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Evaluation of chrono-amperometric signal detection for the analysis of genotoxicity by a whole cell biosensor

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ABSTRACT

Electrochemical signal detection can be readily integrated in biosensors and is thus an attractive alternative to optical detection methods. In the field of environmental chemistry and ecotoxicology there is a growing demand for lab-independent devices based on whole cell biosensors for the detection of genotoxic compounds. Because of the broad occurrence of pre-genotoxic compounds that need to be bio-activated, the integration of a system for metabolic activation into such a biosensor is important. The present study evaluates a chrono-amperometric detection method in which para-aminophenyl β-D-galactopyranoside is used as substrate for a reporter gene assay based on the bacterial SOS-response in comparison to a test system for the determination of genotoxicity in water that is standardized according to the International Organization for Standardization (ISO). The evaluation was done in order to analyze the potential of the electrochemical signal detection to be used as a complementary method for the standard test system and thus to evaluate the usability of electrochemical biosensors for the assessment of genotoxicity of environmental samples. In the present study it is shown that the chrono-amperometric detection of para-aminophenol is specific even in the presence of electro-active species generated by the enzymatic system used for the external bio-activation of contaminants. Under optimized conditions electrochemistry is sufficiently sensitive with a limit of detection that is comparable to the respective ISO-standard.

1. Introduction

The genotoxic potential of pure compounds and environmental samples can be evaluated by a large number of different techniques using eukaryotic cells (Comet assay [1], formation of micronuclei [2]) as well as by prokaryotic test systems like the AMES-test [3,4] and reporter gene assays based on the bacterial SOS-response (e.g. SOS-*umu*-test [5] or chromotest [6]). A number of SOS-dependent genes the gene products of which are involved in DNA-repair-mechanisms are activated by the occurrence of DNA single strands [7]. Such DNA lesions are induced by compounds that e.g. form DNA-adducts (bulky adducts) like benzo[a]pyrene or DNA-crosslinks like mitomycin C. The expression of the SOS-genes is regulated by the LexA protein that specifically binds to SOS-responsive promoter sequences [8]. In order to detect the SOS-response as an indicator for DNA-damage, SOS-sensitive promoters like that of the *recA* or *umuDC* genes are fused to reporter gene(s) (e.g. *lacZ* or *phoA* encoding the enzymes β-galactosidase or alkaline phosphatase) the expression of which is thereby induced by genotoxic stress. For the bacterial test systems it is of crucial importance to mimic the metabolism of xenobiotics that takes place in the liver of vertebrates and which can lead to a formation of bio-activated and thus genotoxic intermediates. Usually, this is done by the addition of the S9-fraction that is prepared from the liver of induced rodents [9,10]. The S9-fraction is composed of a complex mixture of enzymes involved in the metabolism of xenobiotics, in particular the microsomal bound cytochrome-P450-dependent monooxygenases. This latter class of enzymes catalyzes the oxidation of organic compounds by molecular oxygen. The cytochrome-P450-dependent monooxygenases are activated by a reduction step concomitantly with the consumption of NADPH [11].

In order to facilitate an easy to use field-applicable test system for the detection of genotoxicity there are several on-going activities for the construction of biosensors based on the bacterial SOS-response [12–17]. These approaches are based mainly on luminescence or fluorescence; Matsui et al. proposed electrochem-

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ical signal detection via para-aminophenyl β-D-galactopyranoside (pAPG) by scanning electrochemical microscopy. The reporter enzyme, β-galacosidase, cleaves the glycosidic bond of pAPG. The reaction product p-aminophenol (pAP) can be oxidized electrochemically to p-iminoquinone [15,18–23]. It is reported that electrochemical detection of pAPG after cleavage is even possible without cell-lysis allowing its implementation in on-line measurements and biosensors [20,24]. Matsui et al. demonstrated electrochemical signal detection after SOS-induction with 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamido (AF-2), mitomycin C (MMC) or 2-aminoanthracene (2-AA) by scanning electrochemical microscopy (SECM) of collagen embedded *Salmonella typhimurium* TA1535 *pSK1002* [15]. 2AA was metabolically activated to a genotoxic compound by the addition of S9-mix.

Direct electrochemical signal detection is preferable to the other detection approaches listed above, since the use of a simple set of electrodes would greatly reduce the complexity, size and costs that are typically associated by other methods such as optical detection (e.g. colorimetry, bioluminescence etc.) or scanning electrochemical microscopy as proposed by Matsui et al. [15]. However, it has yet to be proven that electrochemistry can also compete in terms of sensitivity with the colorimetric signal detection that is specified in the respective ISO-standard [25]. This is of special interest because of the mandatory presence of the uncharacterized mixture of potentially electro-active enzymes and metabolites (S9-fraction of liver homogenate), as well as several cofactors (e.g. NADP) in the standard assay reaction, that are added to metabolically activate pre-genotoxic substances. These compounds might interfere with the electrochemical signal detection and decrease its sensitivity. Hvastkovs et al. report the use of electrochemiluminescent arrays for genotoxicity testing of metabolites of benzo[a]pyrene that are generated *in situ* by various immobilized cytochrome (cyt) P450 enzymes [26]. Later on the same group published the use of imbedded microsomes as a source of cyt P450 enzymes [27]. In contrast to the present study the aforementioned electrochemiluminescent arrays detect DNA-damage without any cellular context, i.e. the formation of adducts with purified DNA and not a cellular response as demonstrated here.

In this communication we characterize chrono-amperometric electrochemical signal detection following the induction of the bacterial SOS-response in the presence of S9-mix. Furthermore, a thorough correlation of the electrochemical results with the ISO-standardized colorimetric detection [25] was carried out in order to evaluate the potential of the electrochemical measurement to become a complementary method for the detection of genotoxic compounds in environmental monitoring. We demonstrate that the unique substrate mediated electrochemical detection is simple to use, can be integrated on a miniaturized whole cell bio chip and yield satisfactory results in comparison to the respective ISO-standard.

2. Experimental

2.1. Materials

Two *S. typhimurium* strains were used in the course of this study, containing two different plasmids differing in both their sensing promoter and their reporter elements. Strain TA1535 *pSK1002* [5,9] (*hisG46, gal, Δ(chl, uvrB, bio)), rfa*, containing plasmid *pSK 1002* was obtained from Dr. Yoshimitsu Oda (Faculty of Science and Engineering, Kinki University, Osaka, Japan). This strain was used for the SOS-*umu*-test. Strain TA1535 *sulA::phoA*, containing plasmid *pBR2TTSsulA::phoA* harbouring the *phoA*-gene coding for the alkaline phosphatase under the control of the SOS-inducible *sulA*-promoter (Biran et al., unpublished), was used as a negative control.

4-Nitroquinoline-1-oxide (NQO) was purchased from *Sigma–Aldrich*, Germany; 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) was purchased from *Wako-Chemicals GmbH*, Neuss, Germany. Stock solutions of 1 mg mL^{-1} were prepared in Me_2SO and stored at −25 °C until usage. The stock solutions were diluted with Me_2SO to 250 μM IQ or 400 μM NQO. Further six double dilutions of these solutions were prepared in Me_2SO. Thirty microliters of the different dilutions were mixed with 970 μL sterile double distilled water of which 180 μL were used as samples for the SOS-*umu*-test. A 2% (v/v) Me_2SO solution in sterile double distilled water was used as solvent control.

The S9-fraction for metabolic activation was obtained from *RCC Cytotest Cell Research GmbH* (Roßdorf, Germany). The S9-fraction was stored at −80 °C and kept on ice after thawing for immediate usage.

2.2. Instrumentation

The screen printed electrodes consisted of a working electrode (Au), a reference electrode (Ag/AgCl) and a counter electrode (carbon graphite) and were purchased from GEM Gwent Electronic Materials Ltd., UK. The PalmSens from Palm Instruments BV, Houten, The Netherlands, was used as the potentiostat. Control of the measurement and data acquisition were carried out with Palm Sens Lite software (version 1.7.3.0, Palm Instruments).

2.3. SOS-umu-test

S. typhimurium TA1535 *pSK1002* was used for the detection of genotoxic effects via the bacterial SOS-response. IQ (2-amino-3-methylimidazo[4,5-f]quinoline) served as a pre-genotoxic standard compound and was tested with metabolic activation (S9-mix) according to a slightly modified protocol of the ISO-standard 13829 "Water quality-Determination of the genotoxicity of water and waste water using the *umu*-test." [25]. The bacteria were stored with 10% (v/v) dimethylsulfoxide (Me_2SO) in liquid nitrogen until usage. Bacteria were re-cultivated for 8 h in 1× TGA medium (10 g L^{-1} bacto trypton, 5 g L^{-1} NaCl, 2 g L^{-1} D-(+)glucose, 11.9 g L^{-1} HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.0). Fifty micrograms per milliliter ampicillin was added to the culture medium using a 1000-fold stock solution as selection marker for the plasmid *pSK1002* that contains the SOS-responsive reporter gene construct.

To refresh the bacteria for the SOS-*umu*-test 2 mL of an overnight culture (8 h incubation) were added to 18 mL of fresh TGA medium. The culture was incubated at 37 °C and 150 rpm for 2 h. After incubation the optical density at 595 nm (E_{595}) of the bacteria was adjusted to 0.44. The bacteria were kept on ice until usage.

The test was performed in 96 well plates. Twenty microliters of a 10× TGA stock solution containing 500 μg mL^{-1} ampicillin and the cofactors and salts for the activation of S9-enzymes (NADP 29 mM, glucose-6-phosphate 33 mM, KCl 240 mM and $MgCl_2$ 59 mM) were added to 180 μL of sample (genotoxicant in double distilled water with 2% (v/v) Me_2SO) or negative control (2% (v/v) Me_2SO in double distilled water). If no external metabolic activation was needed, 20 μL of pure 10× TGA was used instead of the cofactor solution. Seventy microliters of the bacterial suspension were added to a total volume of 270 μL. In case of external metabolic activation, freshly thawed S9-fraction was added to a final concentration of 0.8% (v/v). After incubation for 2 h at 37 °C and 900 rpm, E_{595} was determined. Subsequently, the exposed bacteria were split for independent colorimetric and amperometric detection of the β-galactosidase-activity.

For the measurement of the activity of the reporter enzyme β-galactosidase, 30 μL of the bacterial suspension were transferred to 150 μL of a reaction mixture containing 103 mM Na_2HPO_4, 39 mM

NaH_2PO_4, 8 mM KCl, 0.8 mM $MgSO_4$, 2.8 mM sodium dodecyl sulfate (SDS), 3 mM oNPG (o-nitrophenyl-β-D-galactopyranoside) and 30 mM β-mercaptoethanol in a new 96-well plate and incubated at 28 °C and 900 rpm for 15 min. Finally, 120 μL 1 M Na_2CO_3 were added. The absorption at 414 nm (E_{414}) was measured for the quantification of p-nitrophenol. The induction factor ($iF_{colorimetric}$) was calculated as mandatory in the respective ISO protocol [25]:

$$iF_{colorimetric} = \frac{(E_{414sample} - E_{414blank})/(E_{595sample} - E_{595blank})}{(E_{414negative\text{-}control} - E_{414blank})/(E_{595negative\text{-}control} - E_{595blank})}$$

2.4. Chrono-amperometric detection

Twenty-five microliters of exposed bacteria were pipetted on top of a screen printed electrode (GEM Gwent Electronic Materials Ltd., Au (working), Ag/AgCl (reference), carbon graphite (counter), connected to a PalmSens (Palm Instruments BV, Houten, The Netherlands)) potentiostat. Eight samples were measured in an alternate mode with a frequency of 2 Hz using a multiplexer. Control of the measurement and data acquisition were carried out with Palm Sens Lite software (version 1.7.3.0, Palm Instruments). After the addition of the samples a potential of 300 mV or 400 mV was applied and the current was monitored for approximately 120 s. 5 μL of the substrate p-aminophenol-β-D-galactopyranoside (pAPG, 36 mM) was added after electrochemical equilibrium was achieved (stable current). The resulting current was monitored for 15 min. For the evaluation of the SOS-induction dI/dt was calculated from 600 to 800 s after the addition of the pAPG. The SOS-induction-factor was calculated by:

$$iF_{amperometric} = \frac{(dI/dt)_{sample} - (dI/dt)_{blank}}{(dI/dt)_{negative\text{-}control} - (dI/dt)_{blank}}$$

This way of calculation differs from the equation for $iF_{colorimetric}$, but by making use of the slope dI/dt instead of the absolute current after a given time, different offsets in the electrochemical signal are directly removed. The other way around the absorbance at 414 nm after 15 min incubation can be interpreted as a rate as well. However, independent of the way of calculation, both induction factors are dimensionless and directly comparable. For the calculation of the $iF_{amperometric}$ the normalization of the slope dI/dt to the E_{595} was excluded because the cell density will not be determined by light scattering in the envisaged biosensor set-up.

2.5. Optimization of pAPG-concentration

Bacterial TA1535 *pSK1002* were exposed to 8 μM 4-nitroquinoline-1-oxide without metabolic activation as described above. The chrono-amperometric signal detection was performed using varying final concentrations of pAPG (1 mM, 1.5 mM, 3 mM and 6 mM) at a constant potential of 0.3 V. The slope dI/dt was calculated from 450 to 550 s after the addition of pAPG.

2.6. Linear sweep voltammetry measurement of pAP and pAPG

S. typhimurium strain TA1535 *pSK1002* was exposed to 5 μM IQ in the presence of metabolic activation as described above. Either 25 μL of the blank sample containing media, genotoxic compound, cofactors and S9-fraction or 25 μL of exposed bacteria (similar to the blank sample except for the addition of bacteria) were transferred on top of a screen printed electrode. Five microliters of a 36 mM pAPG-solution in ddH_2O were added to either the exposed bacteria or the blank sample, each in duplicates. Furthermore, 5 μL of a 15 mM pAP-solution in ddH_2O or 5 μL ddH_2O was added to exposed bacteria in duplicates on separate electrodes. After incubation at 20 °C for 15 min on the screen printed electrodes linear sweep voltammetry was done using a scan rate of 50 mV s^{-1} (potential range 0–800 mV).

2.7. Monitoring pAPG-cleavage with linear sweep voltammetry

Either *S. typhimurium* TA1535 *pSK1002* or TA1535 *sulA::phoA* were exposed to 5 μM IQ in the presence of metabolic activation as described above. Twenty-five microliters of the complete reaction mixture containing media, bacteria, genotoxic compound, cofactors and S9-fraction were transferred on top of a screen printed electrode. A linear sweep voltammetry measurement between 0 and 0.4 V and a scan rate of 50 mV s^{-1} was performed in four replicates for each strain in consecutive mode 1 min, 5 min and 10 min after the addition of 5 μL 36 mM pAPG.

3. Results and discussion

3.1. Optimization of substrate concentration for electrochemical signal detection

Optimization of pAPG concentration was carried out in order to avoid substrate limitation. Bacteria were exposed to 8 μM 4-nitroquinoline-1-oxide (NQO), a strong genotoxic agent that is DNA-reactive without metabolic activation. The SOS-induction factor as determined by the standardized SOS-*umu*-test was >17, indicating a strong expression of the β-galactosidase (data not shown). Using these bacteria, different concentrations (1 mM, 1.5 mM, 3 mM and 6 mM) of pAPG were tested for the detection of the β-galactosidase-activity in terms of the slope dI/dt. The results are shown in Fig. 1. Under the assumption that the cleavage of the pAPG is the rate limiting step of the whole reaction sequence pAPG → para-aminophenol → para-iminoquinone, the data were fitted according to the Michaelis–Menten equation ($v = dI/dt = v_{max} \cdot [pAPG]/(K_M + [pAPG])$) with a nonlinear least squares regression. The Michaelis–Menten constant (K_M) was determined to be 1.78 mM (standard error = 0.44 mM). This is a substantial higher K_M value than that of para-nitrophenyl-β-D-galactopyranoside (0.03 mM) [28] but in the range of other artificial substrates of the β-galactosidase such as phenyl-β-

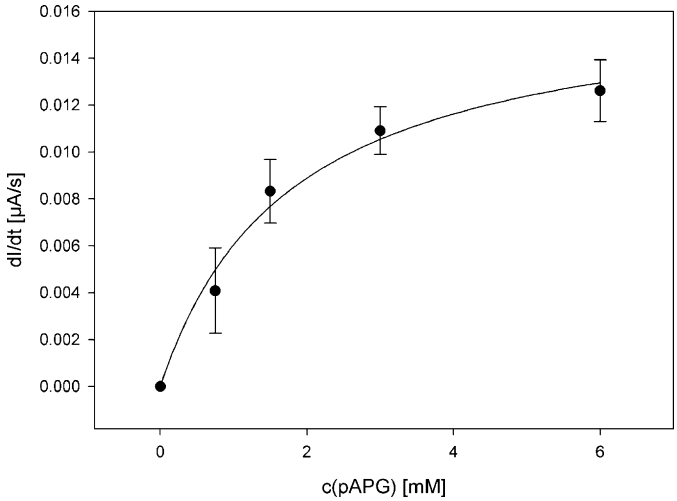

Fig. 1. Optimization of the pAPG-concentration. *S. typhimurium* strain TA1535 *pSK1002* was exposed to 8 μM NQO. Measurement of the β-galactosidase-activity is done amperometrically with pAPG (final concentrations as indicated) at 300 mV vs. Ag/AgCl. dI/dt values were calculated from 500 to 600 s after the addition of pAPG. The data was fitted with $dI/dt = v_{max} \cdot [pAPG]/(K_M + [pAPG])$.

D-galactopyranoside with a K_M = 1.47 mM [29]. Because of the *rfa*-mutation in strain *S. typhimurium* TA1535 its cell wall is highly permeable, especially for hydrophobic compounds [30,31]. However, one must consider diffusion processes of the substrate pAPG and the product pAP across the bacterial cell wall and cell membrane that influence the apparent K_M. It is evident from the measured reaction rates that for pAPG-concentrations around 2 mM substrate limitation can occur when the *umu*-promoter is strongly induced. To achieve a wide linear range, a pAPG concentration of about 6 mM seems to be sufficient to guarantee a reaction of pseudo zero-order even at high concentrations of β-galactosidase.

3.2. Characterization of pAP and pAPG by linear sweep voltammetry

In order to characterize the electrochemical properties of pAPG and its cleavage product pAP in the presence of the complex reaction conditions of the SOS-*umu*-test, linear sweep voltammetry was carried out in a potential range of 0–800 mV. pAPG was measured in the presence and absence of exposed bacteria expressing the reporter enzyme β-galactosidase. The cleavage product pAP was analyzed in the presence of exposed bacteria without further addition of pAPG. As a control exposed bacteria without pAPG or pAP were measured. The results are shown in Fig. 2. In the absence of pAP and pAPG (filled circles) no distinct peak is observable, indicating the absence of electro-active species in the potential range of 0–800 mV. In case of pAP (hollow circles) the peak current potential is located at 395 mV and for pAPG (hollow triangles) at 600 mV. The oxidation of pAPG starts around 400 mV. To simulate the conditions during the assessment of the β-galactosidase-activity via pAPG-cleavage, the linear sweep voltammetry was performed with pAPG (final concentration 6 mM) in the presence of exposed bacteria (filled triangle). Beside a defined peak current potential at 630 mV, presumably caused by uncleaved pAPG, a local maximum at 430 mV is visible. Thus, a peak current potential for the oxidation of pAP is visible despite the overlapping signal of pAPG that is present in high excess. Based on the findings presented here for the tested conditions, a potential range from 300 to 400 mV seems to be suitable for the chrono-amperometric detection of pAP in the presence of pAPG. The electrochemical properties of para-aminophenol (pAP) are characterized by others as well. The oxidation of pAP to para-iminoquinone was demonstrated at potentials of 0–50 mV vs. saturated calomel electrode with a rotating disk electrode [32], or at 60 mV vs. Ag/AgCl in a stirred Tris–HCl buffer (pH 8.0) [33]. The optimum detector potential for the detection of pAP was determined by hydrodynamic voltammetry. It was found that a potential of 0.38 V vs. Ag/AgCl is suitable to detect p-aminophenol [34]. This is in good accordance to the present finding.

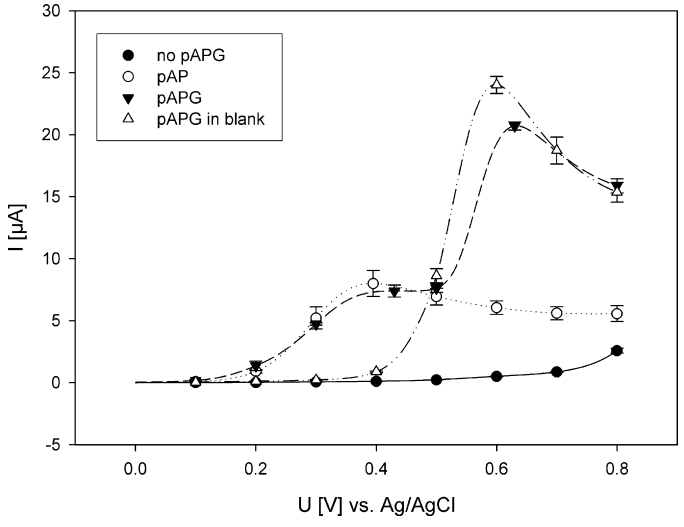

Fig. 2. Linear sweep voltammograms of pAP and pAPG. The bacterial strain TA1535 *pSK1002* was exposed to 5 μM IQ with metabolic activation. In parallel a blank of identical composition but without bacteria was prepared. Linear sweep voltammograms in the range from 0 to 800 mV were measured for the following samples: (a) exposed bacteria without further supplementation (filled circles), (b) p-aminophenol (2.5 mM final concentration) in the presence of exposed bacteria (hollow circles), (c) pAPG (6 mM final concentration) either in the presence of exposed bacteria (filled triangles) or in the blank (hollow triangles). The standard deviation of four replicas is shown at selected potentials.

3.3. The presence of S9-mix does not interfere with electrochemical signal detection

The importance of metabolic activation for the detection of genotoxic compounds in environmental samples is explained in Section 1. The S9-mix that is widely used for bio-activation of pre-genotoxic compounds in order to mimic the liver-metabolism contains electro-active species like NADP or redox-enzymes (e.g. cytochrome P450-dependent monooxygenases). The NADPH that is needed for activation of the P450-dependend monooxygenases is generated by the oxidation of supplemented glucose-6-phosphate, presumably by the activity of glucose-6-phosphate dehydrogenase [35]. Thus, NADPH is constantly generated from NADP until depletion of glucose-6-phosphate or the denaturation of the glucose-6-phosphate dehydrogenase. Two different types of experiments were performed in order to prove that this or other redox-systems, potentially present in the S9-mix do not influence the chrono-amperometric detection of the β-galactosidase-activity. Fig. 3 shows linear sweep voltammograms from 0 to 400 mV after the induction of bacterial reporter cells with the pre-genotoxic compound 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) in the presence of S9-mix. Two different *S. typhimurium* TA1535 strains were used. The first strain (Fig. 3A) contains the plasmid *pSK1002* harbouring a fusion of the *umuDC*-gene cluster to *lacZ* that is used as the reporter gene under the control of the SOS-sensitive *umu*-promoter [5]. The second strain that is used here as a negative control, contains the plasmid *pBR2TTSsulA::phoA* in which the SOS-promoter *sulA* is combined with the *E. coli phoA* gene. Linear sweep voltammograms were measured prior to pAPG-addition and 1 min, 5 min and 10 min after the supplementation of 6 mM pAPG. The successful induction of the SOS-response under these conditions was independently verified by colorimetric signal detection (data not shown). Prior to the addition of pAPG a local maximum in current was visible at ~320 mV (Fig. 3A). After addition of pAPG the measured current rises with potential starting at 200 mV with no visible maximum up to 400 mV. In the case of reporter plasmid *pSK1002*, an increase in current over time was evident at potentials >200 mV vs. Ag/AgCl. In contrast, the current do not increase with time after the addition of pAPG to the strain that contains the reporter plasmid *pBR2TTSsulA::phoA* (Fig. 3B), which expresses alkaline phosphatase instead of the β-galactosidase. Because of the absence of β-galactosidase, pAPG can not be specifically cleaved to pAP. This finding indicates that there is no artificial signal at a potential <400 mV which may be caused by other electro-active species present in the reaction mixture. Furthermore, no artificial cleavage of pAPG to pAP was detectable. Taken together, it can be concluded that the increase in current over time in case of reporter plasmid *pSK1002* was caused only by the specific cleavage of pAPG to pAP by the reporter enzyme β-galactosidase, expressed under the control of an SOS-inducible promoter. Thus, the increase in current is directly linked to the genotoxic stress caused by the sample.

In an alternative experiment, the slope dI/dt at potentials of 100 mV, 200 mV, 300 mV and 400 mV was calculated 15 min after the addition of pAPG to the exposed reporter strains TA1535

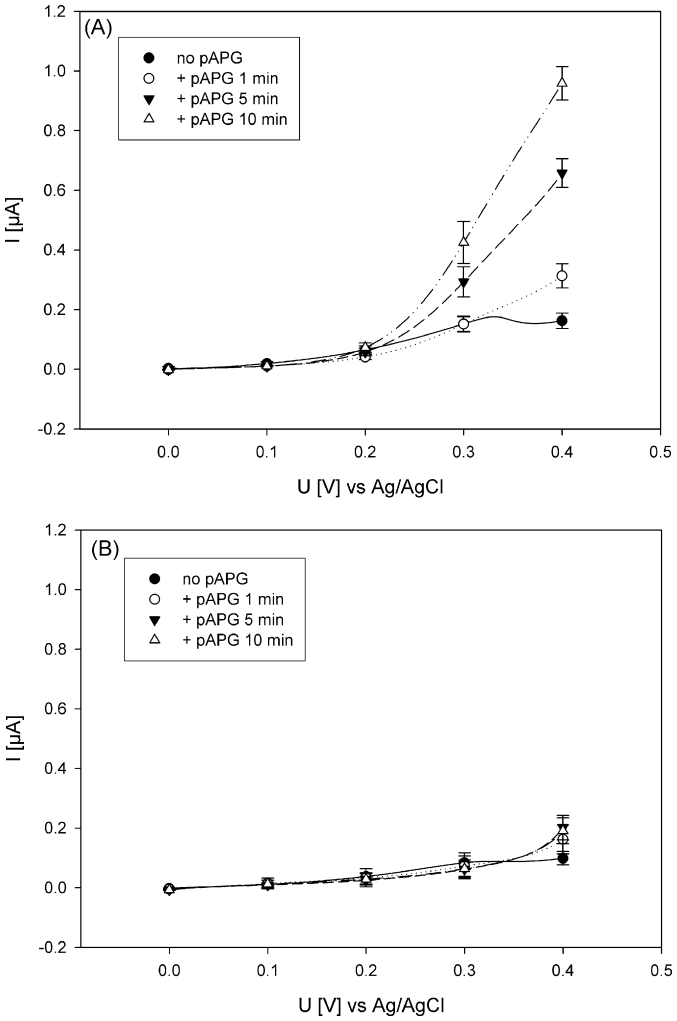

Fig. 3. Monitoring of pAPG-cleavage with linear sweep voltammetry. Bacterial strains TA1535 *pSK1002* (A) and as negative control TA1535 *sulA::phoA* (B) were exposed to 5 μM IQ with metabolic activation. Linear sweep voltammograms from 0 to 400 mV were measured in the absence of the β-galactosidase-substrate pAPG and after the addition of 6 mM pAPG (final concentration) and further incubation for 1 min, 5 min and 10 min as indicated.

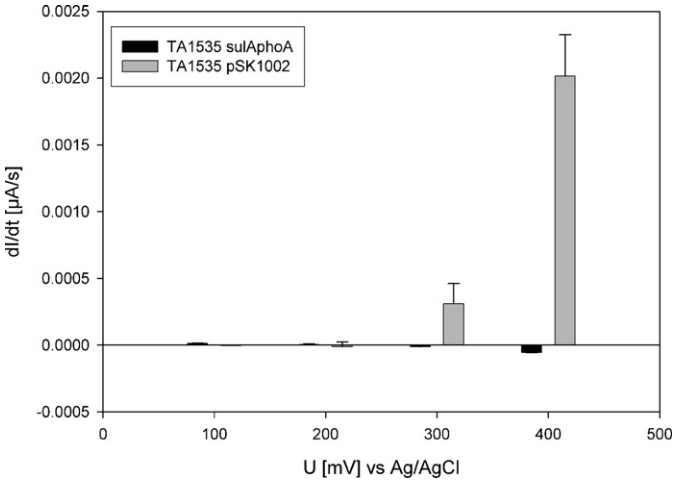

Fig. 4. Amperometric detection of β-galactosidase-activity. Bacterial strains TA1535 *pSK1002* and as negative control TA1535 *sulA::phoA* were exposed to 5 μM IQ with metabolic activation. Samples were placed on electrodes and incubated for 15 min after the addition of pAPG. The current was monitored at 100, 200, 300 and 400 mV vs. Ag/AgCl over ~5 min.

pSK1002 or TA1535 *pBR2TTSsulA::phoA*. The results are shown in Fig. 4. For all conditions, the slope dI/dt was ~0 μA s^{-1} at 100 mV and 200 mV vs. Ag/AgCl. In case of the reporter strain TA1535 *pSK1002*, a positive slope dI/dt was calculated at 300 and 400 mV indicating a constant formation of pAP (the educt for the oxidation reaction) that exceeds the rate of consumption by the electrochemical reaction. In contrast near-zero or even small negative slopes were calculated in case of the strain TA1535 *pBR2TTSsulA:phoA*, which indicates that pAPG is not cleaved by unspecific reactions and furthermore, that no interfering electro-active species are generated by the supplemented compounds for the metabolic activation of xenobiotics.

3.4. Comparison of colorimetric and electrochemical signal detection

To evaluate the potential of electrochemical signal detection to become a complementary method to assays based on colorimetric reactions for the analysis of genotoxic compounds in environmental samples, the effects of SOS-inducing chemicals were determined with both detection methods. *S. typhimurium* TA1535 *pSK1002* was exposed to IQ in several concentrations (5 μM, 2.5 μM, 1.25 μM, 0.62 μM, 0.31 μM and 0.16 μM). Subsequently, the exposed bacteria were split and analyzed either colorimetrically or electrochemically. Separate experiments were performed for measurements at 300 and 400 mV. In Fig. 5 the SOS-induction factors that were calculated using data of the colorimetric signal detection were plotted against the respective SOS-induction factors derived from the electrochemical measurements (Fig. 5A at 300 mV, Fig. 5B at 400 mV). The parameters of a linear regression are shown in Table 1.

For both potentials used for the chrono-amperometric signal detection, the calculated SOS-induction factors show a high correlation with the respective values derived from the colorimetric measurements. This indicates a virtually similar dose–response relationship that is independent from the signal detection method. Thus, in principle electrochemical signal detection is suitable for the characterization of dose–response relationships of genotoxic compounds using SOS-inducible systems. To estimate the noise levels of the different methods, six negative control samples were measured in parallel. The mean of the resulting data was normalized to 1 for each method, i.e. $(\Sigma dI/dt)/n = 1$ for the electrochemical detection or $(\Sigma E_{414})/n = 1$ in case of the colorimetric signal detection. The standard deviation and the resulting standard error are shown in Fig. 6. The standard deviation within the set of negative controls was 0.051 for the colorimetric detection and virtually identical for the electrochemical signal detection at 400 mV (0.061) but substantially higher for the detection at 300 mV (0.13). The limits of detection (LOD) and limits of quantification (LOQ) in terms of SOS-induction that were calculated based on these parameters are listed in Table 2 [36]. In the present context these parameters denote the detection of an effect and not the detection of a compound.

The determined LOQ of the SOS-induction that is 1.51 in case of the colorimetric detection is consistent with the threshold that

Table 1
Parameter for linear regression SOS-induction factors electrochemically vs. colorimetric.

Regression with $y = ax + b$	300 mV		400 mV	
Parameter	Value	Standard error	Value	Standard error
a	0.610	0.053	0.740	0.031
b	0.350	0.053	0.250	0.060
r^2	0.9990		0.9997	

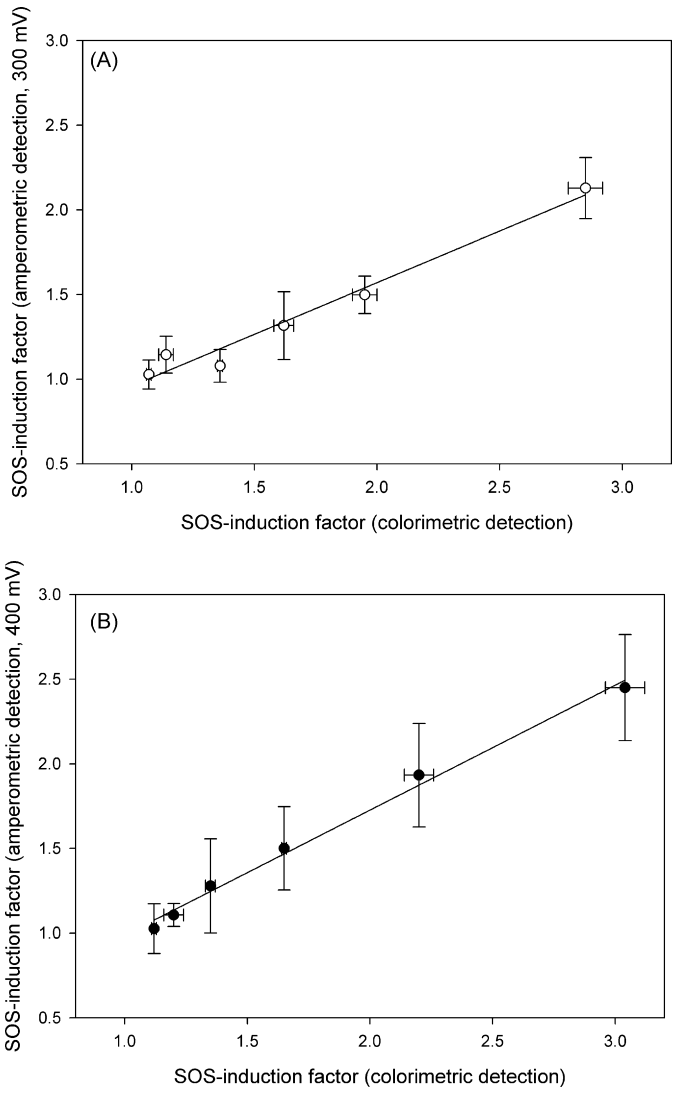

Fig. 5. Correlation between colorimetric and amperometric signal detection. *S. typhimurium* TA1535 *pSK1002* was exposed to several concentrations of the pre-genotoxic compound IQ (5 μM, 2.5 μM, 1.25 μM, 0.62 μM, 0.31 μM and 0.16 μM) with metabolic activation. The activity of the reporter enzyme β-galactosidase was measured colorimetrically by making use of oNPG or amperometrically at potentials of (A) 300 mV or (B) 400 mV by making use of pAPG. The determined SOS-induction factors for each concentration level are plotted against each other. The correlation was determined by linear regression.

is defined in the ISO-standard for the determination of the lowest dilution level of a sample that shows no genotoxic effect (SOS-induction factor <1.5). In case of the electrochemical signal detection at 300 mV the respective LOQ is 2.3 and does not fulfil the demands for a sensitive detection of genotoxic effects. In contrast, detection at 400 mV has a noise level that is identical to the colorimetric signal detection. Thus, the amperometric detection at 400 mV meets the quality standard that is defined in the respective ISO norm. Nevertheless, if environmental samples are to be analyzed, the possible presence of other electro-active compounds like dissolved minerals and salts has to be considered. The artificial amperometric background caused by the presence of electro-active compounds is comparable, in principle, to coloured or turbid samples in classic colorimetric assays. For this reason, a second cultivation step after the exposure of the bacteria was implemented in the ISO-standard, resulting in a further dilution of the sample and consequently a reduction of the background absorption. Such a dilution step would not be feasible for a biosensor device but there are other strategies for the assessment or removal of electrochemical background as follows:

Table 2
Limits of detection (LOD) and limits of quantification (LOQ) in terms of SOS-induction factors for the different detection methods.

	LOD	LOQ
Colorimetric	1.153	1.51
Electrochemical (400 mV)	1.183	1.61
Electrochemical (300 mV)	1.39	2.3

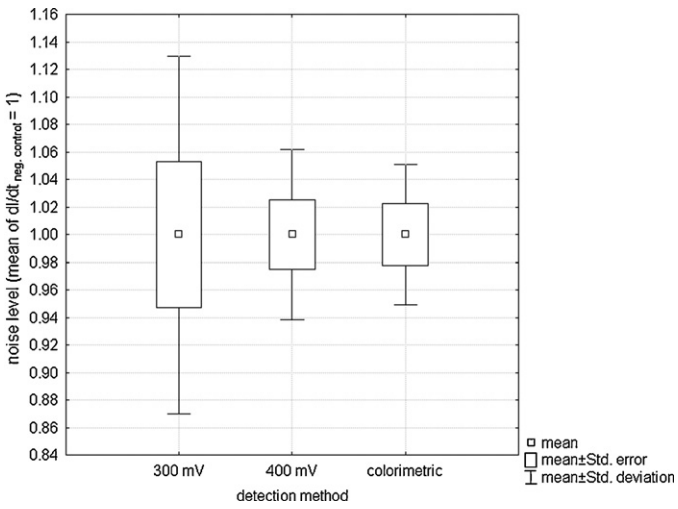

Fig. 6. Noise-level of the detection methods. The mean of six negative controls was normalized to 1 ($dI/dt = 1$ for amperometric detections or E_{414}-blank = 1 in case of colorimetric detection). The box-plot shows the standard error and the standard deviation of the group of negative controls for each detection method.

1. Only reductions with apparent potentials that are above or in turn oxidations with potentials that are below the applied potential of the working electrode can occur, i.e. the reduction of Na^+ (standard reduction potential of −2710 mV) or the oxidation of Cl^- (standard reduction potential 1360 mV) is not interfering with the amperometric signal detection. In this respect the presence of e.g. Fe^{3+} can result in the detection of an artificial background current because the reduction potential of the reaction Fe^{3+} to Fe^{2+} lies above the potential of the working electrode.
2. As shown in Fig. 2, no oxidation of pAP occurs at potentials <200 mV against Ag/AgCl. Therefore, it is possible to remove e.g. adjunct reducible compounds with electrochemical potentials above 200 mV in a pre-treatment step before the measurement is started at potentials ≥300 mV. This step will result in a decrease of the background signal.

The authors are aware that the discussed aspects of the possible presence of electro-active compounds are not exhaustive because the apparent electrochemical potential of such compounds depends upon other factors (e.g. concentration of ions, pH). However, for each sample the background current, which might be caused by the presence of adjunct electro-active compounds, will be characterized by an amperometric measurement in the absence of the reporter gene expression. A correlation of the sample measurement with its respective background measurement will allow the separation of the increasing electrochemical signal that is generated by the expression of the reporter gene from the background signal that is continuously decreasing due to the consumption of adjunct electro-active compounds. By a thorough evaluation of environmental samples, criteria will have to be defined (e.g. tolerable background current) for an assessment of the test validity.

4. Conclusion

Chrono-amperometry which is attractive as a signal detection method to be implemented in biosensors was compared with a standardized colorimetric assay for the detection of genotoxic samples by reporter gene induction (*lacZ*) via the bacterial SOS-system. This was done to evaluate the potential of the electrochemical method to be used in the assessment of environmental samples. The amperometric method using screen printed electrodes was optimized in terms of substrate concentration for the reporter gene β-galactosidase that cleaves p-aminophenyl-β-D-galactopyranoside (pAPG) to p-aminophenol (pAP) which in turn is oxidized to p-iminoquinone at the electrode. It was found that a final concentration of 6 mM pAPG is suitable to guarantee its cleavage by pseudo zero-order kinetics even if the reporter enzyme is strongly induced. By means of linear sweep voltammetry it was shown that a potential range of 300–400 mV is most suitable for the detection of pAP in a potential whole cell-based biosensor even in the presence of a large excess of pAPG. Furthermore, it could be proved, that the potentially electro-active species of the system for metabolic activation of pre-genotoxic compounds do not interfere with the amperometric signal detection of pAP between 300 and 400 mV and that the increase in current with time is caused solely by the specific cleavage of pAPG by the β-galactosidase. A comparison of the colorimetric and electrochemical detection methods shows a high correlation of the determined SOS-induction factors indicating the usability of the amperometric signal detection in principle. But the noise level of the electrochemical detection at 300 mV is substantially increased compared to the colorimetric assay limiting its potential for the assessment of environmental samples because of a decrease in sensitivity. In contrast, the noise level of the amperometric detection of pAP at 400 mV is very similar to the colorimetric standard method. Admittedly, the chrono-amperometric signal detection method has to be evaluated further with a broader spectrum of genotoxic compounds and later on with real environmental samples with high complexity (e.g. presence of metal ions). But the present study is the first that evaluates electrochemical signal detection against a standardized method that is used routinely in the assessment of environmental samples. The envisaged biosensor will contain bacterial reporter strains and, if needed, all necessary compounds for the metabolic activation of xenobiotics (S9-fraction and cofactors), lyophilized on top of the electrode [37,38] in a small reaction chamber. The freeze-dried biological compounds will be dissolved by the sample, followed by the eventual induction of the SOS-response. The electrodes with the biological compounds are mounted on a measuring device [39] and will be exchanged after the measurement.

Taken together, electrochemical detection of β-galactosidase-activity by the determination of the slope dI/dt is shown to be specific, even in the presence of other electro-active species (S9-mix) and, in case of the detection at 400 mV, sensitive enough in order to be utilized for the detection of genotoxins via the bacterial SOS-induction. By this means it is a complementary method to colorimetric assays with the potential to be implemented in a biosensor.

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