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Short communication

The use of single walled carbon nanotubes dispersed in a chitosan matrix for preparation of a galactose biosensor

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Abstract

Chitosan was chosen as a natural polymer for dispersion of single walled carbon nanotubes (SWNT) based on its ability to efficiently solubilize SWNTs to form a stable dispersion. Moreover, chitosan films deposited on a surface of a glassy carbon (GC) electrode are mechanically stable. Further stabilisation of the chitosan film containing SWNT (CHIT–SWNT) was done by chemical crosslinking with glutaraldehyde and free aldehyde groups produced a substrate used for covalent immobilisation of galactose oxidase (GalOD). Different galactose biosensor configurations were tested with optimisation of composition of inner and outer membrane; and enzyme immobilisation procedure, as well. Detection of oxygen uptake by GalOD on CHIT–SWNT layer at −400 mV is robust and, when flow injection analysis (FIA) was applied for assays, a low detection limit (25 μM) and very high assay throughput rate (150 h^{-1}) was achieved. This new galactose biosensor offers highly reliable detection of galactose with R.S.D. well below 2% and it has been successfully applied to assaying galactose in a blood sample with recovery index between 101.2 and 102.7%.

Keywords: Carbon nanotube; Nafion; Chitosan; Galactose oxidase; Interferents; Blood plasma

1. Introduction

Carbon nanotubes have been recognized as one of the most promising electrode materials in the field of electroanalysis because many electroactive species can be detected at lower overvoltage compared to ordinary electrodes resulting in increased selectivity of detection in a complex matrix (Davis et al., 2003; Katz and Willner, 2004; Gooding, 2005; Wang, 2005; Wildgoose et al., 2006). However, unmodified carbon nanotubes are extremely hydrophobic and assemble into bundles and ropes of individual nanotubes. For applications in electrochemistry, it is often necessary to make them soluble. Different approaches to solubilizing carbon nanotubes have appeared in literature, but frequently dimethylformamide (Gooding et al., 2003; Landi et al., 2004; Tsai et al., 2004; Kim et al., 2005), Nafion (Wang et al., 2003; Lin et al., 2005; Tsai et al., 2005; Viswanathan et al., 2006) and recently chitosan (Zhang and Gorski, 2005a,b; Jiang et al., 2005; Luo et al., 2005; Kandimalla and Ju, 2006) have been used for dispersion.

We have previously shown that the choice of dispersing agent is an important factor in the preparation of high performance biosensors exhibiting high detection sensitivity (Tkac and Ruzgas, 2006). Chitosan fulfils many of the requirements for preparing a robust, electrochemically active film composed of nanotubes (Tkac and Ruzgas, 2006). Moreover, chitosan is a natural polyelectrolyte containing free amino groups ($pK_a \approx 6.5$) and is suitable for the convenient preparation of membranes and films (Krajewska, 2004; Yi et al., 2005; Kumar et al., 2004). Due to its biocompatibility, chitosan has been extensively used for the immobilisation of biomolecules (Krajewska, 2004).

There are many strategies for designing an immobilised-oxidase biosensor based on carbon nanotube films. Most commonly, the release of hydrogen peroxide or a consumption of oxygen is monitored during oxidase turnover (Tsai et al., 2005; Lim et al., 2005). Alternatively, the use of artificial electron acceptors may permit higher sensitivity of detection

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and eliminate the dependence on oxygen (Guan et al., 2005; Joshi et al., 2005). Detection of hydrogen peroxide can be improved by co-immobilisation of horseradish peroxidase (Ruzgas et al., 1996; Gorton et al., 1999).

Galactose oxidase (GalOD) has been a subject of extensive fundamental research (Shleev et al., 2005; Whittaker, 2003) and has been successfully used for preparation of a variety of biosensors (Tkac et al., 2000, 2001; Manowitz et al., 1995). The aim of the present study was to optimise the performance of a GalOD biosensor based on nanotube films by using different immobilisation protocols and biosensor configurations. The objective was to develop a biosensor capable of detecting very low galactose concentrations with high selectivity in complex biological mixtures. We report here for the first time an extremely robust, sensitive and selective galactose biosensor suitable for high throughput detection. The biosensor is based on GalOD immobilised on a CHIT–SWNT modified electrode with detection of oxygen depletion upon action of the enzyme. The biosensor has been successfully applied in the analysis of galactose in a blood plasma.

2. Experimental procedures

2.1. Reagents and material

Single walled carbon nanotubes ($d = 1.1$ nm, $L = 0.5$–100 μm, >90% purity) were obtained from Fluka. Nafion (20% solution in low molecular weight alcohols) was purchased from Aldrich. Chitosan (degree of deacetylation of 85%) was provided from Sigma. A dialysis membrane (cut-off 6–8 kDa) was purchased from Serva. Recombinant galactose oxidase expressed in a *Pichia pastoris* was prepared according to published procedure (Whittaker and Whittaker, 2000). The specific activity of enzyme of 500 U mg^{-1} (20 g l^{-1}) was detected by oxygen electrode in the presence of 2 mM ferricyanide. All other chemicals used were of high purity and were used without any purification/pretreatment. All aqueous solutions were prepared in highly pure water (Millipore system). Glassy carbon (GC) disk electrodes (CH Instruments, $d = 3$ mm) were used for casting of a dispersion of carbon nanotubes. For batch electrochemical measurements a BAS equipment (CV-50W, Bioanalytical systems, West Lafayette, IN, USA) was used with GC as a working electrode, saturated calomel electrode (SCE) as a reference electrode and platinum plate as a counter electrode. In flow injection experiments an Ag/AgCl electrode was used as reference one.

2.2. Dispersion of SWNT and electrode modification

A dispersion of SWNT in chitosan (CHIT–SWNT) was prepared by sonication of 1 mg of SWNT in 1 ml of a 0.1% chitosan in 1% acetic acid. Sonication was performed in an ultrasonication bath (Branson 5510, Branson Ultrasonic Corp., CT, USA) for 170 min (if not specified otherwise). GC electrodes were polished using 1 and 0.1 μm alumina/diamond slurry (Struers A/S) and sonicated in distilled water for 3 min. In all cases, GC electrode was modified by casting of 5 μl of dispersion on a polished GC electrode and allowed to dry at ambient temperature. To optimise the performance of the biosensor the CHIT–SWNT layer was further modified by application of the following films/layers in different order (e.g. CHIT–SWNT/NAF(E)/GalOD/NAF(P)): 10 μl of 0.5% Nafion in 50 mM phosphate buffer pH 7.4, NAF(P); 10 μl of 0.5% Nafion in absolute ethanol, NAF(E); 10 μl of 0.1% chitosan in 1% acetic acid, CHIT; a dialysis membrane; 5 μl of a 10 g l^{-1} GalOD solution in 20 mM phosphate buffer pH 7.4, GalOD. CHIT–SWNT layer was in some cases stabilised by dipping of the electrode into 2.5% glutaraldehyde solution for 5 min and extensively washed by distilled water. The films/layers were cast on the electrode in a liquid form and left to dry before application of a next layer. In some cases, a GalOD solution stored in the fridge for couple of days was used with lower specific activity during optimisation steps, but the final characterisation of the biosensor and assay of a plasma sample were done with a fresh portion of a GalOD solution.

2.3. Flow injection experiments

In case of flow injection analysis, the electrodes were mounted into a flow-through amperometric cell of wall-jet type (Appelqvist et al., 1985) containing the modified GC electrode, a platinum wire counter electrode and an Ag|AgCl (0.1 M KCl) reference electrode. Samples were injected with an injector (type 7125 LabPRO, Rheodyne, Cotati, CA, USA) supplied with an injection loop of 50 μl. A 50 mM phosphate buffer pH 7.4 containing 0.1 M KCl was pumped at a flow rate of 0.55 ml min^{-1} (Minipuls 2, Gilson, Villier-le Bel, France), if not mentioned otherwise. Connections between the various parts were made with Teflon tubing; i.d. 0.5 mm and Altex screw couplings. The potential of the working electrode versus the reference electrode was kept at the required value using a potentiostat (Zäta Electronics, Lund, Sweden) the current registered with a recorder (model BD 112, Kipp & Zonen, Delft, The Netherlands).

3. Results and discussion

3.1. The effect of sonication time

Preliminary work has shown that the dispersion of long SWNT in chitosan matrix is more efficient and faster compared to Nafion and dimethylformamide dispersions (Tkac and Ruzgas, 2006). Sonication times up to 120 min were previously tested and longer sonication times (up to 300 min) were examined in the present study. The sensitivity towards galactose increased 2.6-fold as the sonication time increased from 60 to 170 min. However, further sonication time had a negative influence on the sensitivity of the biosensor (data not shown). The same effect was observed for Nafion dispersion and most likely reflects a decrease in sensitivity as a result of polymer degradation upon prolonged sonication (Tkac and Ruzgas, 2006). Moreover, the electrode noise increased with longer sonication time and for further study a sonication time of 170 min was used for preparation of dispersion of SWNT in chitosan.

Table 1
Comparison of different overcoatings (e.g. Nafion, chitosan or a dialysis membrane) on the performance of a GalOD biosensor

	Chitosan film	Nafion film	Dialysis membrane
Sensitivity (nA mM^{-1})	128	429	272
Detection limit (μM)	611	49	44

CHIT–SWNT modified GC electrode was modified by a GalOD layer and on the top of the biorecognition layer films were applied. Batch measurement was run at −400 mV in 50 mM phosphate buffer pH 7.4 containing 0.1 M KCl.

3.2. Optimisation of a biosensor composition

In order to get the highest sensitivity towards galactose, several biosensor configurations were tested. First, the effect of the type of an outer film/layer deposited on the dry CHIT–SWNT/GalOD film was investigated. Three different kinds of membranes were tested including NAF(P), CHIT and a dialysis membrane. Covering the CHIT–SWNT/GalOD layer with a NAF(P) film afforded a detection limit of 49 μM and the highest sensitivity (429 nA mM^{-1}) for the galactose biosensor (Table 1). It was also found a NAF(P) coating has a positive effect on enhanced selectivity of the biosensor towards galactose in presence of interfering compounds (ascorbate, AA, urate, UA and acetaminophen, AAP).

Second, the inner layer of the biosensor was optimised, while using a NAF(P) film outer layer. NAF(P) and CHIT used as an inner layer deposited on CHIT–SWNT film had a negative effect on the performance of the biosensor. The selectivity ratio galactose/AA of the biosensor with an inner layer decreased 20 times compared to the configuration without the inner film (CHIT–SWNT/GalOD/NAF(P)). When NAF(E) was used as an inner layer the selectivity of the biosensor remain unchanged, but a decreased sensitivity of the biosensor towards galactose was observed compared to the unmodified configuration. Thus, for further work a simple configuration of the biosensor without inner layer was used, e.g. CHIT–SWNT/GalOD/NAF(P).

When the GC electrode modified by CHIT–SWNT matrix was dipped into 2.5% glutaraldehyde for 5 min, a higher response towards galactose was observed (900 nA mM^{-1}) compared to the biosensor without further stabilisation (744 nA mM^{-1}). Thus for further optimisation a biosensor with a glutaraldehyde stabilised layer of a CHIT–SWNT layer was used. The use of NAF(E) as an outer layer instead of NAF(P) further decreased interference of ascorbate by 30%, but also decreased sensitivity of galactose detection by 40% and the use of NAF(E) as an outer layer was excluded.

3.3. Optimisation of flow rate and applied potential

To achieve the highest performance of detection it is important to optimise the effect of working potential on both the sensitivity of detection and on the noise level. Lowering the working potential from −200 to −400 mV dramatically increased the sensitivity towards galactose (28-fold) (Fig. 1). The influence of working potential on the noise level and thus on detection limit had a parabolic dependence (Fig. 1). For assays of real sample a working potential of −400 mV was used because at this potential high selectivity of galactose detection in presence of ascorbate was found (see below).

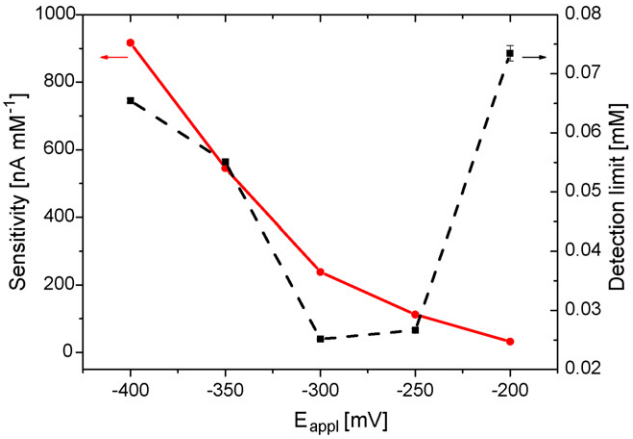

Fig. 1. The influence of applied working potential on the sensitivity of the galactose biosensor and on the detection limit of the biosensor. During the measurement the flow rate was set to 0.55 ml min^{-1} with 0.5 mM galactose injected into the buffer stream containing 50 mM phosphate buffer pH 7.4 with 0.1 M KCl.

Flow rate has a tremendous effect on the sensitivity of detection and sample throughput (Fig. 2). The time needed to attain the same baseline after galactose addition as it was before galactose injection is considered to be a response time at given flow rate. The response time was then inverted and expressed in samples/standards, which can be measured per hour at given flow rate. In order to perform measurements at high speed (>150 injections per hour) and reasonable sensitivity a flow rate of 0.55 ml min^{-1} was used for further work.

3.4. Elimination of interferences

An application of outer NAF(P) membrane led to interference-free detection of galactose in the presence of UA and AAP at applied potential of −200 mV, but ascorbate still interfered with detection of galactose (Fig. 3). By lowering of

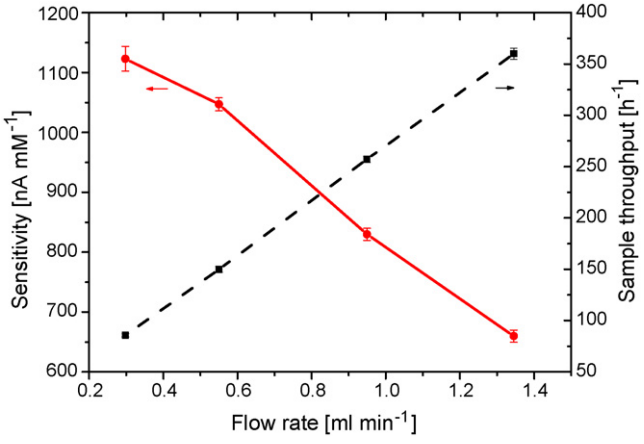

Fig. 2. The effect of a flow rate on the response of the biosensor towards 0.5 mM galactose solution and on the sample throughput in a flow injection mode. Working potential of −400 mV was applied.

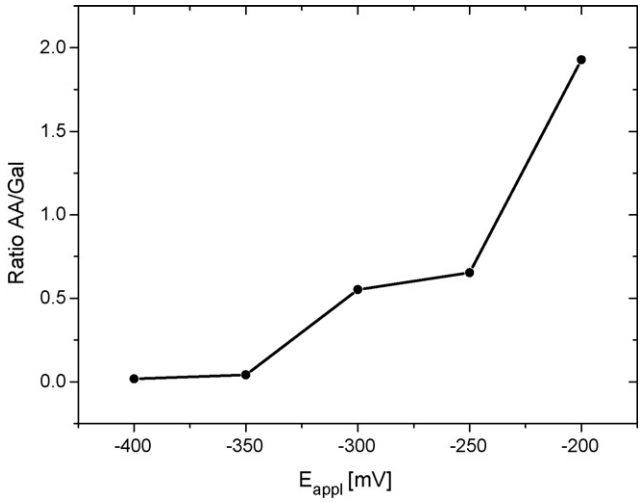

Fig. 3. The influence of applied working potential on the selectivity of galactose detection by the biosensor. Ascorbic acid (AA) and galactose (Gal) were injected at concentration of 0.1 mM (a physiological concentration of ascorbic acid). Current obtained by injection of AA was divided by current obtained by injection of Gal. During the measurement the flow rate was set to 0.55 ml min^{-1}.

working potential from −200 to −400 mV, an AA/galactose interference ratio decreased 104 times from 1.93 to 0.02 and the biosensor was virtually insensitive towards interferents commonly present in a blood plasma at a working potential of −400 mV (Fig. 3).

Carbon nanotube layer is an effective matrix for reductive detection of both oxygen and hydrogen peroxide, but sensitivity towards oxygen is higher at applied potential of −400 mV, what practically means depletion of oxygen in the biorecognition layer upon oxidation of galactose by GalOD is measured. The current is then proportional to galactose concentration in the standard/sample.

3.5. Calibration curve and stability of the biosensor

All of optimised values were applied for characterisation of linear and dynamic range of the biosensor. The detection limit of the biosensor is 25 μM (S/N = 3) with linear range up to 1 mM galactose ($R^2 = 0.9999$) with a sensitivity of 1126 nA mM^{-1}. Galactose can be detected up to 20 mM, but at higher substrate concentration inhibition of the enzyme was observed. The sensitivity of the biosensor is stable (∼1000 nA mM^{-1}) with a decrease to 98% of initial sensitivity after continuous use for 2.5 h even in the presence of a blood sample (dilution factor 1 + 3). The sensitivity dramatically fell down to approximately 600 nA mM^{-1} after exposure to galactose concentration of 20 mM and then it stayed stable again. During blood plasma assay, 6.8 times higher flow rate was applied to wash the system and the sensitivity of the biosensor remained stable, which underscores the high mechanical stability of the enzyme-nanotube film. It is worth noting that the glutaraldehyde stabilised SWNT–CHIT film was so adherent to the GC electrode, that prolonged mechanical polishing (4–6 min) using an alumina slurry was required to completely remove it. The biosensor described here offers a highly reliable output signal with R.S.D. in range from 0.3 to 3.7% (average R.S.D. of 1.4%).

Table 2
Analysis of blood plasma for the content of galactose

Added (mM)	Found (mM)	R.S.D. (%)	Recovery index (%)
0	–	–	–
0.33	0.34	1.1	101.8
0.66	0.68	1.9	102.7
1	1.01	1.6	101.2

3.6. Real sample analysis

Galactose is a clinically important sugar that is usually not detectable in body fluids. A physiological concentration of up to 0.28 mM galactose is considered normal, whereas higher concentrations resulting from disturbed galactose metabolism are associated with cataract formation in adults. In neonates (less than 5 days old) with undiagnosed galactosemia, death can occur (Manowitz et al., 1995). Recently, it was found higher galactose levels in neonates can inteterfere with detection of glucose by hand-held glucose meter, calling for a simple and robust method of galactose detection (Newman et al., 2002). Thus, it is of great importance to detect non-physiological concentration of galactose in blood. In our study a blood plasma diluted (1 + 3) in a running buffer was used for detection of galactose. The initial concentration of galactose in the sample, representing the physiological concentration of galactose in the blood, was below the detection limit of the prepared biosensor (25 μM). When the blood was spiked with a known concentration of galactose almost ideal recovery index (101.2–102.7%, Table 2) was observed, indicating the reliability of the biosensor.

4. Conclusion

This work demonstrates that it is possible to construct a robust and selective galactose biosensor by careful optimisation of biosensor composition and physicochemical parameters. The use of dispersed carbon nanotubes as a transducer allows very rapid detection of galactose with sample throughput of 150 h^{-1} while retaining high selectivity and sensitivity. Moreover, the biosensor is mechanically robust, reliable and has been successfully used for detection of galactose in a blood plasma.

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