conditions at 4°C. The sensitivity was almost the same as the first electrode after measuring for 170 h. Thus, there was almost no influence whether the electrodes were stored or in use.

Calibration data obtained from standard solutions by a stabilized glucose electrode during a cultivation of T-lymphocytes are shown in Fig. 7. Glucose monitoring started after a cultivation time of 90 h. Within the first 4–6 h of use an increase in signal was observed, which remained almost constant during the following 140 h. Later the activity decreased, and after 180 h the signals were too low so that the sensor had to be exchanged.

The comparison of the on-line data for lactate and glucose obtained with the FIA device and the off-line data obtained by manual sampling and determination with Yellow Springs analyzers during the cultivation of T-lymphocytes showed a good correlation (Fig. 8). Four times of cell harvesting are shown indicated by a rapid increase of the glucose concentration caused by the addition of fresh medium into the fermenter. The FIA device measured automatically during the whole cultivation time.

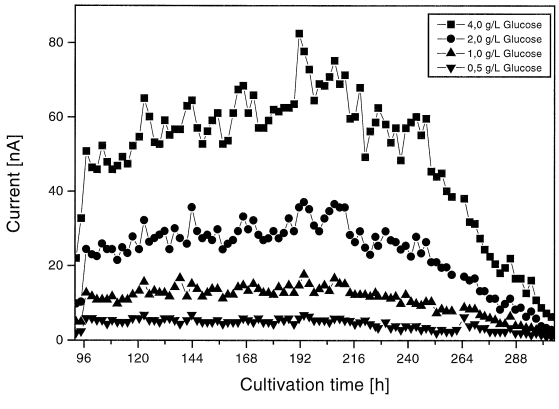

Fig. 7. Currents obtained by glucose standards with screen-printed glucose electrode during animal cell cultivation; immobilization matrix contains 100 mg UV-polymerizable paste, 25 μl GOD solution (100 mg/ml), 25 μl of polylysine solution (20% w/v) and 25 μl of BSA solution (100 mg/ml).

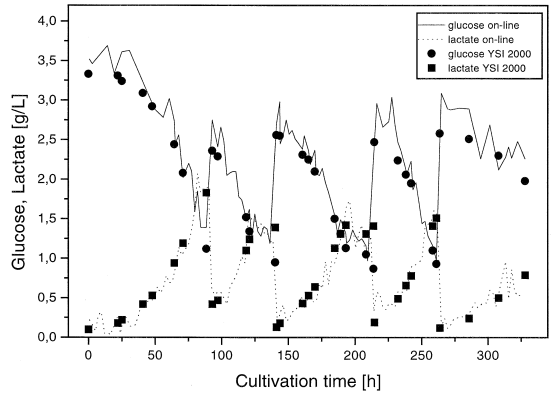

Fig. 8. Comparison of on-line results achieved with FIA system and off-line data obtained by standard analysis during a cultivation of T-lymphocytes.

4. Discussion

It is known that the inactivation of enzymes is due to different effects depending on the structure and type of enzyme. Examples are the loss of weakly bound cofactors or essential metal ions, oxidation of side chains or unfolding of the protein structure. By the addition of suitable reagents to the enzyme solution, e.g., an excess of cofactor or metal ion or easily oxidizable compounds, inactivation may be prevented or at least slowed down [24]. Moreover, specific interactions between additives and suitable domains on the protein surface may stabilize the protein structure and prevent its unfolding.

In our investigations, we used four different enzymes specific for substrates of different chemical nature (charged and uncharged compounds) and differing in molecular weight (ranging from 44 kDa for HRP to 150 kDa for XOD), isoelectric point, degree of glycosylation and required cofactors or metal ions, e.g., FAD in GOD and XOD, FMN in LOD and a heme group and Ca ions in HRP. The crystallographic structures are published only from GOD [25] and HRP [26], from which it could be taken that

GOD is mainly negatively charged on its surface, whereas HRP is mainly positively charged. In both cases the glycosylation of the enzymes is not considered. Among the additives there were high- and low-molecular weight compounds, those which were probably charged and others which were uncharged, and BSA as a catalytically non-active protein. Thus, a number of different interactions between additives and enzymes were possible leading to possible stabilizing or activating effects. As an example, the influence of some additives on dissolved HRP was investigated and activating and stabilizing effects of Gafquat 755N and DEAE-dextran were found. Both polymers have probably positively charged side groups and a more or less unpolar backbone. As the enzyme itself also has positively charged domains on its surface together with sugar residues, the interactions between both polymers and the protein may be due to the polymer backbone and not to the polymer side chains with the positive charge perhaps preventing the loss of the essential cations from the protein.

In the case of the enzyme electrodes additional effects have to be considered: the enzymes and additives are entrapped in a polymer-matrix, which may also lead to interactions. Moreover, the sensitivities of the sensors may be influenced not only by the activity of the enzymes but also by the availability of the active sites of the enzymes for the substrates or the local concentrations of the enzyme substrates, though usually the sensors were not in the diffusion controlled regime (data not shown here). However, interactions between the substrate and the immobilisation matrix, e.g., between the negatively charged lactate and positive additives, could increase or decrease the local substrate concentration. Moreover, additives may protect the enzyme from denaturation due to the UV irradiation used for generation of the enzyme membrane leading to an increase in the initial enzyme activity. This may be the reason for the 'activating' influences of most of the polymeric compounds in the case of the xanthine sensors, where no stabilizing effects were observed. This is supported by the fact that the covalent attachment of XOD to controlled pore glass by glutaraldehyde immobilization (without UV irradiation) resulted in rather stable enzyme reactors. However, due to the complexity of possible interactions and the limited detailed knowledge of the chemical structures of the proteins and the polymers, sound theoretical explanations of the observed effects cannot be given at present and further experiments with respect to a separation of the different effects and computer modelling of the interactions are required.

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