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Quantitative analysis of immobilized penicillinase using enzyme-modified AlGaN/GaN field-effect transistors

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ABSTRACT

Penicillinase-modified AlGaN/GaN field-effect transistors (PenFETs) are utilized to systematically investigate the covalently immobilized enzyme penicillinase under different experimental conditions. We demonstrate quantitative evaluation of covalently immobilized penicillinase layers on pH-sensitive field-effect transistors (FETs) using an analytical kinetic PenFET model. This kinetic model is explicitly suited for devices with thin enzyme layers that are not diffusion-limited, as it is the case for the PenFETs discussed here. By means of the kinetic model it was possible to extract the Michaelis constant of covalently immobilized penicillinase as well as relative transport coefficients of the different species associated with the enzymatic reaction which, *exempli gratia*, give information about the permeability of the enzymatic layer. Based on this analysis we quantify the reproducibility and the stability of the analyzed PenFETs over the course of 33 days as well as the influence of pH and buffer concentration on the properties of the enzymatic layer. Thereby the stability measurements reveal a Michalis constant K_M of (67 ± 13) μM while the chronological development of the relative transport coefficients suggests a detachment of physisorbed penicillinase during the first two weeks since production. Our results show that AlGaN/GaN PenFETs prepared by covalent immobilization of a penicillinase enzyme layer present a powerful tool for quantitative analysis of enzyme functionality.

1. Introduction

Since the concept of enzyme-modified field-effect transistors (EnFETs) was demonstrated by Janata and Moss (Janata and Moss, 1976) there has been a continuously increasing interest in fabrication and characterization of such devices (Turner, 2013). Indeed, the utilization of enzymes as biochemical recognition elements offers a high specificity and optimum performance for biosensor application in physiological conditions (Thévenot et al., 2001). However, most of the reported work focuses on the preparation of EnFETs and the demonstration of their functionality (Baur et al., 2006; Caras and Janata, 1980; Diallo et al., 2013; Kharitonov et al., 2000; Pijanowska et al., 2003; Poghossian et al., 2001; Zayats et al., 2000) while issues of reproducibility and stability are only sparsely addressed (Baur et al., 2006; Kharitonov et al., 2000; Zayats et al., 2000). Even though most of the FETs used for EnFETs were prepared using standard silicon processing technology, a quantitative comparison of the different devices or a quantitative characterization of the immobilized enzyme layer is not possible. This is due to the fact that different functionalization protocols have been employed for enzyme immobilization, such as physisorption (Poghossian et al., 2001), covalent binding with pyrroloquinoline quinine (Zayats et al., 2000) or glutaraldehyde on a hydrated Si_3N_4 surface (Pijanowska et al., 2003) or aggregation with bovine serum albumin (Broun, 1975; Caras and Janata, 1980; Diallo et al., 2013).

The quantitative analysis of the EnFETs response characteristics beyond their means of pure substance detection can open a route to optimizing the device performance and to characterizing the immobilized enzymatic layer. The requirement for rigorous quantitative analysis, as shown here, is the availability of a reproducible and stable immobilization and preparation process.

In Ref. Baur et al., (2006) the preparation of PenFETs with high reproducibility and high stability was demonstrated. To achieve this, the degradation of the enzymatic layer immobilized via silanization with (3-aminopropyl)triethoxysilane (APTES) and crosslinking with glutaraldehyde in humid environments was avoided by stabilization with cyanoborohydride. Due to submonolayer coverage of the enzymatic layer the response behavior of those devices did not show any indication for being limited by

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diffusive processes in the enzymatic layer, *id est* a fast exchange of substrate molecules and reaction products across the interface to the electrolyte solution could be assumed.

In the present work we demonstrate that quantitative evaluation of the response characteristics of such AlGaN/GaN PenFETs using the kinetic model suggested by Glab (Glab et al., 1991) is possible and yields quantitative information on the enzyme functionality and on basic properties of the enzymatic layer in terms of the Michaelis constant K_M and three relative transport coefficients of the different species associated with the enzymatic reaction. Based on these results we quantify the reproducibility and the stability of the analyzed PenFETs and discuss the influence of pH and buffer concentration on the properties of the enzymatic layer.

2. Materials and methods

2.1. Kinetic model for pH-sensitive EnFETs

In Ref. Glab et al., (1991) , suggested a kinetic model for the quantitative description of pH-sensitive EnFET devices that relates the response characteristics to basic properties of a thin layer of immobilized enzymes (Supporting information). Besides the requirement that the targeted enzymatic reaction produces an acid or a base, the foundation of this kinetic model is the Michaelis–Menten equation (Michaelis and Menten, 1913; Johnson and Goody, 2011), schematically expressed for the case of the enzyme penicillinase (E) converting the substrate benzylpenicillin (S) to penicilloic acid (HA) in Eq. (1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + HA \tag{1}$$

This equation describes the reversible formation of the enzyme-substrate complex (ES), with the rate constants k_1 and k_{-1}, which is converted into the acidic reaction product and the enzyme with the rate constant k_2.

Dissociation of the acidic product results in a decrease of pH which can be monitored by a change in the gate-source voltage (ΔU_{GS}) of a pH-sensitive solution-gated FET (SGFET). The general concept of this model is visualized in Fig. 1, where all processes and species that contribute to the EnFET signal are schematically shown. The EnFET response characteristics are governed by the following quantities:

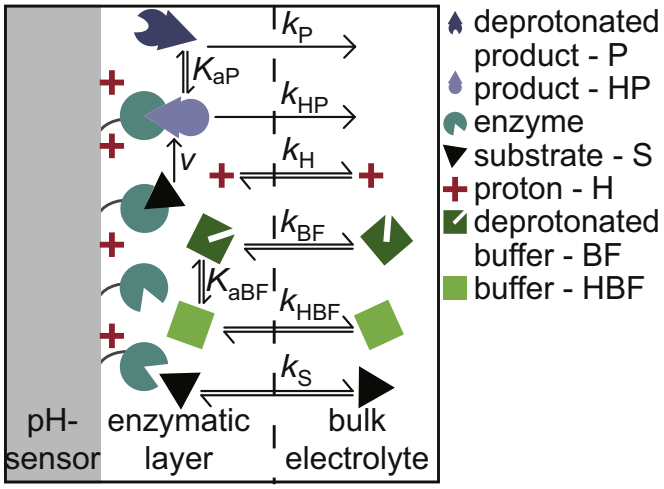

Fig. 1. Schematic representation of a pH-sensitive EnFET whose enzyme produces an acid, where k and K_a, with corresponding subscripts, are transport rate constants and acid dissociation constants, respectively, for species indicated by the subscripts; v is the reaction rate of the enzymatic reaction.

I. The enzymatic reaction rate v, given by the Michaelis–Menten kinetics in Eq. (2)

$$v = \frac{v_{max}[S]}{((k_{-1} + k_2)/k_1) + [S]} = \frac{v_{max}[S]}{K_M + [S]} \tag{2}$$

Here, v_{max} denotes the maximum reaction velocity of the enzymatic reaction and [S] the substrate concentration on the EnFET surface.

II. The dissociation constant of the acidic reaction product (P), K_{aP}, and of the buffer (BF), K_{aBF}.

III. The transport rate constants of protons (H) k_H, buffer k_{BF} and substrate k_S between the enzymatic layer and the bulk electrolyte. For simplicity, normalized rate constants, $k_V = v_{max}/k_S$ in M (mol l^{-1}), $\bar{k}_H = k_H/k_S$ and $\bar{k}_{BF} = k_{BF}/k_S$, both dimensionless quantities, are introduced.

For steady-state conditions a direct mathematical relation of the EnFET response ΔU_{GS} and the substrate concentration in the bulk electrolyte ($[S]^E$) can be derived (Eq. S2, Glab et al., 1991). Hence, the characteristic parameters of the enzymatic layer, K_M, k_V, $\bar{k}_H$ and $\bar{k}_{BF}$, can be extracted when the EnFET response characteristics $\Delta U_{GS}([S]^E)$ are fitted using the kinetic model (Glab et al., 1992a) and reveal information on the enzymatic activity (K_M) as well as the transport properties across the respective interfaces of the EnFET/enzyme/electrolyte system (k_V, $\bar{k}_H$, $\bar{k}_{BF}$). The simulated dependence of the EnFET response characteristics on all four parameters is shown in Fig. S2.

2.2. Preparation of SGFETs

pH-sensitive SGFETs were prepared using a GaN/$Al_{0.27}Ga_{0.73}N$/GaN (800 nm/18 nm/1 nm) high electron mobility transistor structure grown by metal organic chemical vapor deposition on silicon (111) substrate and a 1800 nm GaN buffer (DOWA, Japan). The sheet carrier density and the carrier mobility at room temperature were determined to $(2.0 \pm 0.2) \cdot 10^{13}$ cm^{-2} and (744 ± 62) cm^2 V^{-1} s^{-1} from Hall-effect measurements. Patterning of transistor mesa-structures with gate dimensions of (1.2×0.5) mm^2, was achieved by argon ion beam etching using a ME 601 system (Veeco Instruments, USA). Ti/Au (30 nm/150 nm) ohmic contacts for source and drain were deposited by electron beam evaporation and annealed for 30 s at 820 °C in vacuum. For measurements in electrolyte solution passivation of electrical contacts was accomplished with silicone rubber, the active gate area remained uncovered.

2.3. Immobilization of penicillinase

Covalent immobilization of penicillinase (*Bacillus cereus*, EC 3.5.2.6, P0389, Sigma-Aldrich) on the gate area of the pH-sensitive SGFETs was carried out following the procedure reported in Refs. (Baur et al., 2005, 2006). In this protocol the wet chemical oxidation in a mixture of sulfuric acid and hydrogen peroxide (H_2SO_4:H_2O_2, 3:1) is followed by silanization with APTES in dry toluene with an APTES concentration of 20 mM. Subsequently, the samples are treated with a 20 mM aqueous glutaraldehyde solution. Crosslinking of the glutaraldehyde treated silane layer and penicillinase is accomplished by formation of a secondary amine via a Schiff base in the presence of cyanoborohydride. During this process the penicillinase concentration is 800 nM. The resulting PenFETs (schematically shown in Fig. S3) were stored in 10 mM phosphate buffered saline (PBS) solution (pH 7) with a sodium

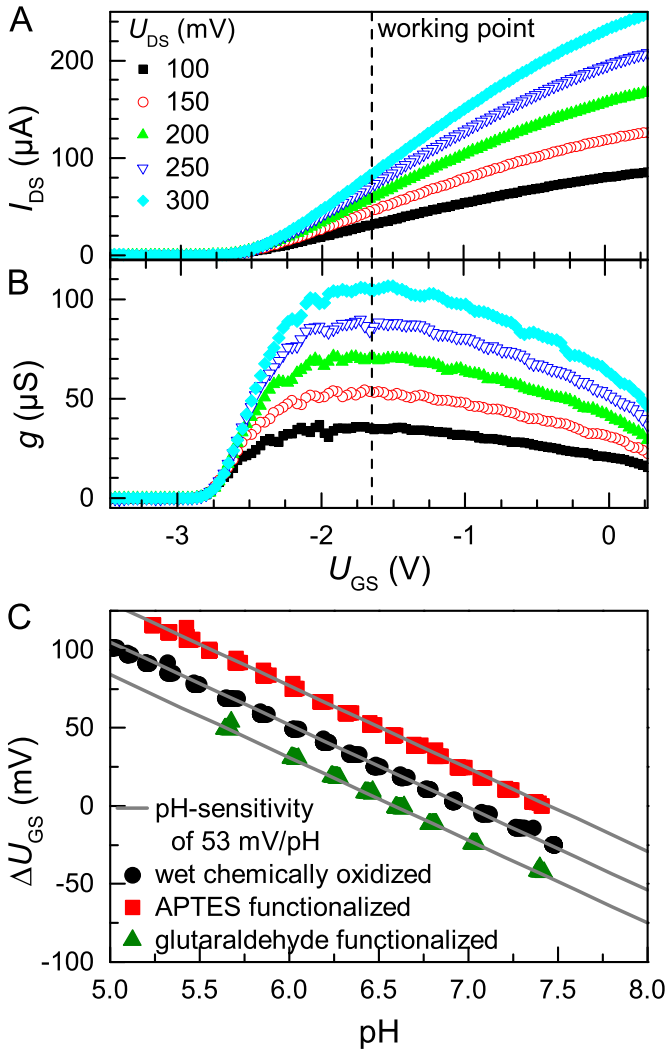

Fig. 2. (A) Transfer characteristics and (B) transconductance of a PenFET; (C) calibration curves for the pH-sensitivity of AlGaN/GaN PenFETs after different steps of the functionalization process. The slope of the solid lines represents a pH-sensitivity of 53 mV/pH.

chloride concentration of 137 mM and a potassium chloride concentration of 2.7 mM at a temperature of 5 °C.

2.4. PenFET characterization

All measurements were conducted at 25 °C in a three-electrode measurement set-up containing stirred PBS solution with a phosphate buffer concentration [BF]=0.5 mM, a sodium chloride concentration of 25 mM and a potassium chloride concentration of 0.1 mM at pH 7, if not stated otherwise. For analysis of the PenFET response, the concentration of the substrate benzylpenicillin was varied by pipetting from a 100 mM stock solution into the measurement volume of 100 ml.

Prior to enzyme immobilization the functionality of the SGFET was assessed by analyzing the transfer characteristics (Fig. 2A) and the transconductance (Fig. 2B). The PenFETs were operated at a constant drain-source voltage (U_{DS}) of 200 mV with respect to the source contact. A gate voltage U_{GS} of 1.65 V was applied with respect to the source contact via an Ag/AgCl reference electrode yielding a transconductance of 60 μS at this working point (indicated by the dashed line in Fig. 2A, B). Changes in the drain-source current (I_{DS}) caused by a change in pH as a result of the enzymatic reaction of benzylpenicillin to penicilloic acid were compensated by adjusting U_{GS} with a computer-controlled potentiostat. The required change in gate voltage ΔU_{GS} is the EnFET signal. To correlate ΔU_{GS} with the change in pH measured by the EnFET the pH-sensitivity between pH 5 and pH 8 was determined after different steps of the immobilization process by pipetting a 10 mM sodium hydroxide solution (Fig. 2C). The results show that for all stages of the immobilization process, a sensitivity of (53.6 ± 1.5) mV/pH is obtained, in reasonable agreement with earlier reports (Steinhoff et al., 2003).

2.5. Evaluation routine

The PenFETs generally exhibit a linear drift in ΔU_{GS} of (0.1–50) μV/s which was determined prior to each measurement and subtracted from the ΔU_{GS} transient. The data was then fitted according to Ref. Glab et al. (1992a) minimizing Φ (Eq. (3)) utilizing the function "NMinimize" with the method option "NelderMead" from Mathematica 9.0 (Wolfram, United Kingdom) for n pH values (pH_i) measured by ΔU_{GS} at a given substrate concentration. $pH_i([S]^E)$ denotes the i^{th} corresponding pH value as calculated by the kinetic model (Eq. S2) with the variable parameters K_M, k_v, $\bar{k}_H$ and $\bar{k}_{BF}$.

$$\Phi = \sum_{i=1}^{n} [pH_i - pH_i([S]^E)]^2 \quad (3)$$

3. Results and discussion

3.1. Variation of phosphate buffer concentration

The applicability of the kinetic model for the PenFETs under investigation was assessed by quantitatively analyzing the influence of variations in [BF] on the PenFET response curves. In all response curves shown in Fig. 3 two regions can be distinguished: the linear region, which defines the sensitivity of the PenFET, and the saturation region, which represents the highest measurable [H] at the gate area and therefore the highest $[S]^E$ for the given measurement conditions. The experimental results (symbols in Fig. 3) show a decrease of the PenFET sensitivity in the linear region as well as a decrease of the saturation signal in the saturation region with increasing [BF]. Furthermore, a fit according to the kinetic model (solid lines) shows excellent agreement with

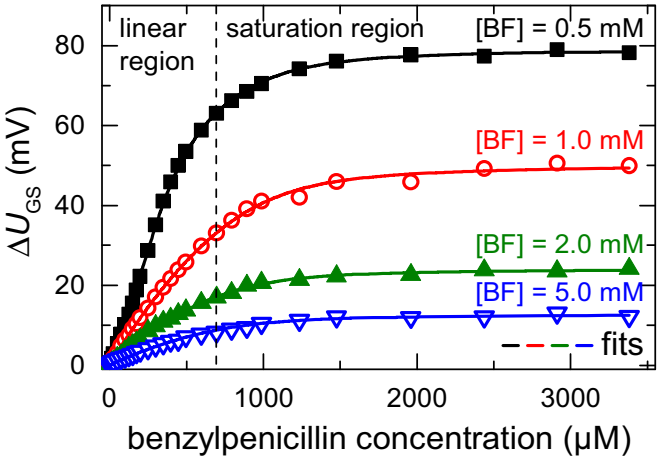

Fig. 3. Influence of [BF] on the response curves of PenFETs. The symbols represent the experimental response curves, solid lines represent fits using the kinetic model.

Table 1
Extracted model parameters for the variation of [BF].

[BF] (mM)	K_M (μM)	k_V (mM)	$\bar{k}_H$	$\bar{k}_{BF}$	Φ
0.5	86	1.11	97	1.79	0.002
1.0	87	1.11	1531	1.02	0.004
2.0	73	1.01	5112	0.02	0.001
5.0	95	0.90	10744	0.001	0.001

the experimental results as indicated by $\Phi < 0.01$ which is shown here representatively for all measurements in Table 1.

In contrast to the work by Glab et al. for the enzyme urease (Glab et al., 1992b), where only two parameters directly related to the enzymatic activity, K_M and k_v, have been systematically investigated and the other two parameters were kept at a constant value of 1, K_M and the three relative transport coefficients were determined by a fit of the experimental data in the present case.

From the fitting results, summarized in Table 1, it is evident that K_M ((85 ± 6) μM) and k_V ((1.03 ± 0.08) mM) are not systematically affected by the change in [BF]. In contrast, a pronounced increase of $\bar{k}_H$ and a simultaneous decrease of $\bar{k}_{BF}$ are observed for increasing [BF]. Hence, only those parameters that are directly related to the buffer system react to changes in [BF], while those that characterize the immobilized enzyme layer remain almost constant.

As K_M is fairly constant it is a reasonable assumption that v_{max} does not vary with [BF] and therefore also k_S is constant, *id est* the increase in $\bar{k}_H$ directly reflects an increasing k_H, the decrease of $\bar{k}_{BF}$ a decreasing k_{BF}. An increase in k_H, *id est* a faster proton transport from the enzyme layer to the electrolyte implies a smaller proton concentration at the pH-sensitive gate surface, resulting in a decreasing saturation signal. In contrast, an increase in k_{BF} results in a decreasing saturation signal as an enhanced exchange of buffer molecules leads to a larger buffer capacity in the enzymatic layer. Hence, k_H and k_{BF} are antagonists, as directly revealed by the results in Table 1.

3.2. Stability and reproducibility

As the results in Section 3.1 show that the kinetic model is a valid tool for the quantitative analysis of PenFET response curves, we have investigated the stability of AlGaN/GaN PenFETs by repeated measurements and quantitative analysis of the resulting response curves. Such measurements were conducted over the course of 33 days counting from the production date (day 0) and storage in 10 mM PBS solution at a temperature of 5 °C was only interrupted for measurements.

The results, displayed in Fig. 4A, indicate a change in PenFET characteristics during the first four measurements on day 0, 2, 6 and 14. During that period the saturation signal decreases continuously by approximately 50% while the slope of the linear region remains almost unchanged from the 2nd measurement onwards. From the 4th measurement (day 14) no further changes in the response curves were obtained. The evolution of the extracted fit parameters K_M, k_V, $\bar{k}_H$ and $\bar{k}_{BF}$ (Fig. 4B) further confirms this trend as all parameters can be regarded almost constant from at least the 14th day on.

It is noticeable that the value for K_M obtained from the 1st measurement is more than five times higher than the values obtained from all other measurements. We assign this behavior to physisorbed agglomerates of penicillinase present on top of the covalently bound enzyme layer that might interfere with the applicability of the kinetic model. Consequently, the parameters from the first measurement are not taken into account in the following discussion.

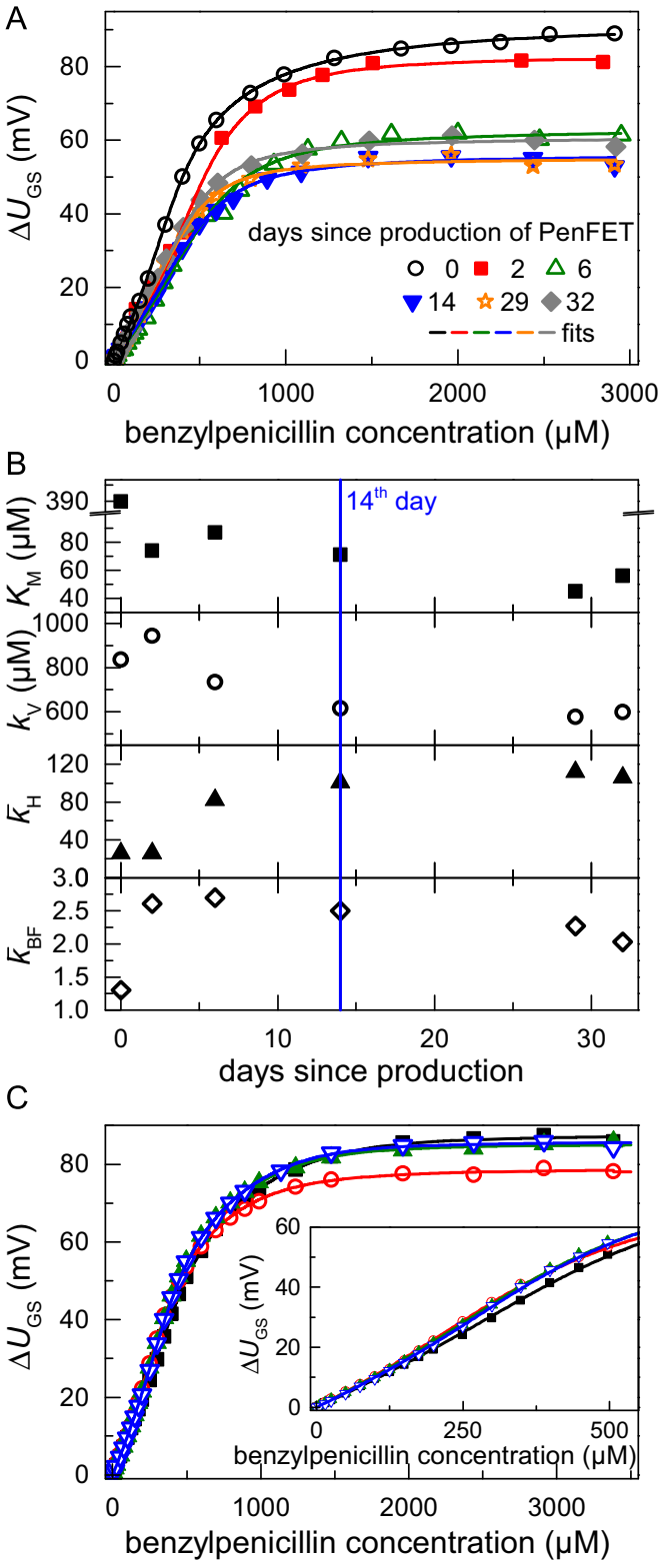

Fig. 4. (A) Chronological development of the AlGaN/GaN PenFET response curves and fits according to the kinetic model; (B) evolution of the extracted model parameters over the course of 32 days. (C) Comparison of four different PenFETs. Inset shows the linear region in detail.

The values obtained for K_M are fairly constant throughout the measurement series, yielding a value of (67 ± 13) μM when averaging from the 2nd to 6th measurement. This mean value is in good agreement to the published value of 60 μM for free penicillinase (Klemens and Citri, 1979), strongly indicating that

the covalent immobilization process applied here does not lead to a sizable deterioration of the enzymatic activity.

Hence, it can be assumed that k_2 (cf. Eq. (2)) is constant, *id est* the catalytic activity of penicillinase is not compromised by the immobilization process. For the saturation region, it follows that $k_V \approx k_2[E]_0/k_S$, *id est* the decrease in k_V from the 2nd measurement on can be attributed to a decrease in the density of enzymes on the gate area $[E]_0$ or/and an increase of the substrate molecule exchange constant k_S.

As $\bar{k}_{BF}$ with an average value of (2.4 ± 0.2) is almost constant throughout all measurements k_S can only increase similar to k_{BF}. Furthermore, the increase in $\bar{k}_H$ points towards an increasing permeability of the penicillinase layer. Hence, the decrease in k_V is attributed to a decrease in $[E]_0$, *id est* a detachment of physisorbed penicillinase, during the first two weeks.

Since the preceding analysis has revealed that reproducible response curves can be expected from at least the14th day on we have compared PenFETs from four different productions runs with respect to the reproducibility of the manufacturing process. Fig. 4C shows those four PenFET response curves, demonstrating that a high level of reproducibility is achieved. The inset in Fig. 4C illustrates separately that the sensitivity of PenFETs in the linear region is reproducible as well.

The reproducibility of the manufacturing process is also reflected by the parameters obtained from the kinetic model (cf. Table S1): The Michealis constant, with an average value of (70 ± 11) µM, lies within the range of the value established from the stability measurements ((67 ± 13) µM) while the parameters k_V ((1.29 ± 0.18) mM), $\bar{k}_H$ (118 ± 21) and $\bar{k}_{BF}$ (1.84 ± 0.03) can be regarded as constant within a small range of deviation.

3.3. Variation of pH

The influence of the pH of the bulk electrolyte, $-\lg([H]^E)$, on the PenFET response curves between pH 6 and pH 8 was also investigated, as depicted in Fig. 5. The corresponding fit parameters according to the kinetic model are summarized in Table 2.

Changing from pH 8 to pH 7 mainly affects the magnitude of the saturation signal, while only small changes are observed in the linear region. As K_M and v_{max} are constant between pH 7 and pH 8 (Waley, 1975) and k_S and k_{BF} should not be affected by a change in $[H]^E$, the only fit parameter that might be affected by changing pH is $\bar{k}_H$ which depends on the proton transport rate constant k_H. But, as the extracted fit parameters at pH 8 and pH 7 are fairly comparable (cf. Table 2), the change in the magnitude of the saturation signal by about 20% cannot be explained by the extracted fit parameters. We rather assign the observed decrease of the saturation signal to the attenuated dissociation of penicilloic acid, reflected by the dissociation constant K_{aP} with -lg(K_{aP}) being 5.2 (Proctor et al., 1982). As a consequence of this, the PenFET signal describable by the kinetic model is expected to vanish for pH < 5.2.

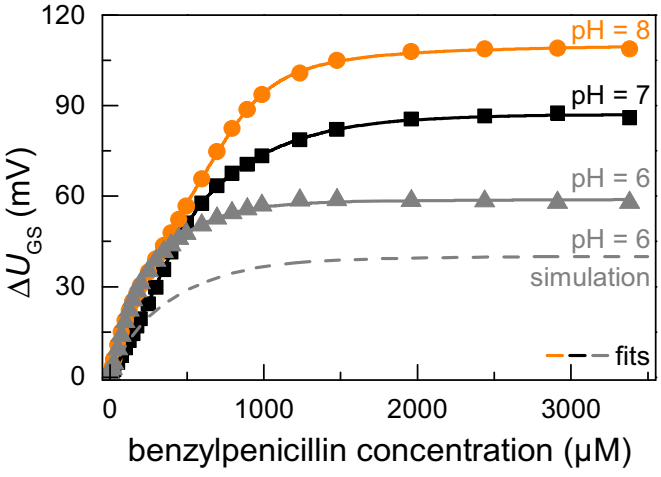

Fig. 5. Influence of the pH in the bulk solution on the response curves of PenFETs. Response curves operated in test solutions with different pH. Solid lines represent fits with kinetic model (cf. Table 2).

Table 2
Extracted model parameters for the variation of pH.

pH	K_M (µM)	k_V (mM)	$\bar{k}_H$	$\bar{k}_{BF}$
8	52	0.92	73	1.72
7	72	1.57	143	1.84
6	–	–	–	–

In contrast, the response curve for pH 6 shows strong deviations from the kinetic model as can be seen by comparing the response curves at pH 6 (gray triangles) and pH 7 (black squares) and their respective fits (solid lines) to the simulation indicated by a dashed line at pH 6 with $K_M = 70$ µM, $k_V = 1$ mM, $\bar{k}_H = 100$ and $\bar{k}_{BF} = 1.8$. The steeper slope in the linear region and the elevated saturation signal compared to the simulation at pH 6 apparently suggest that penicillinase is a faster working enzyme with a higher reaction rate at pH 6. However, according to the results in Ref. Waley (1975) this is not a valid explanation. The reason for this behavior is the protolytic degradation of benzylpenicillin in acidic solutions, yielding the same products as the enzymatic reaction (Hou and Poole, 1971).

Hence, the acid-induced degradation of benzylpenicillin adds up to the enzymatic degradation, resulting in a response curve at pH 6 that cannot be explained by the kinetic model alone. Therefore, the extracted parameters for the variation of pH are only shown for pH 8 and pH 7 in Table 2.

The extracted values for K_M are in the range of the K_M values obtained for PenFETs during their chronological development ((67 ± 13) µM). The value $\bar{k}_{BF}$ is fairly stable for the sample used in this measurement series, while the values k_V and $\bar{k}_H$ differ by approximately a factor of 2. The reason for this deviation might be that benyzlpenicillin is not only degradable by acidic solutions but is as well prone to degradation by nucleophilic attack (*exempli gratia* hydroxide ions) (Hou and Poole, 1971). However, this degradation path plays a minor role at a weak basic pH of 8. Due to this reason the kinetic model is still considered applicable at pH 8 and the deviation of the values k_V and $\bar{k}_H$ is neglected.

4. Conclusions

In conclusion we have shown that AlGaN/GaN PenFETs prepared by covalent immobilization of penicillinase represent an exceptional analytical platform for characterizing the functionality of the immobilized enzymes under various experimental conditions. Quantitative evaluation of the PenFET response using a kinetic model reveals parameters that describe the functionality of penicillinase and the transport properties across the enzymatic layer as well as its dependence on experimental parameters. At the same time the strength of the kinetic model suggested by Glab in 1991 is revealed. An average Michaelis constant of (67 ± 13) µM could be determined for covalently immobilized penicillinase and the decrease of the PenFET signal during the first measurements was assigned to a loss of physisorbed enzymes from the gate surface.

Beyond the application as enzyme-based biosensors these results demonstrate that in combination with a suitable kinetic model the reproducibility and stability of covalently functionalized AlGaN/GaN EnFETs makes them a promising adaptable platform for in-situ analysis of covalently immobilized enzymes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.062.

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