DOI: 10.1002/cphc.200700279

Label-Free Electrical Determination of Trypsin Activity by a Silicon-on-Insulator Based Thin Film Resistor

Petra A. Neff,[a] Andreas Serr,[b] Bernhard K. Wunderlich,[a] and Andreas R. Bausch*[a]

A silicon-on-insulator (SOI) based thin film resistor is employed for the label-free determination of enzymatic activity. We demonstrate that enzymes, which cleave biological polyelectrolyte substrates, can be detected by the sensor. As an application, we consider the serine endopeptidase trypsin, which cleaves poly-L-lysine (PLL). We show that PLL adsorbs quasi-irreversibly to the sensor and is digested by trypsin directly at the sensor surface. The created PLL fragments are released into the bulk solution due to kinetic reasons. This results in a measurable change of the surface potential allowing for the determination of trypsin concentrations down to 50 ng mL^{-1}. Chymotrypsin is a similar endopeptidase with a different specificity, which cleaves PLL with a lower efficiency as compared to trypsin. The activity of trypsin is analyzed quantitatively employing a kinetic model for enzyme-catalyzed surface reactions. Moreover, we have demonstrated the specific inactivation of trypsin by a serine protease inhibitor, which covalently binds to the active site of the enzyme.

Introduction

Recently, numerous field-effect devices have been presented among the variety of different concepts for the realization of label-free biosensors. Silicon-based devices possess several advantages, such as small size and weight, fast response, high reliability, on chip integration of biosensor arrays and the prospect of low-cost mass production.[1] The determination of enzymatic activity is widely used, for example, for medical diagnostics, for the study of metabolic cycles or for the monitoring of biotechnological processes. The characterization of enzymes regarding their catalytic properties requires the measurement of their turnover rate at different conditions. Optical methods such as photometry and fluorimetry are the most frequently used techniques in enzyme analysis.[2] These reactions are usually carried out in bulk. Biocatalytic biosensors are based on enzymes, which combine the specific binding with a specific biochemical reaction. In most cases, surface immobilized enzymes have been used,[3–6] where a substrate in solution reacts to create a detection signal. Accordingly, in a classical enzyme field-effect transistor (ENFET), immobilized enzymes are used for the direct analysis of substrates or inhibitors.[7] Here, the SOI sensor is employed as a biosensor for the determination of enzymatic activity, where the enzyme is not used as the biological recognition element, but is the analyte to be detected. Therefore, not the enzyme but a suitable substrate must be immobilized on the sensor surface. This substrate serves as the biological recognition element. In the course of the enzymatic reaction, a change occurs at the surface, which can be transduced by the sensor into a measurable signal. Here, enzymes are used which cleave biological polyelectrolyte substrates. The adsorption of polyelectrolytes can be readily observed by field-effect devices. These devices are highly sensitive to changes of the potential at the sensor surface caused by the binding of charged molecules. For example, the build-up of polyelectrolyte multilayers has been observed by the use of field-effect devices.[8–12] When the adsorbed polyelectrolytes are enzymatically cleaved into smaller fragments, these fragments desorb from the sensor surface changing the surface charge. This change is transduced by the SOI sensor into an increase or decrease of the current, depending on the polyelectrolyte charge. Thereby, the activity of the enzyme can be directly monitored in real time allowing for a kinetic analysis. Previously, field-effect based biosensor applications have already made use of the specific binding of biological polyelectrolytes. Namely, the hybridization of nucleic acids,[8,9,13] the aptamer based analysis of small molecules[14] and the detection of products of the polymerase chain reaction (PCR)[15] have been demonstrated. It has been found that PCR products can be detected over nucleotide monomers indicating that permanent electrostatic adsorption requires stronger multivalent interactions. Herein, we detect the activity of the serine endopeptidase trypsin, which cleaves the polyelectrolyte substrate poly-L-lysine (PLL), by the desorption of polyelectrolyte frag-

[a] *Dr. P. A. Neff, B. K. Wunderlich, Prof. Dr. A. R. Bausch*
Lehrstuhl für Biophysik–E22
Technische Universität München (Germany)
Fax: (+49) 89 289 14469
E-mail: abausch@ph.tum.de

[b] *Dr. A. Serr*
Physik-Department–T37
Technische Universität München (Germany)

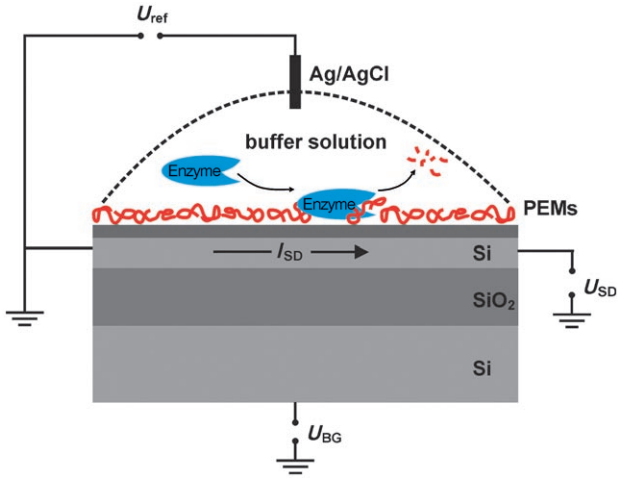


Figure 1. Setup and measurement geometry. Silicon is shown in light grey, silicon oxide in dark grey. From top to bottom: native oxide (1–2 nm), conducting top silicon (30 nm), buried oxide (200 nm), bulk silicon (675 μm). A voltage U_{SD} is applied between the source and the drain contacts and the resulting current I_{SD} is measured yielding the sheet resistance of the device. The carrier concentration in the top silicon layer is tuned by the backgate voltage U_{BG}. The potential of the electrolyte solution is controlled by a Ag/AgCl reference electrode. A microfluidic device allows the rapid exchange of buffer solution. The sensor surface is covered with PLL, which is enzymatically digested.

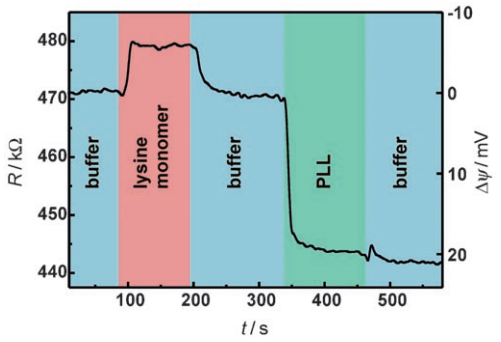


Figure 2. Lysine monomers at 0.01 % (w/v) in Tris buffer are injected into the fluid chamber. Afterwards, the system is rinsed with buffer. No significant change of the sheet resistance is observed. Next, PLL at the same concentration [0.01 % (w/v)] is injected and the system is rinsed with buffer. The resulting decrease of sheet resistance corresponds to an increased surface potential due to the binding of positively charged PLL molecules to the sensor surface.

ments from the sensor surface. Trypsin is a pancreatic enzyme, which is secreted into the small intestine for protein digestion; it is also widely used for biotechnological processes, for example, in the food industry. It was found by paper chromatography that mainly dilysine and trilysine are produced in the digestion of PLL by trypsin. As the end bonds next to a carboxyl or amino group are not split,[16] no monolysine is formed. An anionic aspartate residue inside the substrate binding pocket of trypsin attracts cationic residues, thereby generating its substrate specificity for lysine and arginine residues. We show that PLL strongly adsorbs to the charged sensor surface whereas small molecules such as lysine monomers or dimers are hardly adsorbed from their solutions. At the same time, monomers and shorter oligomers desorb faster from the sensor surface, which is also supported by theoretical considerations. We make use of this priniciple for the determination of trypsin activity. Adsorbed PLL, which is enzymatically digested into short oligomers, is readily released into pure buffer solution.

Results and Discussion

Monomer vs Polymer Adsorption

In our detection scheme, we employ PLL as a substrate for trypsin. The positively charged PLL readily adsorbs to a negatively charged silicon oxide surface and can easily be detected by the SOI sensor. The adsorption of the positively charged polymer results in an increase of the surface potential, which corresponds to a decrease of the measured sheet resistance. On the contrary, the adsorption of lysine monomers to the sensor surface cannot be detected. This can be seen in Figure 2, where both the sheet resistance R and the change in surface potential $\Delta\psi$ with respect to the start of the measurement are given. First, the sensor is equilibrated with Tris buffer. Next, a 0.01 % (w/v) solution of lysine in Tris buffer is injected into the flow chamber. As the SOI sensor also exhibits a pH-sensitivity, an increase of the sheet resistance is observed, which is caused by the basicity of lysine. After the injection of pure buffer solution, the sheet resistance decreases again to the previous value. Hence, no permanent binding of lysine monomers to the sensor surface can be detected. Now a 0.01 % (w/v) PLL solution in Tris buffer is injected into the flow chamber leading to a decrease of the sheet resistance. When the chamber is rinsed with buffer solution, the signal remains unchanged indicating the irreversible binding of the positively charged PLL. This demonstrates that lysine is not adsorbed by the sensor surface while in contact with a solution of lysine monomers as opposed to solutions containing polymeric PLL. This preferential adsorption of longer polymers in contrast to monomers is in accordance with simulation results of (poly)-electrolyte adsorption to charged surfaces[19] and can be understood in terms of thermodynamic considerations. For the adsorption of long polymers, less translational and configurational entropy per monomer is lost when bringing the polymer from the bulk to the surface as compared to the shorter ones. The overall adsorption free energy per monomer is smaller (more negative) for a longer polymer.[20] In our case, the monomer concentration in solution and the monomer-surface attraction are too low to allow for the adsorption of lysine monomers.

Determination of Trypsin Activity

We exploit this preferential binding of longer polymers as compared to shorter ones. The trypsin cleavage of PLL into small peptides is employed for the electrical detection of trypsin activity. For this, we add trypsin solution to a PLL covered sensor surface and observe the desorption of the cleaved polyelectrolyte. Predominantly, dimers and trimers are obtained,[16]

which are too short to efficiently bind to the sensor surface, as we have shown for lysine monomers. For the real-time detection of trypsin activity, we start with a PLL coated sensor chip as shown in Figure 3. After equilibration with buffer, a

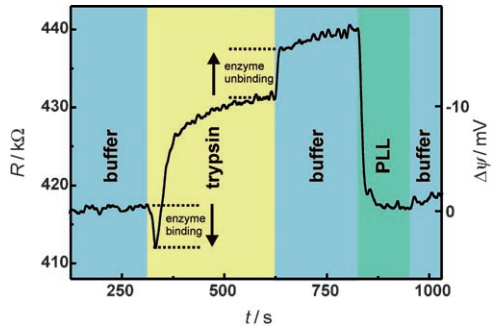


Figure 3. A PLL covered sensor chip is equilibrated with buffer. Next, a trypsin solution of 0.5 mg mL^{-1} is injected. The enzyme cleaves the PLL substrate. The fragments are released into the solution which decreases the surface potential as positively charged molecules desorb from the surface. The chip is rinsed with buffer and PLL can be readsorbed to the surface. Arrows indicate the binding and unbinding of the enzyme.

5 mg mL^{-1} trypsin solution is injected. The enzyme starts cleaving the polyelectrolyte and the fragments are released into the solution. Thus, positively charged molecules desorb rapidly from the surface, which decreases the surface potential and therefore increases the measured sheet resistance. This desorption of PLL fragments can be understood in terms of kinetic factors. Next, the chip is rinsed with buffer, which further increases the sheet resistance. This could be caused by the release of surface bound trypsin as indicated by the arrows in Figure 3. At a pH of 7.4, the net charge of trypsin is positive as it shows a p*K* value of 11,[21] which is in agreement with a decrease of the sheet resistance caused by the binding of the enzyme and an increase of the sheet resistance with its unbinding. Additionally, this effect vanishes for smaller trypsin concentrations, where PLL digestion is slower and the surface is not saturated with trypsin. The sensor can be reused for trypsin determination by reloading it with PLL, which demonstrates that the polyelectrolyte has efficiently been removed from the surface. For the understanding of the desorption of polyelectrolyte fragments we have to consider kinetic factors instead of thermodynamics. Under long enough timescales both long polymers as well as short oligomers desorb into the pure solvent. The electrostatic interaction of a molecule with the surface is determined by the attractive Debye–Hückel potential. Now, the desorption times are determined by this interaction as well as by the diffusion of the molecules. For longer polymers, we not only have a stronger interaction but also a longer diffusion time as compared to shorter oligomers. Therefore, the PLL used here ($N \geq 1\,000$) is quasi-irreversibly adsorbed to the surface on the timescale of the experiment. By contrast, it is well justified to assume an instantaneous desorption of digested polymers in the time resolution of the experiment. The initial velocity of the trypsin digestion in terms of surface potential change per second can be extracted from the graph using the calibration data. We assume that a certain change in surface charge is proportional to the corresponding change in surface potential, which holds in the Debye–Hückel limit. Then the initial velocity can be measured in terms of surface potential change per time unit. This initial velocity is plotted for different trypsin concentrations in Figure 4. Concentrations down to 50 ng mL^{-1} were detected corresponding to a molar concentration of 2 nM.

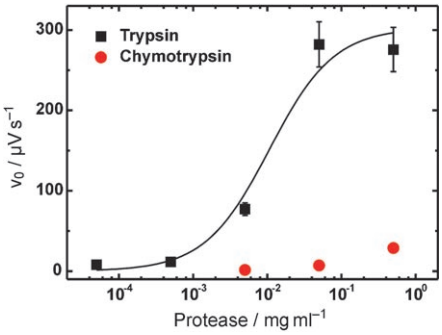


Figure 4. The initial velocity of the PLL digestion, as measured in terms of surface potential change per second, is plotted for different trypsin and chymotrypsin concentrations. Trypsin activity could be detected down to 50 ng mL^{-1}, chymotrypsin concentration down to 50 μg mL^{-1}. The solid line represents a fit by the surface Michaelis–Menten equation [Eq. (1)].

Proof of Specificity

To control the specificity of the digestion, a second protease with different specificity was studied. Chymotrypsin is a serine endopeptidase, which preferentially cleaves peptides at the C-terminal side of tyrosine, tryptophan, and phenylalanine. However, it has been shown that also the amide bonds in PLL are slowly hydrolyzed by chymotrypsin,[22] yet not as efficiently as the digestion by trypsin. In our experiments, this was observed as a considerably lower initial velocity detected by the field effect device as shown in Figure 4. In contrast to trypsin, chymotrypsin could only be detected down to 50 μg mL^{-1} corresponding to a molar concentration of 2 μM, which demonstrates the specificity of trypsin towards its substrate PLL.

Surface Enzyme Kinetics

Conventional Michaelis–Menten kinetics cannot be applied for surface enzyme reactions as considered here. In contrast to solution kinetics, it is not the substrate concentration but the enzyme concentration which is varied. A quantitative model for enzyme-catalyzed surface reactions coupling both adsorption kinetics and enzyme kinetics has been proposed in ref. [23]. In the case of trypsin digestion, the surface reaction can be divided into three steps: First, trypsin adsorbs onto the surface bound PLL forming an enzyme-substrate complex. Next, the enzyme-substrate complex reacts to form the surface bound products. In the last step, short enough PLL fragments will desorb during the timescale of the experiment as opposed to the quasi-irreversibly bound undigested PLL polymers. If the surface enzyme reaction is slow compared to the adsorption of

the enzyme and to the release of fragments into the solution, a steady state solution for the enzyme kinetics can be found.[23] Interestingly, it has the same functional form as obtained in solution Michaelis–Menten kinetics. However, the surface reaction rate is a function of the solution enzyme concentration [E] instead of the substrate concentration [Eq. (1)]

$$v_0 = \frac{v_{max}[E]}{K_M + [E]} \quad (1)$$

with the initial velocity v_0, the maximal velocity v_{max} and the surface Michaelis–Menten constant K_M. We have applied Equation (1) to the concentration dependent data of the trypsin digestion as shown in Figure 4. The fit represented by the solid line yields a maximal velocity $v_{max} = 300 \pm 20\ \mu V s^{-1}$ and a surface Michaelis–Menten constant of $K_M = 400 \pm 150$ nM. Trypsin shows a high catalytic efficiency already at small enzyme concentrations combined with a high affinity for the substrate as can be seen from the small K_M value.

Inhibition of Trypsin

Next, we want to demonstrate the specific inactivation of trypsin by an enzyme inhibitor. Phenylmethylsulfonylfluoride (PMSF) is a serine protease inhibitor, which covalently binds to the active site serine thereby inactivating the enzyme.[24,25] Figure 5 shows the inactivation of trypsin. A PLL coated sensor chip was equilibrated in buffer solution. PMSF was added to a 0.5 mg mL^{-1} solution of trypsin resulting in a final inhibitor concentration of 10 mg mL^{-1}. The solution was injected into the flow chamber, leading to a decrease of the sheet resistance, which could be ascribed to the high concentration of PMSF. After a rinse with buffer solution, only a slight increase in sheet resistance as compared to the previous buffer level is observed due to almost complete inactivation by PMSF. This can also be seen in the following reloading of the sensor with PLL solution: Only a small amount of PLL is adsorbed as the surface was already almost fully covered with PLL.

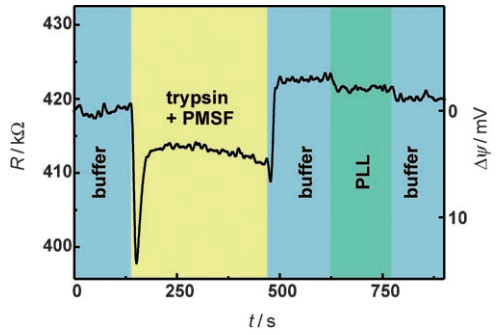


Figure 5. The inhibition of 0.5 mg mL^{-1} trypsin by the serine protease inhibitor PMSF is shown. After equilibration with buffer, a 0.5 mg mL^{-1} trypsin solution containing 10 mg mL^{-1} PMSF was added to a PLL coated sensor chip. Due to the inactivation of trypsin by PMSF, only a slight increase in sheet resistance is observed after a rinse with buffer solution.

Conclusions

We show that the recently introduced field effect device based on SOI can be employed as a biosensor for enzyme determination. The activity of the serine proteases trypsin and chymotrypsin can be monitored electrically employing the polyelectrolyte PLL as a substrate. The higher specificity of trypsin as compared to chymotrypsin towards a lysine containing substrate was demonstrated and analyzed quantitatively. Trypsin activity can be inhibited by the serine protease inhibitor PMSF. The determination of the enzymatic activity is based on the desorption of PLL fragments from the sensor surface upon addition of the enzyme solution.

Experimental Section

All chemicals including poly-L-lysine (PLL, MW 150 000–300 000), trypsin (from bovine pancreas, 9000 U mg^{-1}) and chymotrypsin (from bovine pancreas, 70 U mg^{-1}) were purchased from Sigma-Aldrich. Buffers were prepared using ultrapure water (Millipore, France) with a resistivity >18 MΩ cm. Enzyme and PLL solutions were prepared by direct dissolution in 10 mM Tris buffer at pH 7.4 containing 50 mM NaCl. All washing and measurement steps were conducted in the presence of the same buffer solution. The sensor chips were fabricated from commercially available silicon-on-insulator (SOI) wafers (ELTRAN, Canon) using standard lithographic methods and wet chemical etching as described in detail elsewhere.[17] The top silicon layer of these wafers was 30 nm thick and slightly doped with boron (10^{16} cm^{-3}). Metal contacts were deposited in an electron beam evaporation chamber (20 nm Ti, 300 nm Au). After evaporation, the sensor chips were cleaned using acetone and isopropanol. The chips were glued into a chip carrier and the contacts were Au-wire bonded to the carrier. Afterwards, the chips were encapsulated with silicone rubber to insulate the contacts from the electrolyte solution. The sheet resistance of the device is measured in a four-point geometry.[18] A flow chamber was mounted on top of the sensor and a Ag/AgCl reference electrode was used to control the potential of the electrolyte solution. In a typical experiment, the sensor is first equilibrated in buffer solution. Before the experiment, a calibration measurement is performed to relate a change in sheet resistance to a corresponding change in surface potential.[18] The setup and the measurement geometry are shown schematically in Figure 1. For PLL deposition a 0.01 % (w/v) solution was injected into the flow chamber. After obtaining a stable sensor signal, the chamber was rinsed with buffer. As soon as a stable signal was obtained, the enzyme solution can be injected. The sheet resistance of the thin film resistor was monitored continuously during the experiment.

Acknowledgement

This work was funded by the Deutsche Forschungsgemeinschaft within the SFB 563, A.S. was supported by the CompInt program of the Elitenetzwerk Bayern and the support of the Nanosystems Initiative Munich (NIM) is gratefully acknowledged.

Keywords: enzyme catalysis · kinetics · polymers · silicon · thin films

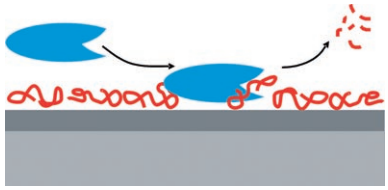
[1] M. J. Schöning, A. Poghossian, *Analyst* **2002**, *127*, 1137 – 1151.
[2] *Bioanalytik* (Eds.: F. Lottspeich, H. Zorbas), Spektrum Akademischer Verlag, Berlin, **1998**.
[3] M. M. F. Choi, *Mikrochim. Acta* **2004**, *148*, 107 – 132.
[4] S. Andreescu, J. L. Marty, *Biomol. Eng.* **2006**, *23*, 1 – 15.
[5] P. V. Bernhardt, *Aust. J. Chem.* **2006**, *59*, 233 – 256.
[6] B. Baur, J. Howgate, H. G. von Ribbeck, Y. Gawlina, V. Bandalo, G. Steinhoff, M. Stutzmann, M. Eickhoff, *Appl. Phys. Lett.* **2006**, *89*, 183901.
[7] S. V. Dzyadevych, A. P. Soldatkin, Y. I. Korpan, V. N. Arkhypova, A. V. El'skaya, J.-M. Chovelon, C. Martelet, N. Jaffrezic-Renault, *Anal. Bioanal. Chem.* **2003**, *377*, 496 – 506.
[8] J. Fritz, E. B. Cooper, S. Gaudet, P. K. Sorger, S. R. Manalis, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14142 – 14146.
[9] F. Uslu, S. Ingebrandt, D. Mayer, S. Bocker-Meffert, M. Odenthal, A. Offenhausser, *Biosens. Bioelectron.* **2004**, *19*, 1723 – 1731.
[10] P. A. Neff, A. Naji, C. Ecker, B. Nickel, R. v. Klitzing, A. R. Bausch, *Macromolecules* **2006**, *39*, 463 – 466.
[11] P. A. Neff, B. K. Wunderlich, R. v. Klitzing, A. R. Bausch, *Langmuir* **2007**, *23*, 4048 – 4052.
[12] A. Poghossian, M. H. Abouzar, M. Sakkari, T. Kassab, Y. Han, S. Ingebrandt, A. Offenhausser, M. J. Schoning, *Sens. Actuators B* **2006**, *118*, 163 – 170.
[13] F. Pouthas, C. Gentil, D. Cote, G. Zeck, B. Straub, U. Bockelmann, *Phys. Rev. E* **2004**, *70*, 031906.
[14] M. Zayats, Y. Huang, R. Gill, C. A. Ma, I. Willner, *J. Am. Chem. Soc.* **2006**, *128*, 13666 – 13667.
[15] C. S. J. Hou, N. Milovic, M. Godin, P. R. Russo, R. Chakrabarti, S. R. Manalis, *Anal. Chem.* **2006**, *78*, 2526 – 2531.
[16] S. G. Waley, J. Watson, *Biochem. J.* **1953**, *55*, 328 – 337.
[17] M. G. Nikolaides, S. Rauschenbach, S. Luber, K. Buchholz, M. Tornow, G. Abstreiter, A. R. Bausch, *ChemPhysChem* **2003**, *4*,1104 – 1106.
[18] M. G. Nikolaides, S. Rauschenbach, A. R. Bausch, *J. Appl. Phys.* **2004**, *95*, 3811 – 3815.
[19] P. Chodanowski, S. Stoll, *Macromolecules* **2001**, *34*, 2320 – 2328.
[20] G. J. Fleer, M. A. Stuart Cohen, J. M. H. M. Scheutjens, T. Cosgrove, B. Vincent, *Polymers at Interfaces*, Chapman and Hall, London, **1993**.
[21] M. Bier, F. F. Nord, *Arch. Biochem.* **1951**, *33*, 320 – 332.
[22] W. G. Miller, *J. Am. Chem. Soc.* **1964**, *86*, 3918 – 3922.
[23] H. J. Lee, A. W. Wark, T. T. Goodrich, S. P. Fang, R. M. Corn, *Langmuir* **2005**, *21*, 4050 – 4057.
[24] R. Kitz, I. B. Wilson, *J. Biol. Chem.* **1962**, *237*, 3245 – 3249.
[25] A. M. Gold, D. E. Fahrney, *Biochem. Biophys. Res. Commun.* **1963**, *10*, 55 – 59.

ARTICLES

*P. A. Neff, A. Serr, B. K. Wunderlich, A. R. Bausch**

■■ – ■■

Label-Free Electrical Determination of Trypsin Activity by a Silicon-on-Insulator Based Thin Film Resistor



Enzyme activity: The polyelectrolyte substrate poly-L-lysine is digested by trypsin directly at the sensor surface. The created fragments are released into the bulk solution which results in a measurable change of the surface potential allowing for the determination of trypsin (see figure).

