Chimia 53 (1999) 62–66

ISSN 0009-4293

Micro- and Nanotechnology in Biosensor Research

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Abstract. Biosensor research is strongly interdisciplinary as it requires experience in chemistry, biochemistry, biology, material science, electronics and engineering. The recent progress in micro- and nanotechnology allows to miniaturize complex systems as well as to address problems at a molecular level. The architecture and even the function of single molecules on a sensor surface have been investigated and can to some extent even be predetermined. At present, microtechnology is well established in the production of micro-fluid transport systems and has a high potential for cell-culturing and monitoring devices in the future.
Three different running projects are presented which illustrate the usefulness of micro- and nanotechnology for biosensor research: *1*) Investigations on amperometric immunosensor devices, *2*) the measurement of binding forces of individual antigen-antibody pairs, and *3*) the fabrication of microchannels suitable for neuron-cell growth and recording. Big efforts, however, will be required to integrate the recognition element of a sensor into a device for an intended application

1. Introduction

15 years ago, biosensors have been expected to be the smart solution for almost every analytical problem. Enzyme sensors based on glucose oxidase have successfully been commercialized, but small, reliable sensors for other analytes than glucose are still rare and do not yet have a market. The key problem of a biosensor is the transduction of the biorecognition reaction into a detectable signal [1]. The three most important parameters of each analytical method are stability, sensitivity and specificity; this is also true for biosensors.

The measurement of a target compound includes usually several steps: transport of the sample to the sensor surface, recognition reaction, signal transfer, amplification, and display of the measured values. In a real biosensor, all these steps run without assistance and within seconds to minutes. For some rather simple substances, disposable analytical tests such as electrodes or paper strips have been realized. For more complex analytes, however, sophisticated systems will often be required. An electronic control of a series of steps can best be achieved by an integration into a miniaturized system [2]. The additional benefit of miniaturization is a low reagent consumption and a short analysis time. Since most analytes are present in aqueous solutions, an integrated fluid-transport system is required. A full integration of the recognition element, the electronic control-unit and the fluid transport in a microsystem will allow effective parallel detection and make biosensor devices attractive for automated analysis [3].

Among the new tools and instruments which arose in the last decade in science, scanning probe microscopy (SPM) has a prominent place [4]. SPM techniques comprise atomic-force microscopy (AFM), scanning tunneling microscopy (STM) and scanning near-field optical microscopy (SNOM). They allow to address nanometer-sized objects on a surface and even to resolve individual atoms on a crystal surface. As a consequence, surface design and analysis in the micro- and nanometer range have become a major topic also in biosensor research. Furthermore, the structure-function relationship can be investigated at a molecular level and then optimized. The progress achieved in understanding how functional molecules interact with the underlying material may contribute to a specific and more effective signal transduction resulting in an improved performance of real biosensors.

2. Current Problems in Biosensor Research

2.1. *Biosensor Applications*

Biosensors are used in drug-development processes, for quality control of food, for clinical and bedside diagnostics and for environmental surveillance. Each of these fields has its own requirements and for each application a specific profile is given to be matched by a suitable biosensor design. Whereas for food control, a biosensor must be fast, cheap and small [5], a device for drinking-water surveillance should run continuously and has to be connected to an information-storage system. Present biosensor research is still mainly technology-driven and focuses on problems related to biorecognition and signal detection. However, a good predictive value of a test often requires the determination of multiple parameters which effectively can be realized by an array of sensing units.

Even before application-oriented research is started, the essential analytical problem must carefully be considered. For instance, a list of antibiotics present in milk are able to prevent the fermentation to cheese and, therefore, a sum parameter would be most predictive. For this problem, a test measuring the inhibition of microorganism growth has a high predictive value, but is cumbersome. On the other hand, a precise detection

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of one specific antibiotic compound is not relevant unless it is a good indicator. This example shows that it is very important to define the final application of the sensor at a very early stage of a development. Finally, a new biosensor device will succeed on the market only if it is clearly advantageous over the existing methods.

2.2. *Biosensor Devices*

The term biosensor is used in a very wide sense. Are the human nose, a test stick or the BIAcore-instrument biosensors? Sensitivity, easiness and automation are important criteria. But it is not as important to achieve a generally accepted definition for a biosensor, as to be aware that design and fabrication of a biosensor device strongly depend on the intended application and that they must be simple. For a long-term use, automated regeneration of the sensor will be required. Furthermore, an effective sampling and fluid-transport system is needed to achieve a high throughput. For these reasons, and since the frequency of maintenance mainly depends on the reagent consumption, miniaturization is highly recommended [3]. Profound experience in engineering is necessary to put all the parts together to a functional device [2].

2.3. *Signal Transfer*

Detectable signals in biosensing are of optical, electrochemical, piezoelectric or calorimetric nature [6]. Most efforts have been made to measure reliable physical signals related to the biorecognition process. From a molecular view, three major factors determine the biorecognition and detection processes: the molecular architecture of the biosensor surface, the size of the involved molecules and non-specific binding (NSB).

Molecular architecture covers the immobilization of biomolecules as well as the structure, and chemical nature of the underlying substrate material. In order to achieve a high stability, covalent-immobilization procedures, which preserve the functionality of the biomolecules, are preferentially used. The topology of the surface, its inertness, and stability in aqueous solution are additional, very important issues, but they are not yet well understood at nanometer dimensions. Local-probe techniques allow the direct investigation of molecular processes and may contribute to an improvement of the sensor surface-design. In the future, nanotechnology will allow to define and produce nanometer-sized surface structures in a sufficient quantity.

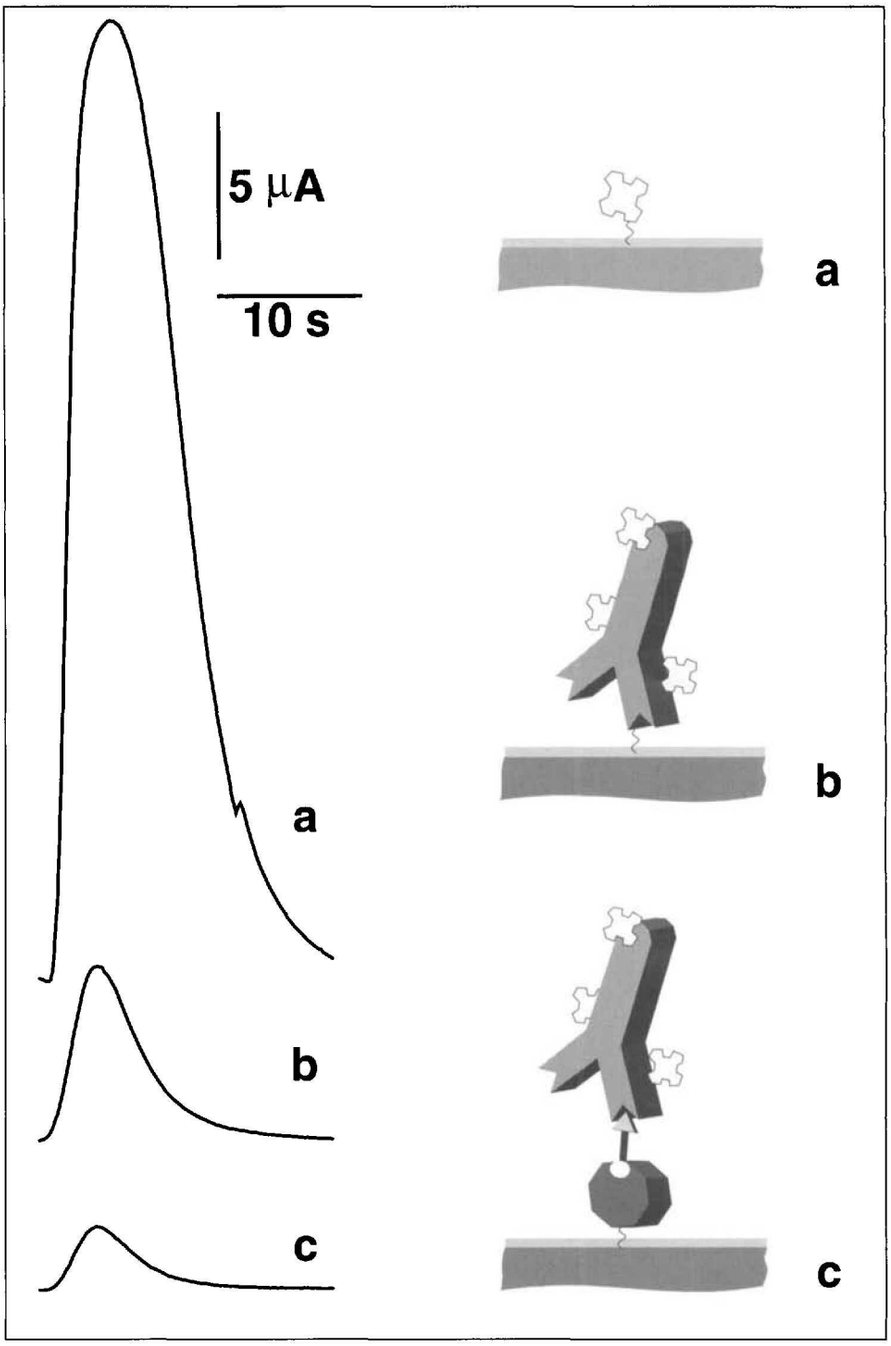

Fig. 1. *The electrochemical signal of a redox center is depending on the molecular environment, as illustrated by currents of the electrochemical reduction of* H_2O_2 (at 180 mV *vs.* Ag/AgCl) *catalyzed by microperoxidase MP-11* a) *immobilized directly on gold,* b) *conjugated to an antibody molecule, and* c) *when the conjugate is bound* via *streptavidin and a biotinylated antigen* [11]

The second factor which is often neglected is mass-transport limitation. Small molecules like glucose can easily diffuse through a polymeric network whereas most proteins cannot. Polymer layers are hardly surmountable barriers preventing a direct contact of proteins to the sensor surface and, therefore, have a limited applicability for amperometric immunosensors.

As mentioned above, a high sensitivity and specificity are essential for a biosensor. Both parameters closely depend on a low non-specific binding. The sample, for instance waste water, blood or milk, is often a complex cocktail containing a lot of substances which bind to surfaces just as well as the analyte, which is usually present only in trace amounts. Especially proteins stick preferentially to any kind of surface, if it is not suitably protected. Blocking agents based on the milk protein casein are commonly used in bioanalytical methods since they effectively reduce NSB [7]. However, as a consequence, a

thin protein layer on the surface is formed which may inhibit the access to the biorecognition element, or the transmission of the signal. Surface modification by small charged molecules is a valuable alternative still allowing a direct contact of the binding partner with the biosensor surface [8].

3. Selected Results

The following selected examples illustrate the facilities of micro- and nanotechnological techniques for the production and characterization of sensor surfaces. It should be kept in mind that these new methods do not replace conventional techniques, but complete them.

3.1. *Molecular Architecture*

Strictly speaking, functional molecules on surfaces are required for any kind of biosensing. In electrochemical sensors, a direct contact of the redox center to the electrode surface is essential. For enzyme sensors, conducting polymers have been used as molecular wires connecting the redox center to the surface [9].

Molecular architecture means: rational design of the sensor surface at nanometer resolution. A modular setup for a sensor has the evident advantage that it can easily be adapted for novel analytes. This can be achieved by using antibodies in combination with immobilized avidin, to which any biotinylated antigen may be bound. For the immobilization of avidin molecules, the following criteria have been considered: *1*) covalent binding on the surface material to guarantee long-term stability, *2*) introduction of a suitable linker to achieve the flexibility needed for the biorecognition reaction, *3*) little or no loss of functionality by a mild derivatization, and *4*) optimal surface density. Using 1-(3-mercaptopropyl)-1,1,1-trimethoxysilane in combination with the heterobifunctional crosslinker 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester, a stable immobilization of functional streptavidin molecules at a defined surface density has been achieved [10].

When the steptavidin-biotin complex is used in an amperometric immunosensor, the protein layer on the gold electrode unfortunately limits a direct signal transfer resulting in a low signal [11] (*Fig. 1*).

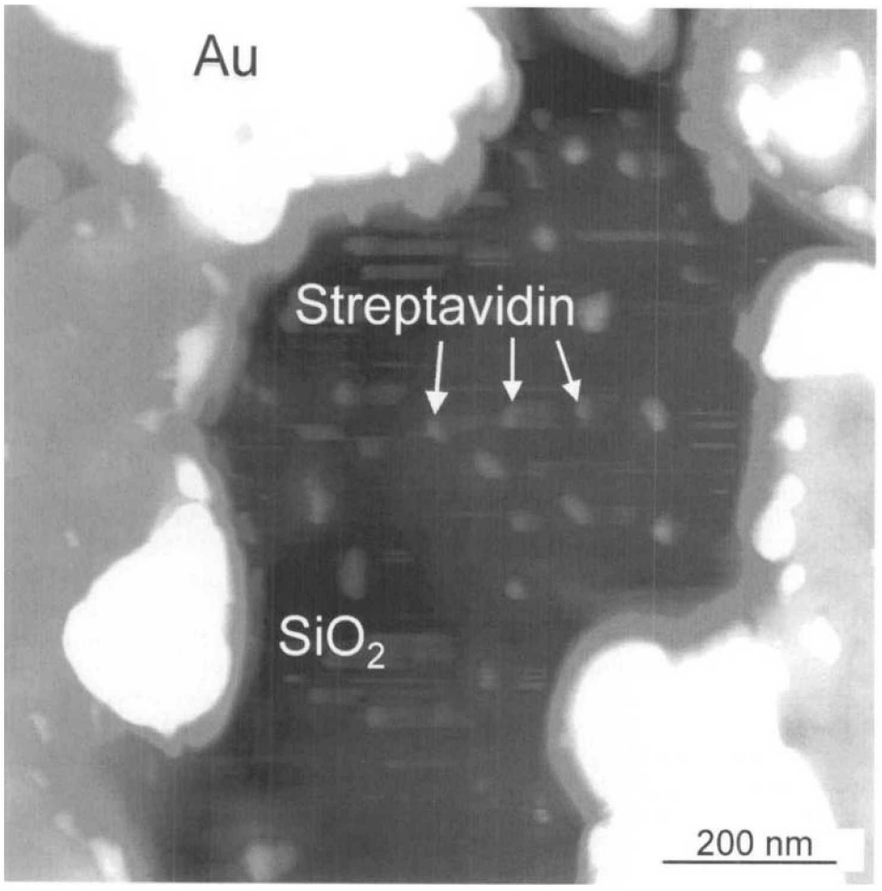

Fig. 2. *Scanning probe methods allow a characterization of sensor surfaces at nanometer resolution.* Individual streptavidin protein molecules, immobilized in a large opening of a nanostructured gold electrode, were imaged by AFM in the non-contact mode [13].

In order to overcome this problem, gold electrodes with openings of about 50 nm diameter have been produced on silicon chips [12]. Redox centers which are conjugated to the bound antibody molecules within the openings should provide a direct contact to the conducting gold. The electron transfer may be facilitated analogously to conducting polymers but without limiting the access of big antibody conjugates to the electrode. By using AFM in the non-contact mode, it could be confirmed that streptavidin molecules are localized within the openings [13] (*Fig. 2*). Non-specific binding of proteins to the gold, as determined by using ^{125}I-labeled IgG, could effectively be prevented by the small charged compound 2-mercaptoethyl sulfonate (MES) which does not impair electrochemical reactions [8]. However, the benefit of nanostructures for the signal transfer has not yet been proved.

3.2. *Functions at a Molecular Level*

Functionality of an antibody molecule means that it specifically binds the corresponding antigen. This immunological recognition is widely used in analytical assays, histology and cell biology using radioisotope, fluorescence or colorimetric labels. The observed signal is the average of a huge number of binding events. It may be interesting to elucidate which individual molecules on the surface are functional and which ones are not. A simple way to visualize function is to attach one binding partner to a nanoscopic object, for instance a gold nanosphere. However, it is not easy to demonstrate whether the presence of nanospheres is due to specific or non-specific binding [13].

Recently, the binding reaction of one individual antigen-antibody pair was directly observed [14]. By molecular-biology techniques, single-chain Fv fragment (scFv) molecules of an anti-fluorescein antibody have been produced which carry an additional flexible peptide sequence with a *C*-terminal cysteine. The thiol-group present in this sequence allows an oriented and stable immobilization of the scFv molecules on a flat MES-protected gold surface at a low surface density. The corresponding antigen fluorescein has been covalently bound through a long flexible linker to a tip of an AFM. Using AFM imaging, single spatially well-isolated scFv molecules were selected to which the tip was then periodically approached and retracted. With a high probability, formation and rupture of the antigen-antibody complex occured and was investigated. Based on a careful calibration, the forces of the so-called unbinding or rupture events

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have been determined with a relatively high accuracy and precision. This experiment even allowed us to discriminate between the wild-type and a mutant scFv molecule (mutant His(H58)Ala) in which histidine at position 58 of the heavy chain in the binding site had been exchanged by alanine (*Fig. 3*). Thus, SPM techniques do not only contribute to better characterization of surface topography, but also open the door for functional analysis of single molecules.

3.3. *Micro- and Nanostructures for Cells*

Eucaryotic cells are objects of micrometer extensions. In order to address single individual cells in long-term experiments, microstructuring of surfaces will be required. By photolithography techniques using masks or photoactive precursors, or microcontact-printing techniques, patterns of biomolecules have been generated on surfaces [15]. Patterns of hydrophilic/hydrophobic areas, of adhesion proteins or of specific peptides, induce the adhesion of cells at predetermined areas on the surface. Beside the chemical nature of the guiding molecules, the size of the features has a direct influence on cell survival [16].

The influence of surface topology on cell behavior has been investigated in detail [17]. Most investigations use neuron cells which form outgrowing axons. The size of topological structures on surfaces was varied and the direction of axon outgrowth has been observed. It seems that vertical structures in dimensions of some micrometers are required to guide axons in a predefined direction.

In a recently started project we combine chemical and structural guidance [18]. By a series of photolithographic steps, microchannels have first been generated in a 10 mm thick polyimide layer on glass, suitable in size to host single neuron cells (*Fig. 4*). The surface at the bottom of the channel is modified by the short laminin-derived peptide Arg-Gly-Asp-Cys (RGDC). It could be demonstrated that this surface modification induces adhesion and growth of dissociated chicken embryonic neurons. Two microelectrodes are ending in the channel allowing the stimulation and recording of individual neurons. Tailor-made neuronal adhesion molecules like axonin-1 [19] are now immobilized locally at these electrodes which may provide a close cell-electrode contact.

For establishing neural networks in the future, an array of interconnected channels will be required to investigate neural

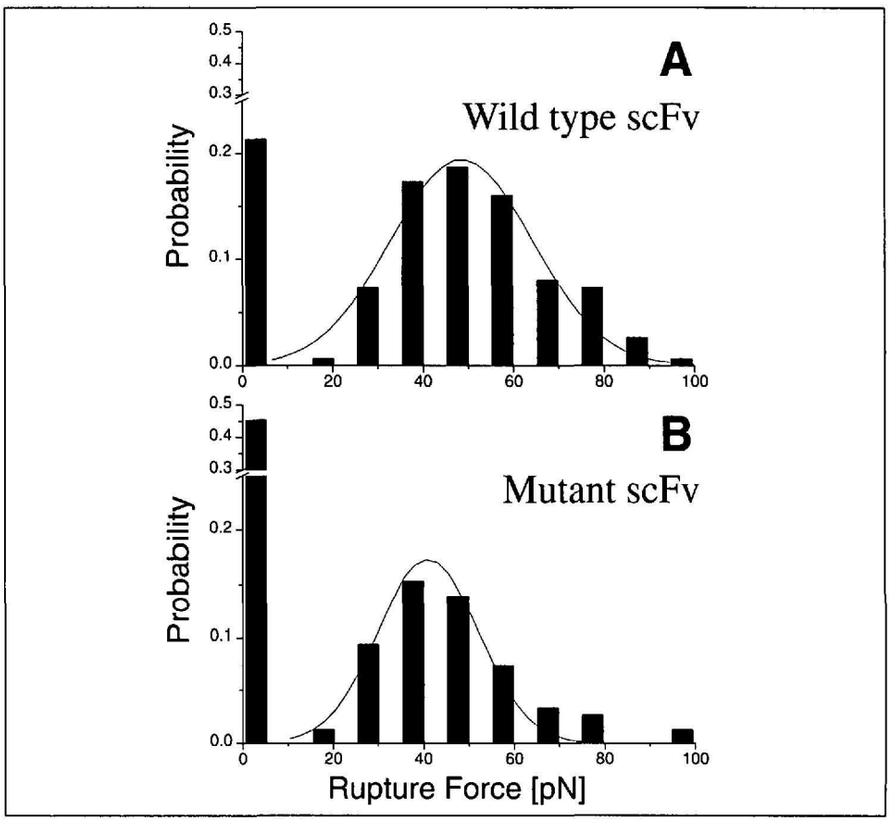

Fig. 3. *AFM yields information on binding forces of antigen-antibody complexes.* A single-chain Fv fragment (scFv) of a fluorescein antibody is bound to a flat gold surface and the corresponding antigen to an AFM tip. The distributions of measured rupture forces of the wild-type scFv (*A*) and of a scFv-mutant (*B*), in which a single amino acid was exchanged, show significantly different mean values [14].

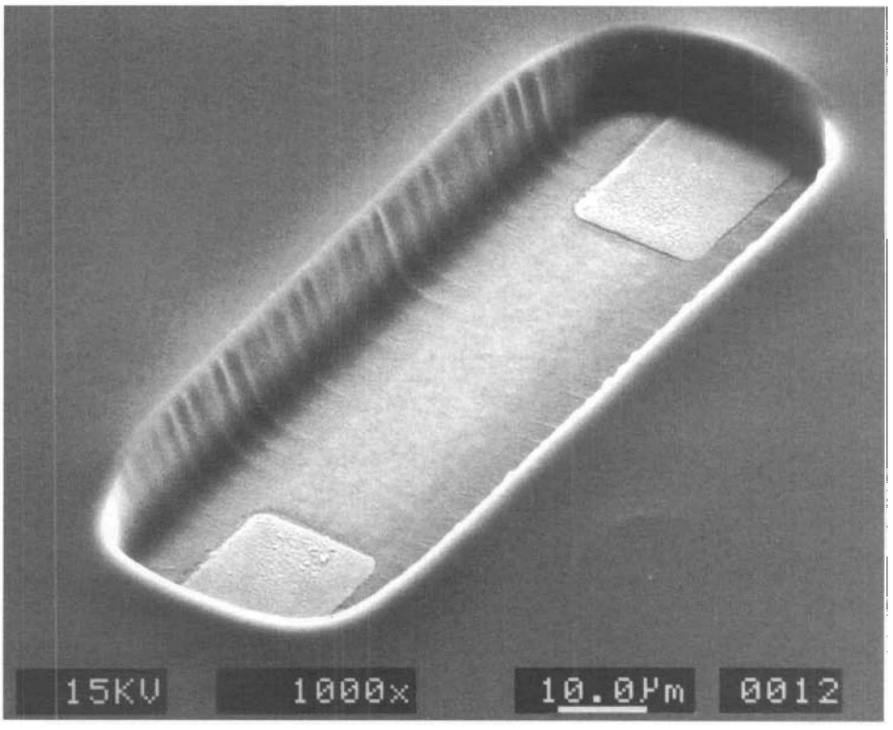

Fig. 4. *Networks of dissociated neurons will be realized on microstructured substrates.* This prototype microchannel (20 × 100 µm) in a polyimid layer on a glass chip is suitable in size for one single neuron cell. Biocompatibility is achieved by the used materials and by a suitable surface modification. Two microelectrodes are inserted into the channel intended to be used for electrical stimulation and recording [18].

information processes under defined conditions. However, they probably also can be used as sensing tools to screen combinatorial chemical libraries for toxic substances which is an important issue in every drug discovery process.

This example shows, how experiences in microstructuring, surface chemistry, molecular biology, electrophysiology, and cell biology are combined for novel biosensor devices.

4. Outlook

Some useful applications of micro- and nanotechnology in biotechnology have been presented. In the future, progress in the following areas will be necessary to achieve commercial success of biosensor devices.

- *Simple Manufacturing:* The production of the sensing part must be simple, versatile and cheap. Parts and procedures established in other mass-production processes (semiconductors or polymers) have to be adapted as far as possible [20].
- *Compatibility of Processes:* Since biomolecules are prone to denaturation, micro- and nanostructuring processes usually have to be completed before the immobilization. However, at certain conditions, proteins can be protected and tolerate non-physiological conditions for short periods. More knowledge about these limits is needed.
- *Stability of Sensors:* Proteins immobilized on surfaces are often more stable than in solution as known from bioanalytics. Nevertheless, the recognition element as the weakest part should be easily exchangeable in a biosensor.
- *Packaging:* The integration of the micro- or nanostructured surfaces into a macroscopic frame is the first and most critical step toward a device. Additionally, a control unit for fluid transport, signal detection, amplification and display is needed. The example of the pen-sized glucose sensor demonstrates that it is possible to build a fast, small and reliable biosensor. For other analytes, a similar, handy design should also be envisaged.
- *Automation:* Depending on the application, repeated analysis will be required. The output signal can optionally be used to control bioreactor processes or give alarm at a certain concentration. A direct interface to the electronic control is required.
- *Sample Preparation:* Especially the matrix of food samples is very complex. Mechanical or extraction treatments will be required, before sensing can be started. A sample pretreatment must be avoided or at least be very simple for disposable sensors, whereas it can be automated for sensors used for the quality control in a lab.

Progress in biosensor research is still not very predictable and early enthusiasm gave way to a certain disappointment [21]. The complexity of biosensor research and development is one major reason for this situation. Furthermore, many good alternatives to biosensors are already on the market for clinical diagnostics. However, market niches for new biosensor devices do exist where a quick on-site analysis is required: in health care for bedside and self diagnostics, in food production and distribution for quality control, and for an automated surveillance of drinking-water quality. Progress achieved in micro- and nanotechnology will contribute to the development of small and automated biosensor devices.

The authors acknowledge the contributions of *A. Grubelnik, J.-J. Hefti, S. Kossek, R. Ros* and *H. Sorribas* and the *Swiss National Science Foundation* (SPP Biotech and NFP36) for the financial support.

Received: November 16, 1998

[1] W. Göpel, *Sensors & Actuators B* **1991**, *4*, 7.
[2] I. Karube, K. Yokoyama, *Sensors & Actuators B* **1994**, *13*, 12.
[3] D.J. Harrison, A. van den Berg, *Proceedings μTAS Workshop Banff*, Canada 1998, Kluwer, Dordrecht.
[4] W. Göpel, P. Heiduschka, *Biosensors & Bioelectronics* **1995**, *10*, 853.
[5] A.O. Scott, *R. Soc. Chem.: Biosensors for Food Analysis* **1998**, *167*, p. 181.
[6] R.S. Sethi, *Biosensors & Bioelectronics* **1994**, *9*, 243.
[7] D.M. Bodmer, L.X. Tiefenauer, *J. Immunoassay* **1990**, *11*, 139.
[8] L.X. Tiefenauer, S. Kossek, C. Padeste, P. Thiébaud, *Biosensors & Bioelectronics* **1998**, *12*, 213.
[9] A. Heller, *Acc. Chem. Res.* **1990**, *23*, 128.
[10] C. Padeste, S. Kossek, L. Tiefenauer, *Proceed. Transducers'95/Eurosenosors IX* **1995**, p. 487.
[11] C. Padeste, A. Grubelnik, L. Tiefenauer, *Anal. Chim. Acta* **1998**, *374*, 167.
[12] C. Padeste, S. Kossek, H. Lehmann, C.R. Musil, J. Gobrecht, L. Tiefenauer, *J. Electrochem. Soc.* **1996**, *143*, 3890.
[13] S. Kossek, C. Padeste, L.X. Tiefenauer, H. Siegenthaler, *Biosensensors & Bioelectronics* **1998**, 31.
[14] R. Ros, F. Schwesinger, D. Anselmetti, M. Kubon, R. Schäfer, A. Plückthun, L. Tiefenauer, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7402.
[15] A.S. Blawas, W.M. Reichert, *Biomaterials* **1998**, *19*, 595.
[16] C.S. Chen, M. Mrksich, S. Huang, G.M. Whitesides, D.E. Ingber, *Science* **1997**, *276*, 1425.
[17] P. Clark, P. Connolly, A.S.G. Curtis, J.A.T. Dow, C.D.W. Wilkinson, *J. Cell Sci.* **1991**, *99*, 73.
[18] H. Sorribas, C. Padeste, U. Gennser, P. Sonderegger, L. Tiefenauer, *Proceed. Biosensors 98, Berlin* **1998**, p. 364
[19] E.T. Stoeckli, T.B. Kuhn, C.O. Duc, M.A. Ruegg, P. Sonderegger, *J. Cell. Biol.* **1991**, *112*, 449.
[20] G.T.A. Kovacs, K. Petersen, M. Albin, *Anal. Chem.* **1996**, *July, 1*, 407A.
[21] D. Griffiths, G. Hall, *TIBTECH* **1993**, *11*, 122.