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Stability of immobilized enzymes as biosensors for continuous application in vitro and in vivo

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Abstract

The surplus of enzyme activity is a main prerequisite for the proper long-term function of enzymatic biosensors based on a diffusion-controlled process at any time. Long-term functional stability in vitro could be reached with sensor preparations using human serum albumin (HSA, Rhodalbumin) and glutaraldehyde (GDA, 25%,) as a mixture with glucose oxidase (GOD, EC 1.1.3.4., *Aspergillus niger*, 300 IU/mg) covered by polyurethane (PUR, Tecoflex EG 80 A) as a membrane with well-defined diffusion qualities. A very rapidly decreasing sensitivity has been observed after sensor implantation. As a reason for this, a reversible enzyme inhibition has been hypothesized, underlined by a slow restoration of the sensitivity up to the original one over a period of 5 days after sensor explantation. The same immobilization procedure on the surface of electrochemical sensors has been used very successful in the case of lactate oxidase (LOD, *Pedicoccus* species, 35 IU/mg). Dependent on the covering membrane lactate measurements in the range of 0.05 up to 50 mM lactate, concentration in milk and products of that can be realized. Further research has been pointed at the development of such immobilization methods which guarantee sufficient enzyme stability at in vivo conditions, too.

Keywords: Biosensors; Enzyme; Immobilization; Glucose; Lactate; Sterilization; In vivo application; In vitro application

1. Introduction

The conventional application of enzymatic sensors in lab analyzers is aimed at the measurement of single samples, separated each other by buffered cleaning solution and recalibrated at predetermined times or sample numbers. Thus, alterations of the sensitivity of the measuring system can be tolerated in a wide range. This is true for those alterations being dependent on a changing activity of the immobilized enzyme, too.

In contrast to that, enzymatic sensors have to be extremely stable if they are assigned to work in the continuous mode. This is the case if sensors are working in vivo or controlling processes, e.g., in the field of food production and storage.

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Especially, the application of enzymatic sensors in vivo, e.g., for continuous measurements of the intracorporal glucose concentration in diabetics, does not allow any real recalibration of the sensor signal and no surface cleaning of the probe is possible. As demonstrated in various experimental applications, the reliability of the in vivo glucose measurement often is affected by progressive decrease of sensor sensitivity [1]. Among others, a reversible enzyme inhibition has been hypothesized as one reason for such signal instabilities. However, additional reference values from venous or capillary blood can be estimated to check and correct the correspondence of the actual blood glucose concentration and the sensed signal, respectively. But, in most cases, this is not acceptable by the patient, at least not very frequently. Consequently, one main prerequisite for the proper long-term function of enzymatic biosensors to be realized is to guarantee a surplus of enzyme activity. In this case, only, one can be sure that the enzymatic reaction represents a diffusion controlled process but not a reaction controlled one at any time of application [2–5]. If this surplus is available, alterations of the enzyme activity or—in other words—a certain instability of the immobilized enzyme may be tolerated. However, if the enzyme instability leads to a decreasing activity which crosses that critical value of activity within the application period, the measuring range will be limited [6].

Since a certain decrease of the enzyme activity cannot be completely excluded so far, there are three essential points to be realized in any enzyme sensor preparation:

- Enzyme activity, as much as possible, has to be fixed on the active (e.g., electrochemical) surface of the technical sensor.
- Enzyme immobilization has to be carried out without larger influence on enzyme activity and a real fixation of the enzyme preparation has to be guaranteed.
- Analyte diffusion from the original, undiluted sample into the enzyme reaction layer has to be limited to make sure a diffusion controlled enzymatic reaction even if the enzyme activity decreases up to a lower limit.

The last point includes the development of a special membrane (or membrane combination) designed for enzyme fixation as well as for controlling the ratio of analyte to co-reactant diffusion into the reaction layer.

Connected with enzymatic biosensors for in vivo use, especially, the sterilization procedure necessary before any application in humans has to be considered. Conventional methods, as heat- or steam-sterilization, are unsuitable since the immobilized enzyme would be destroyed by denaturation. Therefore, on one hand, hydrogen peroxide, gamma irradiation, and combinations of both components at subtoxic doses have been investigated concerning their influence on the enzyme activity [7,8]. On the other hand, similar investigations after changing from gamma to ultraviolet irradiation have been started successfully both in realizing sterility and undisturbing the functional stability of enzymes.

2. Experimental

Based on modified electrochemical transducers, we have developed enzymatic biosensors for continuous application in medicine and in food technology [9,10]. So far, this research has been concentrated on biosensors estimating glucose in diabetics for diagnostic and therapeutic purposes, and lactate to characterize the actual

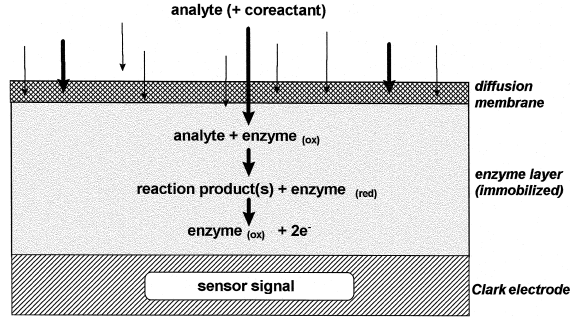

Fig. 1. Schematic structure and basic reactions of enzymatic biosensors.

state of food products concerning freshness, maturity and decay, and to control production lines in future. Structure and basic reaction processes of these sensors are shown in Fig. 1. The geometrical shape of sensors is different depending on the kind of application, e.g., for in vivo application, a needle type sensor has been used with an outer diameter of 0.5 mm, only. The geometry of these thin electrochemical sensors is characterized by a central working platinum electrode, 0.1 mm outer diameter surrounded by a silver/silver chloride coil serving as a reference electrode (oxygen measuring electrode, MSME 02, ELBAU Berlin, Germany). The electrodes have been cleaned electrochemically in a very careful manner before covering with the enzyme layer. Polarographic curves (i/u-diagram) of these electrodes are marked by a typical plateau in the potential range from 500 to about 800 mV, where the electrode current is nearly independent of alterations of the polarization voltage.

To immobilize glucose oxidase (GOD), we have used both covalently bound as well as physically entrapped enzyme to prepare needle type glucose sensors for in vivo use. In the case of covalent immobilization, sepharose was used as an enzyme carrier [11,12] but the fixation of the enzyme loaded sepharose spheres on the electrochemically active electrode tip is a technological problem in the case of needle-type sensors.

Therefore, sensor preparations based on human serum albumin (HSA, Rhodalbumin) and glutaraldehyde (GA, 25%,) with GOD (EC 1.1.3.4., *Aspergillus niger*, 300 IU/mg) as a mixture have been used. After dipping the electrochemical sensor into that mixture (GOD, HSA, GA) at least three times with a break of 1 h between the single dipping procedures to allow the solution to dry on the surface, it has been covered by polyurethane (PUR, Tecoflex EG 80 A) solved in dimethylformamide (DMF) and tetrahydrofurane (THF) forming a membrane with well-defined diffusion qualities. The membrane formation procedure has been carried out at predetermined and well-controlled environmental conditions at 36°C and a relative humidity of 76 to 80%.

The same immobilization procedure via Bovine Serum Albumin (BSA, Hoechst-Behring, Behringwerke, Marburg, Germany) and GA (10 μl, 2.5%, GA, Merck, Darmstadt, Germany) on the surface of electrochemical sensors has been applied very successfully in the case of lactate oxidase (6 mg LOD, *Pedicoccus* species, 34 IU/mg, Sigma, Steinheim, Germany), solved in 100 μl phosphate buffer, pH 6.25. Electrodes used here have a cylindric–symmetric geometry, too, but an outer diameter of about 2.5 mm (ELBAU). To overcome the problem of oxygen deficiency connected to the anaerobic process of lactate formation and for controlling and limiting the analyte diffusion into the enzymatic reaction layer [9], both an analyte door formed by a perforated polyethylene membrane and a co-immobilization of ferrocene (FeCp2R, 33 mg, Sigma), acting as an electron mediator, have been used. In any case, this enzyme-mediator-membrane-system has been covered by another membrane from regenerated cellulose to make sure that no proteins, cells and other substances or particles from the biological sample medium could reach the enzyme layer.

To sterilize implantable enzyme biosensors, the antimicrobial effectivity of low concentrated hydrogen peroxide was combined with low dose gamma irradiation [7] or with a universal homogeneous ultraviolet irradiation (UHUV) [12]. The main objective of the use of such adapted two-step procedures is to guarantee an effective sensor sterilization without disturbing its functionality.

3. Results

Using enzyme preparations of GOD and GA with HSA as described above, long-term functional stability in vitro was reached over more than 600 days (Fig. 2). During that time, enzyme sensors were stored at room temperature

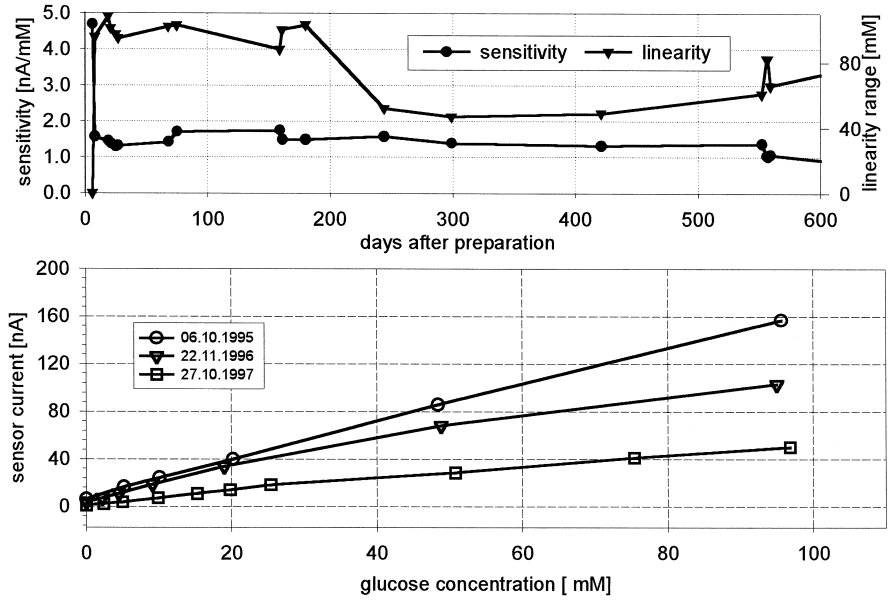

Fig. 2. Long term functional stability of a glucose sensor over 600 days with enzyme immobilized via glutaraldehyde and human serum albumin. Upper panel: sensitivity (filled circles) as well as range of linear glucose measurement (filled triangles) dependent on the storage time; lower panel: various calibration curves estimated during the storage period.

in imidazole buffer (Imidazole, Serva, Boehringer Ingelheim Bioproducts, Heidelberg, Germany). All in vitro measurements were carried out at 37°C in glucose standards based on imidazole buffer, too. Nevertheless, this excellent stability of the sensing system at all has not been a guarantee for a similar long-term function in vivo. Sensors were implanted subcuta-

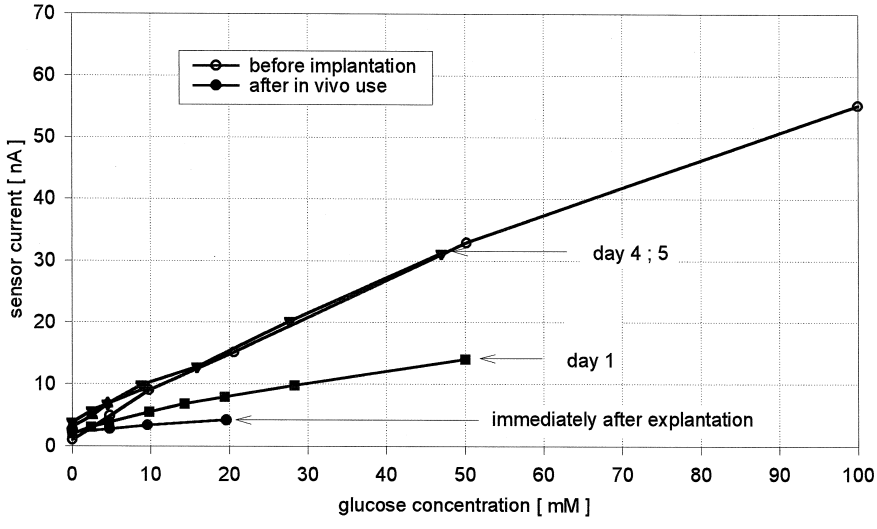

Fig. 3. Calibration characteristics of a needle type glucose sensor before (open symbols) and after (filled symbols) in vivo residence over 8 h in subcutaneous tissue, measuring the interstitial glucose concentration in a human volunteer.

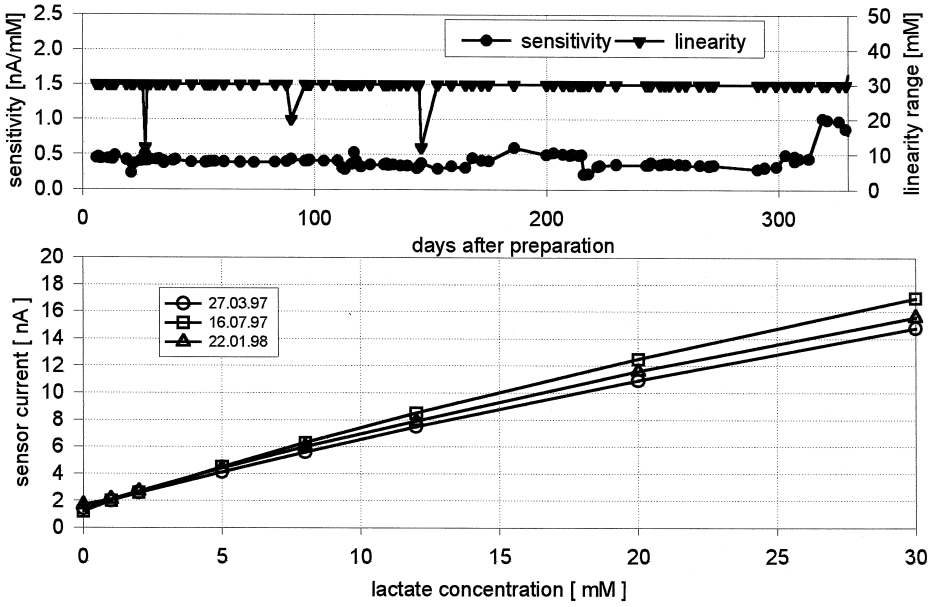

Fig. 4. Long-term functional stability of a lactate sensor over 340 days with enzyme immobilized via glutaraldehyde and human serum albumin. Upper panel: sensitivity (filled circles) as well as range of linear lactate measurement (filled triangles) dependent on the storage time; lower panel: various calibration curves estimated during the storage period.

neously using a tunnelling technique. After a run-in of about 2 to 3 h, an excursion of the intracorporal circulating glucose concentration was provoked by an oral or an intravenous

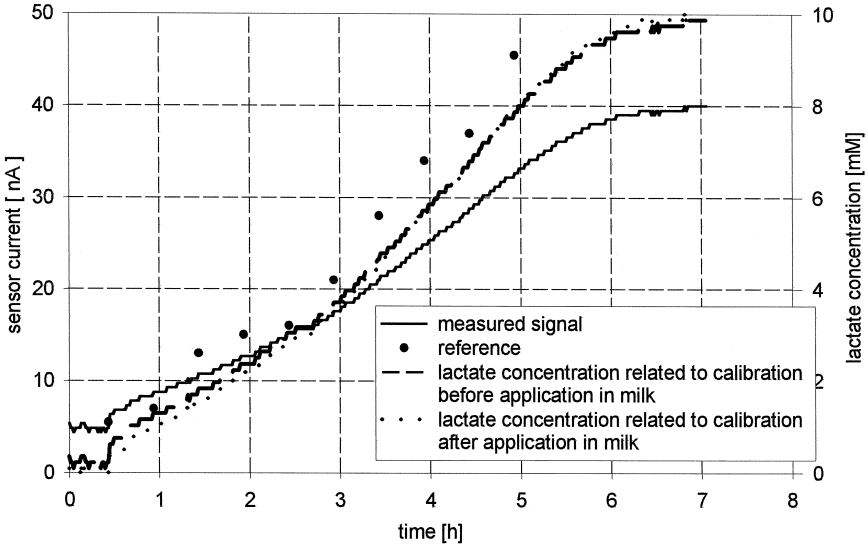

Fig. 5. Continuous measurement of lactate in potable milk: measured sensor signal (continuous line); lactate concentration based on the sensor signal related to the calibration characteristics before (- - -) and after (. . .) application in milk; reference values (•) estimated with the BIOSEN lab analyzer (EKF).

glucose load. Reference values from plasma samples were measured every 30 min during normal rest intervals but much more often during the glucose load periods. As shown in Fig. 3, obviously there was a rapidly decreasing sensitivity during sensors' in vivo residence being fully reversible during the first 4 to 5 days after explantation.

In contrast to the often discussed instability of LOD, after immobilization, a sufficient long-term in vitro stability of lactate sensors could be reached as shown in Fig. 4 making these sensors suitable for different applications. In this case, sensors were stored in phosphate buffer pH 6.25 at room temperature and driven in lactate standards based on the same buffer or in milk of different concentration.

Among others, the process of lactate formation in potable milk could be reflected continuously by the enzyme sensor (Fig. 5) showing an excellent correlation with the reference values estimated by the enzymatic laboratory analyzer BIOSEN (EKF, Magdeburg, Germany). Above all, there was no difference between the continuously measured lactate concentration calculated from the sensor signal related to the calibration characteristics checked before and that related to the calibration parameters checked after sensors' residence in milk. This underlines the stability of the functional characteristics of the sensor and, consequently, of the immobilized LOD during the whole period of lactate measurement (Fig. 5).

Gamma irradiation is a well-established method to sterilize medical products which are not allowed to be sterilized by means of heat. The sterilization efficacy as well as the remaining functional stability of the enzymatic system has been sufficiently realized by this method [13], but a decentralized use of this sterilization method is very complicated and specially long-term irritation of polymeric materials cannot be excluded. Those complications are cancelled using a new developed universal homogeneous ultraviolet irradiation (UHUV) instead of gamma irradiation. The combination of UHUV and hydrogen peroxide action as an effective method for sterilization has resulted in both sterility and undisturbed sensor function including the long-term stability of the sensor materials [13].

The direct UHUV irradiation of aqueous enzyme preparations leads to a drastic decrease of enzyme activity of both non-immobilized and immobilized GOD, even if the sepharose immo-

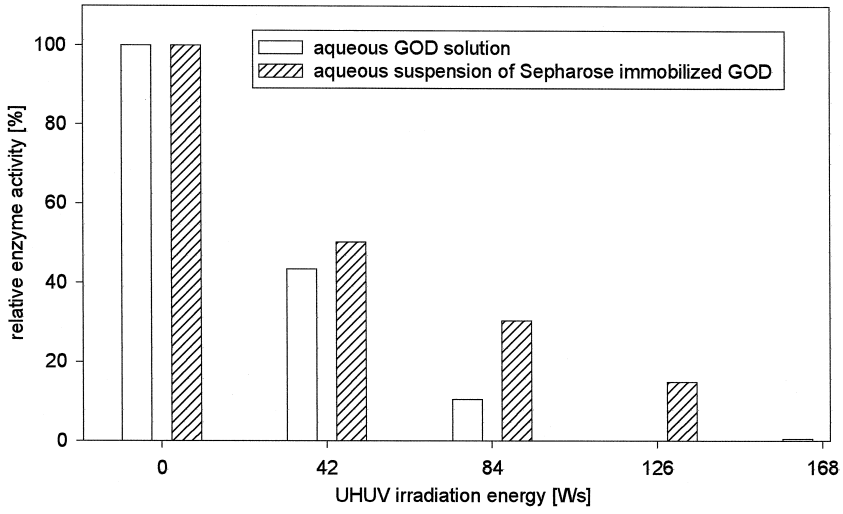

Fig. 6. Influence of ultraviolet radiation on the enzymatic activity of GOD in aqueous solution non-immobilized (open columns) and immobilized to sepharose (hatched columns).

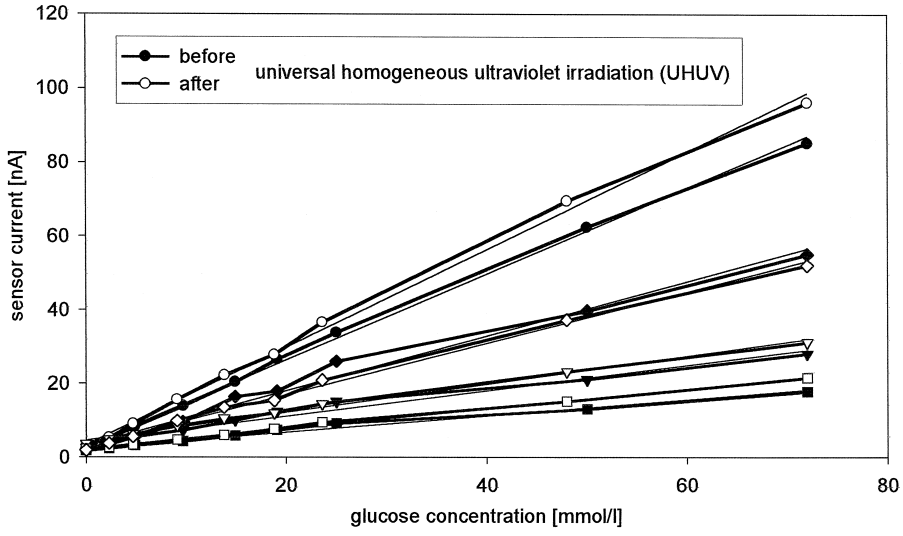

Fig. 7. Individual calibration characteristics of six glucose sensors before (open symbols) and after (filled symbols) universal homogeneous ultraviolet radiation (UHUV) with 360 Ws.

bilized GOD seems to be slightly more stable (Fig. 6). However, if the enzyme was fixed on the sensor tip covered by a diffusion membrane, UHUV irradiation obviously had no influence on the sensor calibration characteristics and thereby on the effective enzyme activity (Fig. 7).

4. Discussion

There are at least two different fields of application of enzymatic biosensors which are to be estimated very differently concerning the enzyme stability.

In the case of single- or multi-channel analyzers, samples have to be measured, which may be prepared depending on the qualities of the probes. Under steady state measuring conditions, recalibrations may be carried periodically at predetermined times on demand using sample-like standards. Thus, functional alterations related to the previous calibration values of the sensing system mainly caused by some kind of instability are compensated to a certain degree. Consequently, a loss of enzymatic activity will not result in mismeasurements in such laboratory analyzers in principle.

This is not true with enzymatic sensors assigned to work in the continuous as well as in the discontinuous mode but without any sample-free period at the sensor surface. In this case, any recalibrations are nearly impossible [14].

The apparently drastically decreasing sensitivity of biosensors especially after intravascular or subcutaneous implantation is hindering a real breakthrough in their routine application for continuous in vivo monitoring up to now.

With regards to the functional stability in vitro, the enzyme sensors fulfil the demands for any long-term application. Consequently, the decrease of sensitivity observed during in vivo residence seems to be caused by inhibiting substances coming from the biological medium into the enzyme layer [15]. This is underlined by the fact that any loss of sensitivity in vivo is fully reversible after explantation of the sensors. Direct biological deposits as proteins or cells can be excluded as a cause of sensors' malfunction since such deposits are not suitable to hinder the analyte diffusion through the membrane at all. Additionally, the time-dependent characteristics

of, e.g., glucose consuming inflammatory cell deposits on the sensor surface should lead to a slower influence on the sensor signal, i.e., it should be observed some days after implantation, only.

A similar decrease of sensitivity was not found using enzymatic lactate sensors in potable milk.

The comparison of long-term stability of the described biosensors over periods in vitro and an apparent instability after short-time usage in biological media lead to the conclusion that immobilization procedures as used in our lab lead to long-term stable preparations for in vitro application but further research has to be carried out to develop such immobilization methods which guarantee enzyme stability in biological media, too. This is the most essential prerequisite on the way to routine application of biosensors in vivo and to monitor processes of food production. A main precondition to stabilize the sensor function in vivo is to guarantee a sufficient surplus of enzyme activity on the sensor to compensate such enzyme inactivations. The extent of enzyme activity needed depends on the proposed application time. Consequently, the main research activities will be concentrated on well-defined and reproducible enzyme immobilization procedures. Investigations of other membranes as a suitable support to solve this problem have to be done, too.

As another main prerequisite, the gentle sterilization of biosensors without short-term or long-term disturbance of the enzymatic stability has been developed based on the combination of hydrogen peroxide action and UHUV as mentioned above. Whereas the direct UHUV irradiation of the enzyme leads to a complete destruction of the enzymatic activity at 126 and 168 Ws, respectively, there is no influence of an UHUV irradiation energy as high as 360 Ws on the enzyme immobilized on the sensor covered by a diffusion membrane. The reason for this extremely different effect is to be seen in the missing depth action of the UHUV irradiation. Consequently, this surface-active UHUV irradiation has to be combined with the action of a depth-effective substance like hydrogen peroxide to fulfil the demands of sterility assurance.

The present state of the art in enzymatic biosensor development is characterized by a very specialized research to solve very detailed problems related to different fields of application. Long-term stability of the enzyme supported by an excellent biocompatibility of all materials used in sensor production and as membranes covering the immobilized enzyme will open the serial application of biosensors in the medical field and as test instrument for food producers and consumers.

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