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In-device enzyme immobilization: wafer-level fabrication of an integrated glucose sensor

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Abstract

Wafer-level fabrication of integrated enzyme-based BioMEMS usually requires high temperature wafer-bonding techniques such as anodic bonding. Enzymes denature at comparatively low temperatures. Thus, enzymes need to be immobilized after wafer bonding. A convenient in-device immobilization method is presented allowing wafer-level patterning of enzymes inside micro-scale flow channels after wafer bonding. Enzymes are entrapped in a poly(vinyl alcohol)-styrylpyridinium (PVA-SbQ) membrane crosslinked by UV exposure through a transparent top wafer. The reaction kinetics of immobilized glucose oxidase is investigated in more detail. A low apparent Michaelis constant of 3.0 mM is determined indicating a rapid diffusion of glucose into the PVA-SbQ membrane as well as an oxygen-limited maximum catalytic rate. The entrapped glucose oxidase preserves its native properties since it is not chemically modified. Furthermore, the active PVA-SbQ membrane can be dehydrated in a vacuum and later rehydrated in buffer solution without significant loss of enzyme activity. An integrated enzyme-based glucose sensor fabricated on a wafer-level using in-device immobilization is described to demonstrate the potential of this novel technique. The sensor is part of a disposable microneedle-based continuous glucose monitor. The stability of glucose oxidase entrapped in PVA-SbQ is sufficient to continuously operate the sensor at 25 °C for 24 h.

Keywords: In-device immobilization; Enzyme immobilization; Protein patterning; Glucose oxidase; Glucose sensor; BioMEMS

1. Introduction

1.1. Fabrication issues of integrated BioMEMS

Integrated enzyme-based BioMEMS often require the enzymes being immobilized inside a micro-scale flow channel; one example is a disposable microneedle-based continuous glucose monitor. The glucose monitor consists of a microneedle array to sample interstitial fluid [1], an electrochemical enzyme-based glucose sensor and fluidic components such as valves and pumps (Fig. 1). An integrated porous polysilicon dialysis membrane [2] separates the interstitial fluid from dialysis fluid, which is pumped through the microneedles. Glucose can diffuse through this membrane into the system while larger proteins are retained improving the sensor long-term stability. The integrated glucose sensor measures the glucose concentration in the dialysis fluid, which is related to the glucose level in the interstitial fluid. The glucose monitor also includes a separate calibration fluid reservoir and pumping system to enable periodic sensor recalibration, which is required due to loss of enzyme activity during continuous operation.

Wafer-level fabrication of such complex BioMEMS usually requires high temperature wafer-bonding techniques such as anodic bonding ($T \geq 350\,^{\circ}C$), which allows bonding of two structured wafers. Enzymes denature at comparatively low temperatures; glucose oxidase for instance denatures above 65 °C [3]. Thus, enzymes need to be immobilized after wafer bonding. A novel in-device immobilization technique has been developed allowing wafer-level pattering of enzymes inside micro-scale flow channels after wafer bonding [4]. The technique is based upon poly(vinyl alcohol)-styrylpyridinium (PVA-SbQ) [5], a water-soluble photosensitive polymer. PVA-SbQ is already successfully employed in many biosensors, which have the enzymes immobilized on uncovered electrodes [6–10]. A mixture of enzymes and PVA-SbQ is either spin-coated or directly deposited onto the electrodes and then UV crosslinked. However, none of these sensors have the enzymes immobilized inside a micro-scale flow channel, as required for many BioMEMS. In-device immobilization takes advantage of the real benefit of photochemical crosslinking which is the integration of biochemical components inside micro-scale flow channels. The enzyme–polymer mixture is filled into

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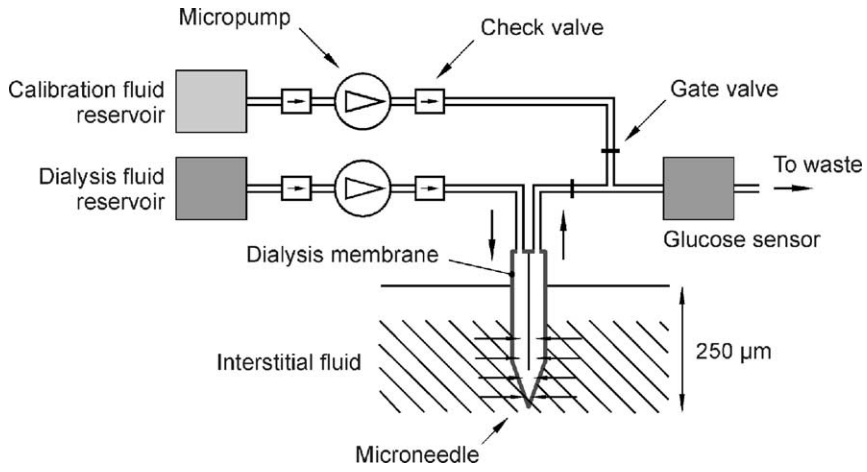

Fig. 1. Schematic diagram of a disposable self-calibrating continuous glucose monitor.

the flow channels after wafer bonding and then selectively exposed to UV light through a transparent top wafer using a shadow mask. Thus, enzymes are entrapped in the locally formed membrane inside the flow channels. The design of auxiliary channels, which connect all sensor devices and allow easy filling and flushing of the entire wafer through a single inlet is critical for wafer-level enzyme immobilization. This simple technique is generally applicable for any biochemical components that can withstand low energy UV exposure and allows wafer-level fabrication of integrated biosensors.

1.2. Michaelis–Menten theory and limitations

Michaelis and Menten proposed a simple model to account for the kinetic properties of free enzymes in solution [11]. Critical feature of their theory is that an enzyme E combines with a substrate S, with a rate constant k_1, to form an enzyme–substrate complex ES, which is a necessary intermediate product in catalysis (1). The enzyme–substrate complex can dissociate, with a rate constant k_2, or the product P is formed, with a rate constant k_3.

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \overset{k_3}{\rightarrow} \mathrm{E} + \mathrm{P} \tag{1}$$

The catalytic rate V of the proposed reaction varies with the substrate concentration [S] following the Michaelis–Menten Eq. (2), which describes the kinetic characteristic of many enzymes including glucose oxidase under specific conditions. The Michaelis constant K_m is equal to the substrate concentration at which the catalytic rate is half of its maximum value. It also measures the affinity of the enzyme–substrate complex in case of $k_2 \gg k_3$, which applies for most enzymes. The Michaelis constant of free enzymes in solution is independent of the enzyme concentration but depends on the particular substrate and also on ambient conditions such as temperature, pH value and ionic strength. The K_m of glucose oxidase from *Aspergillus niger* with respect to glucose is reported to be 33 mM in sodium phosphate buffer (25 °C, pH 5.6, in air) [12].

$$V = V_{\max}\frac{[\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{m}}, \qquad K_\mathrm{m} = \frac{k_2 + k_3}{k_1} \tag{2}$$

The maximum catalytic rate $V_{\max}$ and the Michaelis constant K_m can be determined by plotting the reaction rate versus various substrate concentrations. Non-linear curve fitting with the Michaelis–Menten equation yields both parameters. However, there are two concerns interpreting the measuring results of enzyme-based sensors using the Michaelis–Menten model.

1.2.1. Diffusion-limited catalytic rate

The kinetic characteristic of immobilized enzymes differs from the Michaelis–Menten model since the reaction rate is limited by the diffusion rate of the substrate into the membrane for low substrate concentrations. In this diffusion-controlled range, a linear correlation between the substrate concentration and reaction rate results (Fig. 2a). The Michaelis–Menten model still applies for high substrate concentrations, where the diffusion rate is much faster than the catalytic rate of the immobilized enzymes. Thus, the effect of immobilization on the enzyme properties can be quantified by fitting the enzyme-controlled part of the curve. Fitting of the entire curve yields an apparent Michaelis constant $K_{\mathrm{m,app}}$ higher than K_m for free enzymes in solution under identical ambient conditions (Fig. 2a). However, this apparent Michaelis constant measures both the effect of substrate diffusion into the membrane as well as the effect of immobilization on the catalytic performance of the enzymes. A large increase in $K_{\mathrm{m,app}}$ combined with inaccurate curve fitting indicates a large diffusion-controlled range rather than a loss in enzyme activity. Correspondingly, the apparent Michaelis constant decreases with an increasing substrate diffusion rate. Furthermore, $K_{\mathrm{m,app}}$ depends on the enzyme concentration since $V_{\max}$ scales with the total

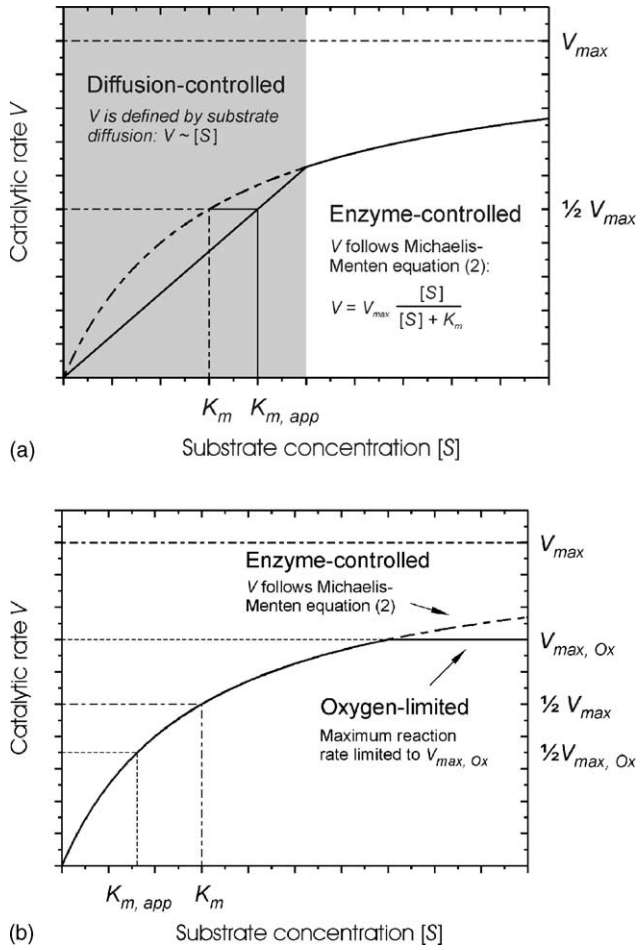

Fig. 2. (a) Effect of the substrate diffusion on the Michaelis constant. (b) Effect of the oxygen concentration on the Michaelis constant.

enzyme activity while the diffusion-controlled part of the curve remains unchanged. Thus, the apparent Michaelis constant decreases with a decreasing enzyme concentration. It equals K_m of soluble enzymes for very low enzyme concentrations.

1.2.2. Oxygen-limited maximum catalytic rate

In the case of a multi-substrate reaction such as the oxidation of glucose (3) another effect on the kinetic characteristic needs to be addressed. The catalytic rate can also be limited by stoichiometric restrictions. Thus, the oxidation rate of glucose depends on the oxygen and glucose concentration as well as on the catalytic efficiency of glucose oxidase. The K_m of glucose oxidase from *A. niger* with respect to oxygen is reported to be 0.51 mM in potassium phosphate buffer (0.01 M, 25 °C, pH 6.6, 0.2 M glucose) [13].

$$\text{glucose} + O_2 \overset{\text{GOD}}{\rightarrow} \text{gluconic acid} + H_2O_2 \quad (3)$$

Low oxygen concentrations or high enzyme concentrations can lead to an oxygen-limited maximum catalytic rate V_{max},O_x. The apparent Michaelis constant $K_{m,app}$ drops below K_m under such conditions (Fig. 2b). Furthermore, the apparent Michaelis constant decreases with an increasing enzyme concentration or decreasing oxygen concentration. However, the maximum catalytic rate is not oxygen-limited at high oxygen concentrations and low enzyme concentrations. The kinetic characteristic is described by the Michaelis–Menten equation under these conditions and the apparent Michaelis constant equals K_m of soluble enzymes.

1.2.3. Linearized forms of the Michaelis–Menten equation and the effect on $K_{m,app}$

Rather than fitting the non-linear kinetic characteristic, a convenient way to determine the Michaelis constant K_m and maximum catalytic rate V_{max} is to plot the experimental data in a linear correlation such as the Lineweaver–Burke (4), Eadie–Hofstee (5) or Hanes form (6) of the Michaelis–Menten equation [14,15]. Linear regression of the data then easily yields both parameters.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S]} \quad (4)$$

$$\frac{V}{[S]} = \frac{V_{max}}{K_m} - \frac{V}{K_m} \quad (5)$$

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \quad (6)$$

However, since the kinetic characteristic is not following the Michaelis–Menten model in case of a diffusion-limited catalytic rate V or an oxygen-limited V_{max}, the effect of linearization itself on the apparent Michaelis constant needs to be considered under these conditions. Linear regression of the experimental data plotted in the Lineweaver–Burke form for instance yields a higher apparent Michaelis constant than non-linear curve fitting since low substrate concentrations are more weighted. This emphasizes the diffusion-controlled part of the curve. Linear regression of the same data plotted in the Hanes form on the other hand yields a lower $K_{m,app}$ since high substrate concentrations are more weighted [14]. Non-linear curve fitting yields an apparent Michaelis constant with a more balanced measure of the effect of substrate diffusion and oxygen limitation.

2. Experimental

2.1. Enzyme immobilization

Enzyme solutions with glucose oxidase (GOD) concentrations of 0.6–45.6 μg/ml were obtained by mixing the initial glucose oxidase solution (5.7 mg/ml, glucose oxidase from *A. niger*, dissolved in sodium acetate buffer solution, 0.1 M, pH 4.0, Sigma–Aldrich, USA) with phosphate buffered saline (PBS) solution (0.01 M, pH 7.4, containing 2.7 mM potassium chloride and 0.137 M sodium chloride, Sigma–Aldrich, USA).

Poly(vinyl alcohol)-styrylpyridinium (SPP-13-H Bio, Toyo Gosei, Japan) was mixed 1:1 with the enzyme

solutions yielding final enzyme–polymer solutions with GOD concentrations of 0.3–22.8 μg/ml. The photosensitive PVA-SbQ was crosslinked under UV light (365 nm, 0.75–15 J/cm^2, notch filter, minimized IR heating). Thus, the enzymes were entrapped in the formed polymer membrane. Since glucose oxidase was not crosslinked to the PVA-SbQ backbone it preserved its native properties [5].

2.2. Photometric measurement of the soluble and entrapped enzyme activity

The activity of soluble and entrapped glucose oxidase was measured by photometrically monitoring the hydrogen peroxide production. The reaction rate of the overall reaction (7) corresponds to the catalytic rate of glucose oxidase since the glucose oxidation is the slowest reaction step [16]. The concentration of the oxidized form of *o*-dianisidine was monitored at 490 nm using a microplate reader (model 550, BIO-RAD, USA). The enzyme activity corresponds to the gradient of the absorption–time characteristic.

$$\begin{aligned}&\text{glucose} + O_2 + \overset{\text{GOD}}{\rightarrow} \text{gluconic acid} + H_2O_2\\ &H_2O_2 + o\text{-dianisidine (reduced)}\\ &\overset{\text{peroxidase}}{\rightarrow} o\text{-dianisidine (oxidized)}\end{aligned} \quad (7)$$

D-Glucose, peroxidase from horseradish and *o*-dianisidine dihydrochloride were all purchased in powder form from Sigma–Aldrich, USA. All glucose solutions (0.48–10% (w/v)) were prepared with deionized water. The dye solution was prepared by dissolving 50 mg of *o*-dianisidine dihydrochloride in 7.6 ml deionized water. One milliliter of this solution was then mixed with 100 ml PBS resulting in the final dye solution. Five hundred forty micrograms of the peroxidase were dissolved in 2 ml deionized water. Both the dye solution and peroxidase solution were prepared immediately before the experiment.

All experiments were carried out in air at 20 °C in natural 96-well polypropylene plates (USA Scientific, USA).

Soluble glucose oxidase experiments: 240 μl dye solution, 10 μl peroxidase solution, 10 μl enzyme solution (GOD concentration of 1.8 μg/ml) and 50 μl glucose solution (0.48–10% (w/v)) were mixed together.

Immobilized glucose oxidase experiments: 60 μl of the enzyme–polymer solution (GOD concentration of 0.3 μg/ml) were filled into a well and crosslinked under UV light. A mixture of 240 μl dye solution, 10 μl peroxidase solution and 50 μl glucose solution (10% (w/v)) was added after exposure.

Enzyme storage and leakage experiments: 60 μl of the enzyme–polymer solution (GOD concentration of 22.8 μg/ml) were filled into a well and crosslinked under UV light. Ten microliter PBS were filled on top and allowed to soak (0–2 h). The buffer solution was removed and added to a mixture of 240 μl dye solution, 10 μl peroxidase solution and 50 μl glucose solution (10% (w/v)). The enzyme concentration in the buffer solution was determined by comparing the measured activity with that of soluble enzymes.

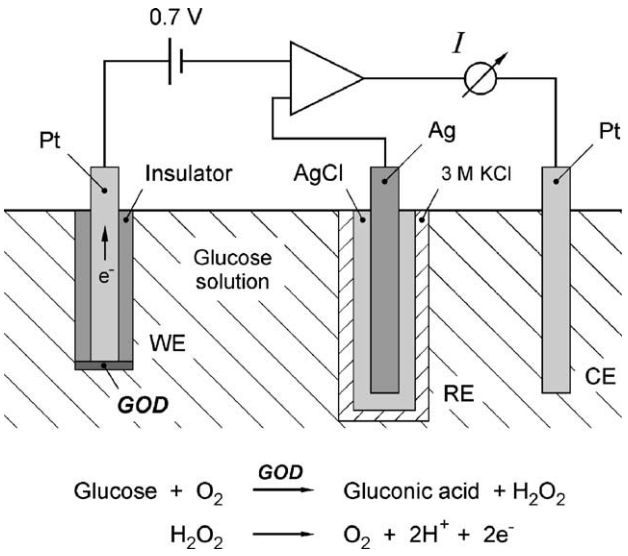

Fig. 3. Schematic diagram of an enzyme-based electrochemical glucose sensor.

2.3. Electrochemical measurement of the entrapped enzyme activity

The catalytic rate of the immobilized glucose oxidase was also measured using an electrochemical glucose sensor consisting of a Pt working electrode (WE, disc electrode, Pt ∅ = 2.05 mm), a Pt counter electrode (CE) and an Ag/AgCl reference electrode (RE) (Fig. 3). All electrodes were purchased from CHI Instruments, USA. Five microliter of the enzyme–polymer solution were applied onto the working electrode and crosslinked under UV light. The working electrode potential was 0.7 V versus the reference electrode. The steady state current I was measured in air at 20 °C with a potentiostat (model 750A, CHI Instruments, USA) and corresponds to the catalytic rate. All glucose solutions (20–600 mg/dl) were prepared with PBS. The same experimental setup was also used to measure the steady state current of the integrated glucose sensor.

3. Results and discussion

3.1. Kinetic analysis of glucose oxidase immobilized in PVA-SbQ

As mentioned in Section 1, the effect of immobilization on the reaction kinetics can be quantified by comparing the apparent Michaelis constant with K_m of soluble enzymes under identical ambient conditions. Since the exact conditions inside the PVA-SbQ membrane are unknown, especially during the glucose oxidation, a pH value of 7.4 and a temperature of 20 °C are assumed to be approximate ambient

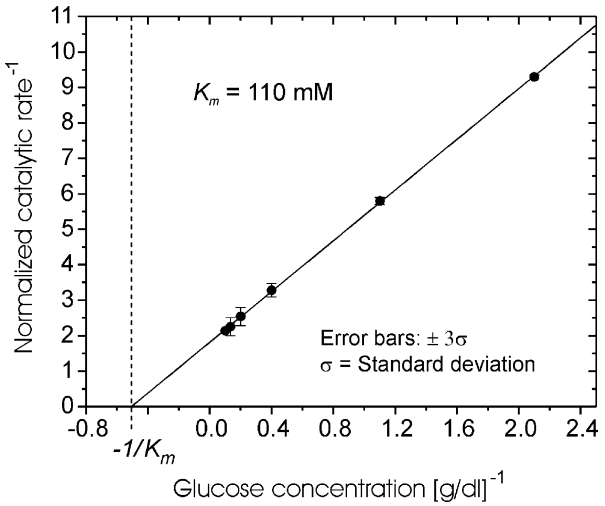

Fig. 4. Photometrically measured catalytic rate of soluble enzymes for various glucose concentrations (Lineweaver–Burk presentation). Error bars are applied to all data points. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 0.48–10% (w/v) glucose and 0.06 μg/ml GOD.

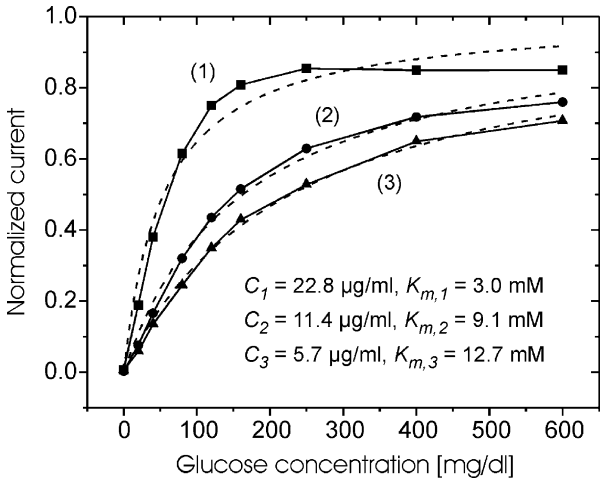

Fig. 5. Electrochemically measured apparent Michaelis constant of the immobilized enzymes for different GOD concentrations. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 20–600 mg/dl glucose, 5.7–22.8 μg/ml GOD, 5 μl gel and 750 mJ/cm^2 exposure energy.

conditions. The pH value of 7.4 corresponds to that of interstitial fluid. The catalytic rate of glucose oxidase is photometrically measured under these conditions for various glucose concentrations up to 10% (w/v). The low enzyme concentration of 0.06 μg/ml prevents an oxygen-limited maximum catalytic rate for high glucose concentrations. Linear regression of the experimental data (Fig. 4) plotted in the Lineweaver–Burk form yields a high Michaelis constant of $K_m = 110$ mM at pH 7.4 and 20 °C. The Michaelis constant is independent of the linearization method since the catalytic rate is neither diffusion-controlled nor oxygen-limited under these experimental conditions. Thus, emphasizing a particular part of the curve has no effect on the Michaelis constant.

The catalytic rate of the immobilized enzymes is measured electrochemically for glucose concentrations up to 600 mg/dl. The steady state current corresponds to the catalytic rate. Non-linear curve fitting of the experimental data (Fig. 5) yields a low apparent Michaelis constant of 3.0 mM for a high enzyme concentration of 22.8 μg/ml. Furthermore, reducing the enzyme concentration increases the apparent Michaelis constant. Both indicate an oxygen-limited maximum catalytic rate as well as a fast diffusion of glucose into the membrane. The latter makes PVA-SbQ a suitable material for bioassay applications, which require a rapid diffusion of substrate into the active gel to maximize the reaction rate. As stated in Section 1, the apparent Michaelis constant approaches K_m of soluble enzymes for low enzyme concentrations. Correspondingly, the measured characteristic almost matches the Michaelis–Menten equation for a low enzyme concentration of 5.7 μg/ml. A very low enzyme concentration of 0.3 μg/ml yields an apparent Michaelis constant of 20.0 mM (Fig. 6). Linear regression of the experimental data plotted in the Lineweaver–Burk form yields a correlation coefficient of $r = 0.999743$ indicating an accurate linear fit. Thus, the oxygen concentration is sufficient and the effect of substrate diffusion negligible. $K_{m,app}$ equals K_m of soluble enzymes under identical ambient conditions. It measures only the effect of immobilization on the enzyme properties. Surprisingly, $K_{m,app}$ is much lower than $K_m = 110$ mM of soluble enzymes in buffer solution indicating totally different ambient conditions inside the gel than in the surrounding buffer solution.

However, sensor applications require a large diffusion-controlled range where the sensor response is proportional to the glucose concentrations and independent of the enzyme activity. A reliable glucose sensor must respond linearly up to 400 mg/dl. Thus, the diffusion-controlled range needs to be extended. One possibility to decrease the diffusion rate

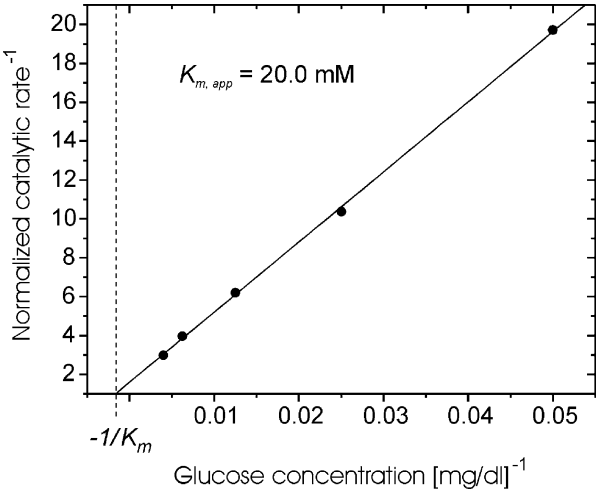

Fig. 6. Electrochemically measured catalytic rate of the immobilized enzymes (Lineweaver–Burk presentation). Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 20–250 mg/dl glucose, 0.3 μg/ml GOD, 5 μl gel and 750 mJ/cm^2 exposure energy.

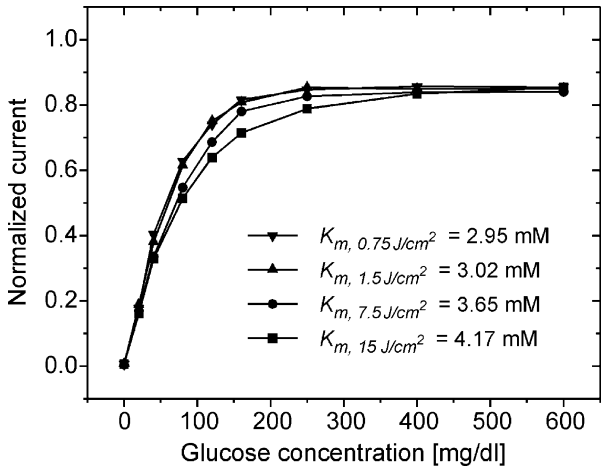

Fig. 7. Electrochemically measured apparent Michaelis constant of the immobilized enzymes for different UV exposure energies. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 20–600 mg/dl glucose, 22.8 μg/ml GOD, 5 μl gel and 0.75–15 J/cm^2 exposure energy.

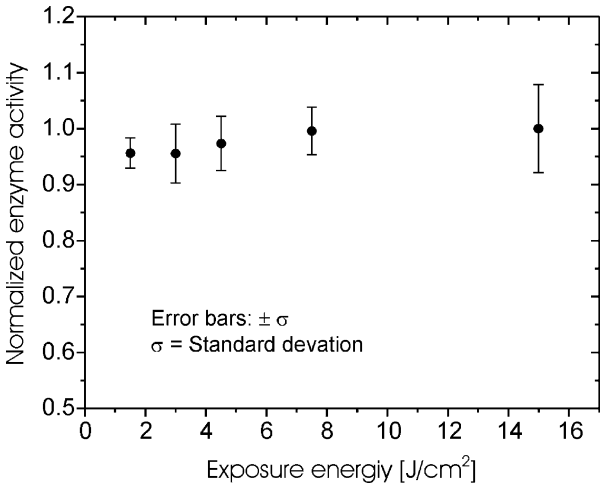

Fig. 8. Photometrically measured enzyme activity of the immobilized glucose oxidase for various exposure energies. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 10% (w/v) glucose, 0.3 μg/ml GOD, 60 μl gel and 1.5–15 J/cm^2 exposure energy.

is to increase the degree of polymerization of the PVA-SbQ by increasing the exposure dose. However, only a minor increase of $K_{m,app}$ was measured for exposure energies up to 15 J/cm^2 (Fig. 7). This indicates only a slight decrease of the diffusion rate if the enzyme activity remains constant. An increase in the PVA-SbQ concentration or SbQ concentration might be more effective but was not investigated. Since the maximum catalytic rate is oxygen-limited for the used enzyme concentration of 22.8 μg/ml an increase in enzyme activity has no effect on the apparent Michaelis constant whereas an exposure induced decrease in activity can significantly change $K_{m,app}$. A decreased diffusion rate combined with a decreased activity can result in an unchanged $K_{m,app}$ since both effects causes the opposite change in $K_{m,app}$. Thus, an unchanged $K_{m,app}$ might be misinterpreted as an unchanged diffusion rate.

No decrease in enzyme activity was measured for exposure energies in the range 1.5–15 J/cm^2 (Fig. 8) indicating glucose oxidase is insensitive to UV light at 365 nm. Thus, increasing the exposure dose does not significantly decrease the diffusion rate since the enzyme activity remains constant. An exposure dose of 750 mJ/cm^2 seems to be sufficient to completely polymerize the PVA-SbQ. In order to increase the linear range of the sensor, an additional membrane such as the dialysis membrane is required to reduce the glucose diffusion.

The sensor long-term stability is mainly limited by aging of the immobilized glucose oxidase, which depends on the ambient conditions inside the gel such as pH value and temperature. Additional activity loss is caused by diffusion of glucose oxidase out of the gel since enzymes are only entrapped but not crosslinked to the PVA-SbQ backbone. Loss of enzymes due to leakage was verified by measuring the enzyme concentration in the surrounding buffer solution. A significant amount of enzymes is diffusing out of the gel. This is a major issue shortening the sensor long-term stability (Fig. 9). The degree of enzyme loss observed is much greater than previously reported by Jaffrezic-Renault et al. [9]. Thus, enzymes need to be crosslinked to the PVA-SbQ backbone using a second crosslinker. Alternatively enzymes can be crosslinked among each other using bovine serum albumin (BSA) and glutaraldehyde. This would reduce the diffusion rate due to an increased molecule size. However, the sensor signal drops by 65% within 24 h of continuous sensor operation at 25 °C due to aging of the enzymes and significant leakage. Nevertheless, a self-calibrating disposable glucose monitor might be usable for 24 h in spite of the activity loss.

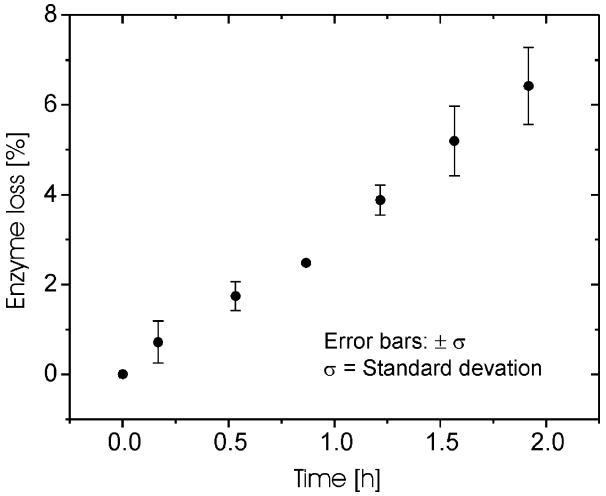

Fig. 9. Photometrically measured enzyme loss due to leakage of enzymes out of the gel. Error bars are applied to all data points. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 22.8 μg/ml GOD, 60 μl gel and 750 mJ/cm^2 exposure energy.

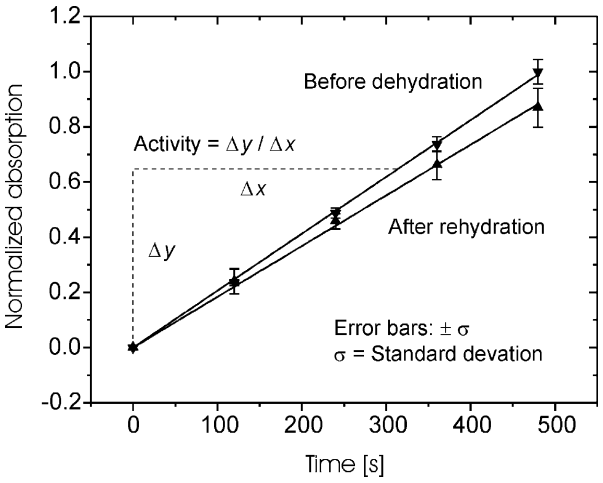

Fig. 10. Photometrically measured enzyme activity before dehydrating the PVA-SbQ membrane in a vacuum and after rehydration in PBS. Experimental: 0.01 M PBS, pH 7.4, 20 °C, in air, 10% (w/v) glucose, 0.3 μg/ml GOD, 60 μl gel and 750 mJ/cm^2 exposure energy.

In order to store a sensor device in a dry environment, the PVA-SbQ membrane can be dehydrated in a vacuum, which reduces its size to 3% of the initial volume. It can be rehydrated later to its original size while the activity drops by 10% (Fig. 10). Repeating dehydration and rehydration is not causing any additional loss of activity. Detailed time temperature storage experiments were not performed.

3.2. Wafer-level fabrication of an integrated glucose sensor

An enzyme-based electrochemical glucose sensor integrated inside a micro-scale flow channel is fabricated on a wafer-level using in-device enzyme immobilization. The glucose sensor is designed as a flow-through device with the enzymes immobilized upstream from the electrodes so that the hydrogen peroxide product is transported to the electrodes when dialysis fluid flows through the sensor [17,18]. The flow channel is structured into the silicon base while the electrodes sit in a shallow trench in the Pyrex cover (Fig. 11). Since the chloride ion concentration in biological fluids remains constant at 0.15 M, the chloride concentration in the dialysis fluid is constant as well. Thus, a planar Ag/AgCl electrode can be used as a pseudo-reference electrode [19]. The details of the sensor fabrication are not subject of this report. Here the technique of wafer-level sensor integration is reported.

3.2.1. Wafer-level enzyme integration

PVA-SbQ is mixed 1:1 with PBS containing the glucose oxidase. This enzyme–polymer solution is filled into the flow channels after anodic wafer bonding. Critical for wafer-level immobilization is to include auxiliary channels in the design (Fig. 12), which connect all sensor devices and allow easy filling of the entire wafer through a single inlet and flushing of the non-polymerized solution after UV exposure. For filling, the wafer stack is partially immersed in the enzyme–polymer solution so that the inlet is completely covered with solution while the air outlet remains open. The entire wafer fills automatically in 2 min by capillary action without any bubble formation.

The PVA-SbQ is then selectively crosslinked by UV exposure through the Pyrex cover using a shadow mask (Fig. 13). Thus, the enzymes get entrapped in the locally formed gel. After exposure a syringe is connected via a capillary tube to the fluid inlet to rinse out the unlinked solution with PBS. The active gel remains inside the flow channels (Fig. 14). Residuals of the unlinked polymer dissolve in the buffer solution since PVA-SbQ is water-soluble. Due to poor wall adhesion of the PVA-SbQ gel, it is most effective to polymerize the PVA-SbQ in side pockets or in the form of sleeves around silicon posts inside the flow channel (Fig. 13).

After in-device enzyme immobilization, the wafer stack is diced into chips (Fig. 15). This opens the auxiliary channels connecting the sensor devices, which are now used as in-plane device fluid ports. The chip design also allows easy

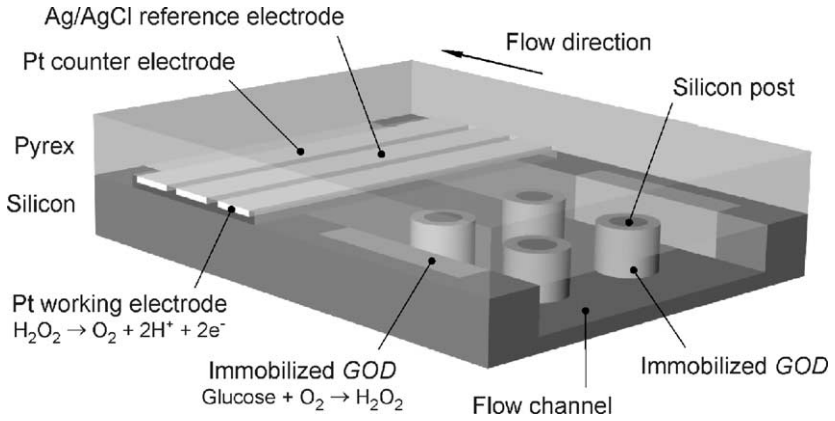

Fig. 11. Schematic diagram of an integrated enzyme-based flow-through glucose sensor. The enzymes are immobilized in side pockets and around silicon posts inside the flow channel. Hydrogen peroxide is transported in flow direction to the working electrode.

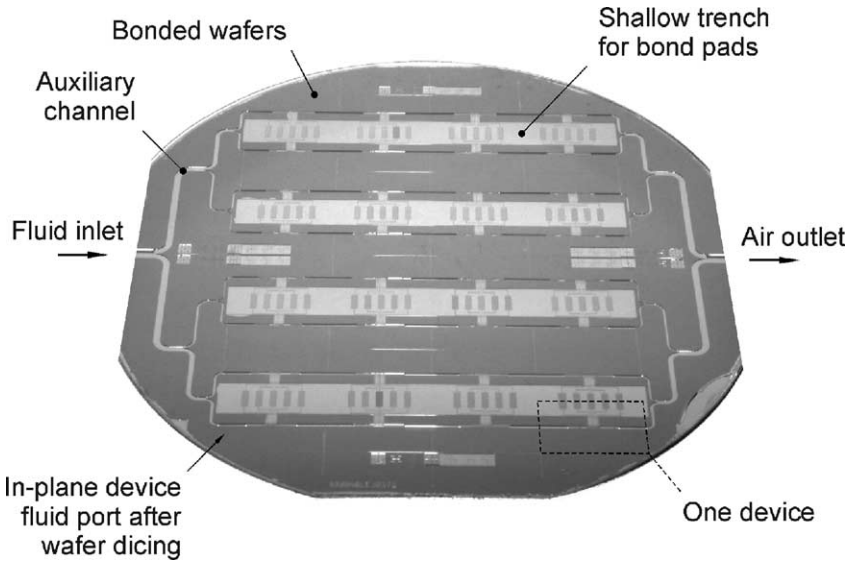

Fig. 12. Photo of the bonded wafer pair showing the fluid inlet and air outlet for wafer-level enzyme loading. The bond pads sit in shallow trenches in the Pyrex cover, which remain unbonded.

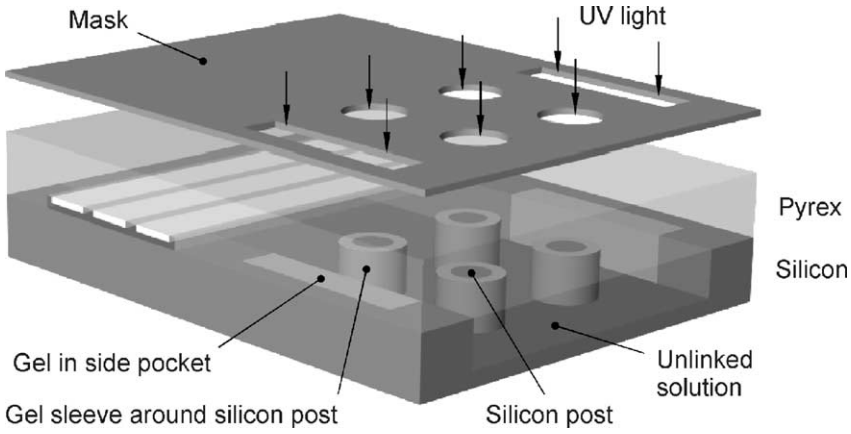

Fig. 13. Schematic diagram of the in-device immobilization technique.

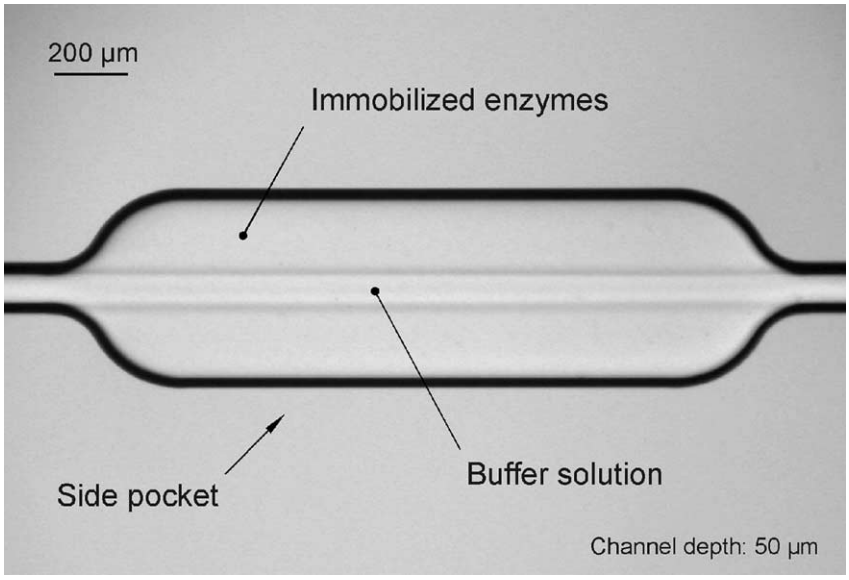

Fig. 14. Photo of a micro-scale flow channel with PVA-SbQ crosslinked in two side pockets.

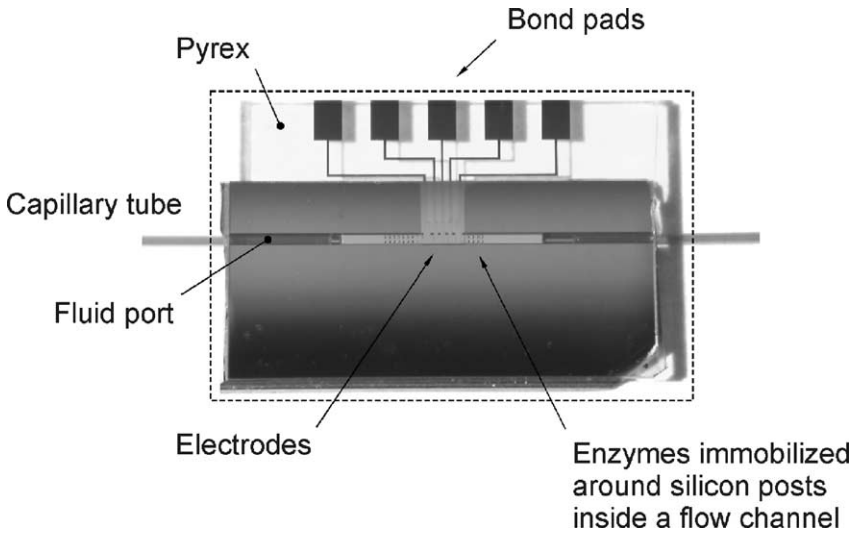

Fig. 15. Photo of a single chip with an integrated glucose sensor inside the flow channel. Wafer dicing opens the in-plane fluid ports (former auxiliary channels). The design also allows easy bond pad access since the unbonded overlapping silicon breaks off readily.

bond pad access. The overlapping silicon breaks off readily and releases the bond pads after wafer dicing. Capillary tubes can be glued into the in-plane fluid ports (400 μm × 400 μm).

3.2.2. Kinetic characteristic of the integrated glucose sensor

The kinetic characteristic of an integrated flow-through glucose sensor is measured at a flow rate of 100 μl/min. No erosion of the PVA-SbQ membrane could be observed. The high flow rate insures a homogeneously distributed glucose concentration along the flow channel even for low glucose concentrations since the glucose consumption is negligible compared to the fast glucose supply. Thus, the kinetic characteristic of the sensor response should be defined by the kinetic properties of the immobilized enzymes rather than by concentration variations imposed by glucose consumption along the channel. The measured kinetic characteristic (Fig. 16) supports this assumption and is quite similar to the results obtained under static conditions with the PVA-SbQ-coated disc electrode (Fig. 5). Both the low apparent Michaelis constant of 7.0 mM and the inaccurate curve fit indicate an oxygen-limited maximum current. Furthermore, a value greater than the static case suggests that this system has a slightly greater diffusional resistance or an increased oxygen concentration. The linear range of the sensor response is 0–160 mg/dl.

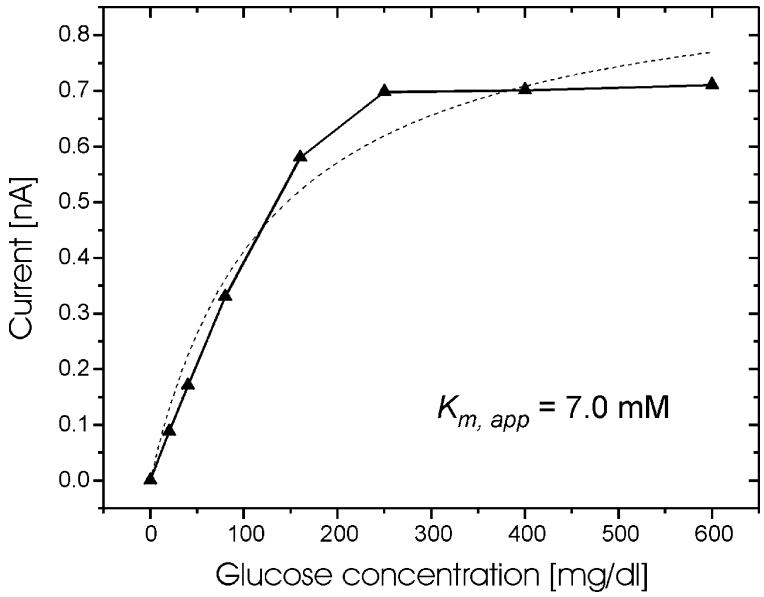

Fig. 16. Kinetic characteristic of an integrated flow-through glucose sensor (50 μm deep and 100 μm wide flow channel, 100 μl/min flow rate). The enzymes are immobilized in side pockets (2 mm long and 200 μm wide). Experimental: 0.01 M PBS, pH 7.4, 20 °C, 20−600 mg/dl glucose, 22.8 μg/ml GOD, 750 mJ/cm^2 exposure energy.

4. Conclusion

In-device enzyme immobilization is demonstrated as a convenient method for wafer-level patterning of enzymes inside micro-scale flow channels. A photosensitive polymer, poly(vinyl alcohol)-styrylpyridinium is mixed with buffer solution containing the enzyme, filled into the channels via capillary action after wafer bonding and then selectively crosslinked by UV exposure through a transparent top wafer using a shadow mask. Thus, enzymes get entrapped inside the locally formed gel. The unlinked solution is rinsed out while the active gel remains inside the channels. Critical for wafer-level integration is to include auxiliary channels in the design ensuring fluid connection between all sensor devices. In-device immobilization is generally applicable to any biochemical component that can withstand a low energy UV exposure. Results indicate glucose oxidase is very insensitive to UV light at 365 nm. No decrease in enzyme activity was measured for exposure energies in the range 1.5–15 J/cm^2. PVA-SbQ seems completely polymerized at an exposure energy of 750 mJ/cm^2. The long-term stability of the immobilized glucose oxidase is reduced due to leakage of enzymes out of the gel. Thus, enzymes need to be attached to the PVA-SbQ backbone using a second crosslinker.

The effect of immobilization on the kinetic characteristic has been investigated for glucose oxidase. The low apparent Michaelis constant of 3.0 mM for an enzyme concentration of 22.8 μg/ml indicates an oxygen-limited maximum catalytic rate as well as a fast diffusion of glucose into the membrane rather than an increased enzyme-substrate affinity. PVA-SbQ is a suitable material for bioassay applications where rapid diffusion of substrate into the active membrane is desired to maximize the catalytic rate. However, to extend the linear response an additional membrane is required to reduce the substrate diffusion.

An enzyme-based electrochemical flow-through glucose sensor integrated inside a flow channel is fabricated using in-device enzyme immobilization. The kinetic characteristic of the sensor response is quite similar to the static experiment with a polymer-coated disc electrode. The sensor response is linear in the range 0–160 mg/dl.

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Biographies

Stefan Zimmermann received his diploma in electrical engineering in 1996 and his PhD in electrical engineering in 2001 from the Technical University Hamburg-Harburg (TUHH), Germany. He worked in the Department of Microsystems Technology at the TUHH from 1996 to 2001. His research focused on MEMS design and fabrication. In 2002, he received the research award “Promotionspreis der Metall- und Elektroindustrie” of the Nordmetall, Verband der Metall- und Elektroindustrie, Germany, for the development of a micro-flame ionization detector and a micro-flame spectrometer. In 2001, Dr. Zimmermann joined the Berkeley Sensor and Actuator Center at the University of California, Berkeley, USA, as a post-doctoral research engineer with support of a Feodor–Lynen Fellowship of the Alexander von Humboldt

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Dorian Liepmann is the vice chair and Lester John and Lynne Dewar Lloyd distinguished professor of bioengineering at UC, Berkeley. He received his BS and MS in chemical engineering from Caltech, and his PhD from the University of California, San Diego. Before coming to Berkeley in 1993, Dr. Liepmann worked in the Advanced Technology Group at SAIC in La Jolla, on projects for the Navy. Previously Dr. Liepmann was a member of the Arroyo Center providing technology and policy analysis for the Army. He joined the Mechanical Engineering Department at UC, Berkeley in 1993 and the Bioengineering Department in 1999. Dr. Liepmann is a director of the Berkeley Sensor and Actuator Center (BSAC). He is currently working on the application of MEMS to biological and medical problems.