Modular biosensor reaction-cell

Hunor Sántha / *Attila* Bonyár

Abstract

An approach to simultaneously monitor the processes of a biosensor transducer surface and the bulk of the sample solution is presented. A novel PDMS (Poly-DiMethyl-Syloxane) based modular biosensor reaction-cell is described, which is suitable for the parallelised electrochemical monitoring of biosensor electrode arrays by means of its dual connector array and for optical spectroscopic monitoring of the bulk of the surrounding sample solution by means of micro transmission dip probes insertable inside the reaction cell.

An experimental setup with 280 and 520 mm^3 reaction-cell volumes was tested and characterized. 2×13 individually addressable electrode connectors were easily realisable in order to meet the requirements of electrochemical functionalities.

For the optical spectroscopic capabilities 0.13 O.D. (Optical Density unit), corresponding to 0.68 pmol/μl concentration of the DNA (Deoxyribo Nucleic Acid) used was found to be the minimum, and 3.43 O.D., corresponding to 20.59 pmol/μl concentration of the DNA used was found to be the maximum of the absorbance range which can be monitored with ca. 5% standard deviation using a certain parameter setting during a certain measurement.

Keywords

electrically active biosensors · biomolecule manipulation · in situ spectroscopy.

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1 Introduction

The first attempts to create a general but still accurate definition of what a biosensor have happened in the early '80s. The most accepted description considers an analytical device as a biosensor device, if it converts the concentration of a target substance, called hereinafter the analyte, into an electrical signal through the combination of a biological or biologically derived analyte recognition system either integrated within or intimately associated with a suitable physico-chemical transducer [1]. The scheme of general structure of biosensor devices and their operation principle is shown in Fig. 1.
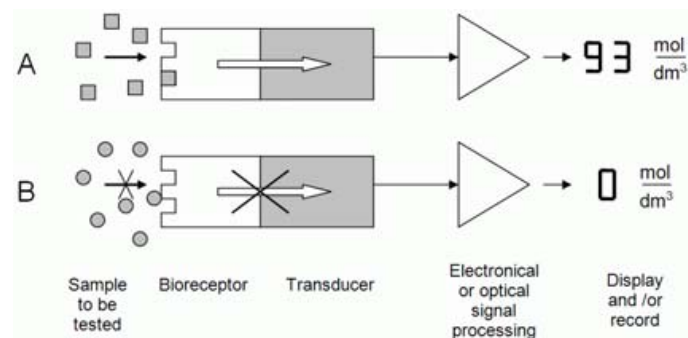

Fig. 1. General structure of biosensor devices and their operation

1.1 RTD Areas of Biosensors

RTD work related to the structure of biosensor devices can focus on 3 areas according to the scheme above:

1 To find or realise new/improved analyte recognition systems (bioreceptors). Basic disciplines for such works are biology and chemistry. Molecular biology, genomics, protein engineering and microbiology are examples for specialized sciences dealing with this field.

2 To find or realise new/improved physico-chemical transducer methods/structures. Materials science, electronics and optics are the main sciences here to build on.

3 To find or realise new/improved methods for the integration of an appropriate analyte recognition system with a certain

physico-chemical transducer. Generally this activity is referred to as immobilisation, since the analyte recognition system has to be prevented from being washed off from the transducer structure during a measurement in the liquid phase. Such issues need knowledge mainly in organic chemistry, biochemistry, materials science and technology.

Regarding the functionality of biosensors a further RTD field exists, namely, to improve/validate operation processes and operation circumstances of biosensors. In this topic both theoretical and experimental researches have room to improve the state of the art, since biosensor based devices are still considered as non-robust, short-lifetime devices with moderate reproducibility and long time-to-result compared to traditional "bio-less" electronic sensors like e.g., a capacitive pressure sensor or a thermistor based temperature sensor [2]. In exchange, biosensors provide unique selectivity and sensitivity which may ensure one-shot testing instead of a lengthy laboratory based analytical method. However the present situation in biosensor research is that, on the one hand, hundreds of biochemical recognition systems and tens of different transducers are available, while on the other hand, there are only a very limited number of biosensors commercially available.[3]

1.2 Mass Transfer Related Limitations of Biosensors

Although many advanced technologies enabling significant miniaturisation were key driving forces in the development of the most advanced biosensor devices of today all current microarray platforms still suffer from severe mass transfer limitations. Rates of analyte transport to the biosensing surface significantly lag behind rates of analyte binding in the case of affinity biosensors and rate of analyte binding + catalysis in the case of biocatalytic sensors. For instance, typical time-to-result of DNA microarrays is 2–8 hours, which is far away from the ideal values demanded by an end-user. Electrochemical immunobiosensors published in the literature need typically 30–120 minutes incubation time for optimum results. Thus, speed of operation of affinity biosensor based devices may be significantly improved yet by improving mass transfer during the operation. The biocatalytic sensor devices may benefit from higher sensitivity if mass transfer can be improved.

To achieve this goal three approaches are straightforward.

1 Minimising diffusion distances of the analyte from the sample to the biosensing surface by miniaturisation of the physical structure.

2 Active mixing/stirring, practically speaking, exploitation of convection.

3 Electrophoretic/dielectrophoretic manipulation of the analyte towards the biosensing surface.

All these three issues have to be considered and addressed during the conceptualisation phase of any biosensor device if one intends to develop a biosensor device superior to the state of the art. But the general design rules of biosensor devices are far from established yet, not to mention the lack of "design-for-optimum-operation" methods. To predict mass transfer in a certain biosensor reaction-cell setup diffusion coefficients of molecules has to be considered as a first step. However, diffusion coefficients for DNA oligomers or other biomolecules/analytes are typically not easily available for aqueous phase in the literature, because lateral diffusion experiments are the best sources of such data but these are performed in agar-agar or other gel matrices. Some authors have used estimated values for their calculations, e.g. 15 000 $\mu m^2/Vs$ for electrophoretic mobility and 20 $\mu m^2/s$ for diffusion constants of a 20 base ssDNA (single stranded DNA) oligo [4]. The most useable equation of the literature we were able to find is

$$D_{aq} = 4.9 \times 10^{-6} \times [bp]^{-0.72} (cm^2/s) [5] \qquad (1)$$

where $[bp]$ is the number of base pairs of the actual DNA oligonucleotide molecule. For other biomolecules one can rely on a rough statement generally accepted in the literature: $D_{aq} \approx 10^{-7} - 10^{-6}$ cm^2/s . Ions compared to this have $D_{aq} \approx 0.6 - 2.0 \times 10^{-5}$ cm^2/s. [6] When considerations of electrophoretic/dielectrophoretic impacts are addressed in certain cases the necessary data about electrophoretic mobility of molecules can be retrieved from CE (Capillary Electrophoresis) literature [7], however, the electric fields applied in CE are very high (500-1000 V/cm) compared to any application in a biosensor reaction-cell so biomacromolecules having in some cases sensitive three dimensional structure may behave different to some extent in CE experiments compared to biosensor reaction-cells deploying much weaker electric fields.

1.3 Experimental Works Focusing on Manipulation of Biomolecules

The aim of our work is experimentally oriented, thus, primarily our efforts are not addressing preparation of mass transfer simulations and the best practices for electrical biomolecule manipulation are even more relevant than values of diffusion coefficients and mobilities of biomolecules. In the case of a biosensor reaction-cell the applied DC voltage, if there is any, must be defined very carefully because unspecific oxidation currents of easily oxidisable components of the sample matrix may appear above few hundred mV DC which may hinder correct measuring. Additionally, electrolysis of water may come into account depending on the pH in the case of DC driver signals above 2.0-2.4 V [8]. For this reason AC driver signals and dielectrophoresis impacting on any polarisable particles and molecules can be more usable then electrophoresis which either way impacts only on charged particles and molecules. Of course for dielectrophoresis a spatially inhomogeneous electric field distribution, thus, certain supplementary electrodes have to be present inside the reaction-cell.

Regarding dielectrophoretic experiments, electrode and gap sizes published in the literature are typically in the tens of μm range and the applied driver signal may be 50 V$_{RMS}$ @ 50 Hz, or 1-3 MV/m @ 1 MHz [9], +/− 2 V DC with peaks of 0.030 V/μm compared to Ag/AgO quasi reference electrode [4], 8 V$_{pp}$ @ 100kHz-100 MHz [10], 2.5 V$_{pp}$ or 7.5 V$_{pp}$ AC in order to ensure 1 MV/m peaks [11] or 16 V$_{pp}$ AC [12]. Direct application of electric potential to speed up the operation of DNA biosensors, or to assist hybridisation and stringency control can be found in the literature [4, 13, 14] and in commercialised DNA microarray products as well [15], however, the information feedback is ensured in these cases either by the functional biosensor surface itself, thus, not in an additional independent way or by means of labelling of the molecules under investigation (e.g., fluorescent or radioisotope) [16].

1.4 Scope of this Paper

In order to support certain RTD works touching the improvement of operation of either an affinity biosensor or a biocatalytic sensor we concluded that a means to observe the processes in the bulk sample solution surrounding the biosensor transducer can add an independent complementary feedback during such experiments. In this work a novel concept has been considered, elaborated and characterised in order to provide biosensor researchers with a tool for the *in situ* monitoring of the bulk of the sample liquid surrounding the biosensor transducer under investigation. The initial expectations/preferences were the following:

1 adaptability to as many forms of transducers and as many biosensor fabrication technologies as possible

2 no need for labelling of the analyte molecules to be observed, because labels may influence the natural behaviour of biomolecules to different extent. E.g., fluorescent dye DNA labelling introduces minor nonspecific binding to gold (i.e., hydrophobic surface absorption) [16]

3 some sort of spatial resolution.

This paper presents our proposed solution and a prototype device in 2 slightly different versions. The testing and characterisation of all the targeted functionalities of the device and our concept are described as well.

2 Experimental
2.1 Materials
Poly-DiMethyl-Siloxane (PDMS)

The PDMS sidewall element was prepared by silicone casting. The raw material was Sylgard 184 silicone elastomer purchased from OMYA AG. Photos of the realised PDMS structures are shown in Fig. 7 and 8.

Deoxy-ribo-Nucleic Acid (DNA) oligomer

DNA oligonucleotides were purchased from Sigma Aldrich (Germany). A dilution line of 24 steps in the range of 330-0.5 μM was prepared by adding distilled water to the 17-mer oligonucleotid 5'-TTC CAA CTT TCG GAA CC-3'. Dilution and subsequent DNA absorbance characterization experiments shown in Figs. 14-16 were carried out in 500 μl Eppendorf tubes, while the described measurement platform and modular reaction-cell were used for electrophoretic and self-diffusion experiments presented in Figs. 13 and 17-20. In the biosensor reaction-cell a solution of 3 μM concentration was used freshly loaded before each measurement.

Avidin

Avidin was purchased from Aldrich (Germany). For the avidin absorbance characterization measurement presented in Fig. 21 solution of 152 μM avidin concentration was used in a 500 μl Eppendorf tube. Preparation of the dilution line and subsequent absorbance vs. concentration measurements presented in Fig. 21 were done in 500 μl Eppendorf tubes as well in one series.

FR4 plates, polystirene plates, glass substrates and other electronic parts

The FR4 (Flame-Retardant-4) glass fibre reinforced epoxy substrates were provided by our in-house PCB (Printed Circuit Board) manufacturing line. Glass sheets, polystyrene sheets of 2 cm thickness and electronic parts, eg. spring-loaded tip needles, wires, Peltier elements, etc. were off-the-shelf available items in a standard shop for electronics and mechanical parts.

2.2 Methods
Preparation of the electrodes

The gold thin film electrodes were produced in-house by vacuum evaporation using chromium adhesion layer and the layout was prepared by laser ablation by means of a frequency tripled Nd:YAG laser. Before experiments the electrodes were cleaned in two steps: 15 min cleaning in an ultrasonic bath while rinsed in 96 % ethanol then another 15 min cleaning in boiling 30 % H$_2$O$_2$. Cleaned electrodes were rinsed in water, dried under steam of filtered air then used instantly.

Electrophoresis and diffusion experiments

Different fast methods (application of tunable light source [17] or fast electronically tunable acoustooptic spectrophotometer [18]) can be used for the spectral investigations of the processes of a biosensor reaction-cell. Because of its simplicity and compact form the electrophoretic and diffusion experiments shown in Figs. 13 and 17-20 the DNA and avidin absorbance characterization measurements were carried out with an Avantes spectrometer, an AvaLight-DHS lightsource and optical fibre micro transmission dip probes with optical pathlengths of 1.0 and 0.5 cm. In all cases the deuterium lamp was used which covers the 215-400 nm wavelength range.

The peak of the DNA light absorbance spectra is at 260 nm so we usually monitored the 230-300 nm range. Before every experiment light and dark currents were measured in water with

200 ms integration time and the average of every 100 readings was saved as reference.

During the electrophoretic experiments the optical fibre micro dip probe connected to the Master channel of the detector was placed in the closest hole to the upper electrode surface which was biased to +1.9 V in its whole area for the first 30 minutes while the optical fibre micro dip probe connected to the Slave1 channel of the detector was placed from the opposite direction in the reaction-cell through the hole closest to the bottom electrode surface which was biased to 0 V (GND - ground) in its whole area for the first 30 minutes as presented in Fig. 13. Both micro dip probes were pushed until their physical gap reached exactly the centerline of the inner space of the electrochemical cell, as shown in Figs. 6 and 10. All the other 8 holes not occupied by the microprobes were sealed with small plastic plugs against leakage of liquid from the inside after filling up the chamber. After running the first 30 minutes of the electrophoretic experiments the potentials were changed to opposite for a next 30 minutes as shown in Fig. 18-20 or switched off in the case of the self diffusion experiments as shown in Fig. 17 without any change in the setup or longer delays.

The integration time of the detector array was set to 200 ms and the averages of every 25 readings were recorded in every 360 seconds. In the electrophoretic experiments at room temperature the average of five, on 37 ^oC the average of three and in the self diffusion experiments also the average of three performed measurements are shown with error bars of the standard deviation.

2.3 Equipment
Spectrophotometer

The optical spectroscopic measurements were performed by a PC controlled Avantes AvaSpec-2048-4-DT UV/VIS/NIR optical fibre probe spectrophotometer device, having 0.7 nm spectral resolution between 220-1100 nm and 4 identical channels. The 'Master' and the 'Slave1' channels have a spectral range of 200-720 nm, while the 'Slave3' and 'Slave4' channels have a range of 600-1100 nm. The micro transmission dip probes used have 1.4 mm outer diameter and an optical pathlength of 1 and 0.5 cm. In the electrophoretic measurements when we needed to use two probes simultaneously we also used a FC-IR400-2 line distributor.

Laser

To create 2.5 × 2.5 cm gold thin film electrodes laser ablation and a frequency tripled Nd:YAG laser were used. The wavelength of the laser was 355 nm, while the focus diameter was 30 μm. The frequency of the Q-switch was 30 kHz and we applied the maximal possible pumping current.

Voltage source

To bias the electrodes in the electrophoretic experiments a GW Instek GPS-4303 voltage source was used. The applied

voltage was 1.9 V against GND.

PDMS casting workstation

PDMS casting was used to create the resilient sidewalls of the modular biosensor reaction-cell. Raw PDMS was prepared by adding Sylgard 184 curing agent to Sylgard 184 silicone elastomer in 1:10 m/m value. Using a mold form glued together of FR-4 and glass parts the PDMS was casted in a homemade casting workstation consisting of a vacuum exsiccator, a water stream based vacuum pump and tubings. The vacuum exposition lasted for 10 min in conditions below 5 kPa vacuum until the air bubbles left the PDMS body.

Softwares

OrCAD Layout Plus 10.5, Solid Edge 15 and Inventor 2008 softwares were used for designing the holder platform and parts of the reaction-cell. Avantes Avasoft 6 was used for photometric experiments. MicroSoft Excel 2003 was used for data evaluation.

3 Results and Discussion

As a result of the above mentioned criteria and scopes, and based on our experiences and survey of the literature the typical construction of experimental setups deployed for biosensor development has been identified. Fig. 3 shows a drawing of the most widespread schematics of biosensor development platforms.

As the majority of biosensor experiments are performed in the liquid phase, the related liquid handling issues like bubble formation, good sealing, easy interchange of liquid content in the reaction-cell, etc., influence strongly the usability of an experimental setup. Based on the idea that a resilient construction instead of the typically rigid reaction-cell structure (typically made of PMMA – PolyMethyl MethAcrylate) could ease the solution of fluidic interconnections for inlet/outlet and additionally can ensure good sealing, a PDMS based modular biosensor reaction-cell concept has been elaborated. This concept is presented in Fig. 4. The O-ring is substituted with a sealing flange since the PDMS can be formed easily in such a way by means of an appropriate mold. The necessary fluidic interconnections can be solved with simply inserting metal tubes in the resilient PDMS wall(s) and deploying resilient tubes for outer piping.

The first innovation makes the assembling process easier and minimises the size of hidden recesses in the reaction-cell which property is advantageous for more homogeneous material distribution during fluidic inlet/outlet and against bubble forming. The second innovation reserves much more space free around the fluidic connectors than the traditional solutions, thus, more interconnections are possible on the same reaction-cell.

3.1 Construction Activities

For the actual requirements of a planned series of experiments in our lab and according to the available laboratory equip-

Fig. 2. Schematics of the internal space and electrical connections of the modular biosensor reaction-cell equipped with gold coated glass sheets on the bottom and upper side for electrophoresis experiments
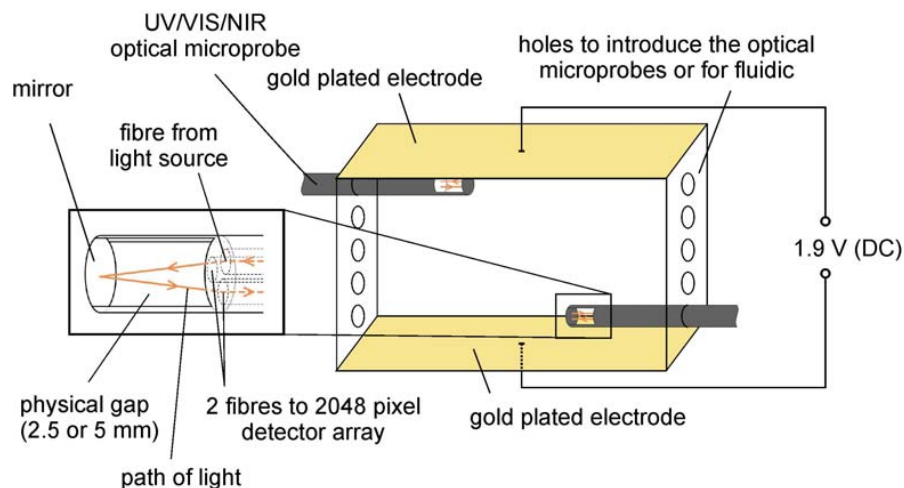
Fig. 3. The most widespread schematics of biosensor development platforms
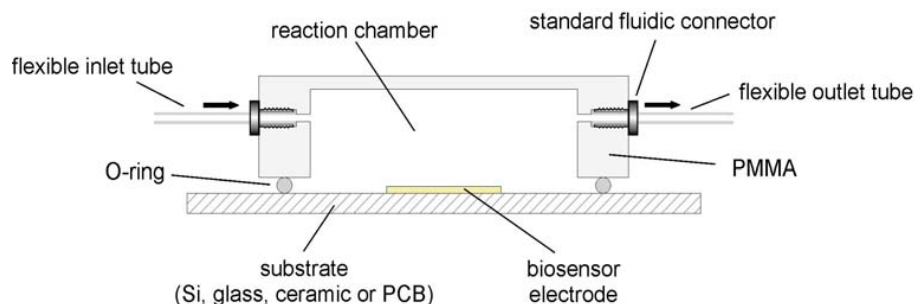
ment, a concrete design has been prepared. This is presented in Fig. 5. In the envisaged electrical biomolecule manipulation experiments, both the bottom and cover plate have to be capable to support biosensor electrodes or supplementary electrodes, thus, just a sidewall element was planned of PDMS, and the bottom and cover plates can be made of any material in the size of 25 x 25 mm^2 area and maximum thickness of 1 mm.

In Fig. 6 the inner space of this modular biosensor reaction-cell is shown. In the drawing we emphasize that theoretically any sort of biosensor arrays can be inserted in this platform within certain size limits, as presented in the bottom of the drawing.

According to the concept above, 2 prototypes of the PDMS sidewall element and a holder framework for easy assembling have been fabricated.

Figs. 7 and 8 present the photos of the prepared two versions of PDMS sidewall elements and a photo of the mold which was used for the preparation. In the second version sealing flanges were realized around the inner space of the reaction-cell for easier usage.

The custom design holder framework which presses together the parts of the modular cell and holds the optical micro dip probes is shown in Figs. 9 and 10.

As different temperature values may be a requirement during biomolecule manipulation experiments or even during any biosensor related experimental work, a thermal insulator box was designed and constructed around the holder framework.

Cooling and heating means (two ca. 50 W Peltier elements) were integrated in the box as well. Figs. 11 and 12 show photos
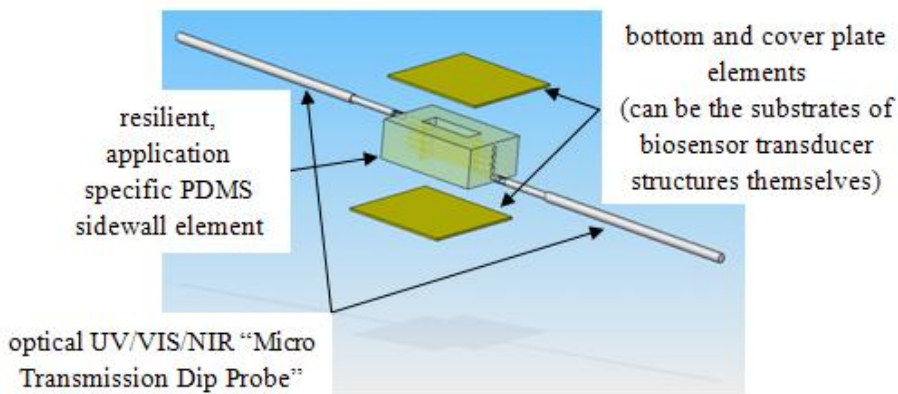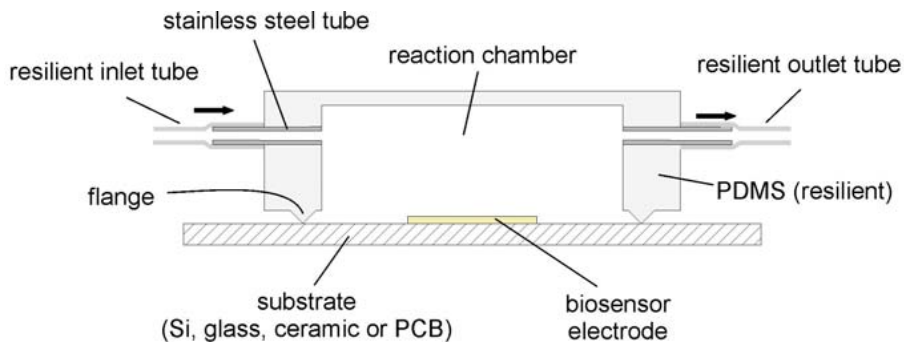
of the thermally controlled insulator box.

3.2 Characterisation

The testing and characterisation of the concept and the prototypes have been performed by using solutions of DNA oligomer molecules. The absorption spectra were recorded over time on two different locations of the inner space of the reaction-cell while electric field was generated according to the description in the *2.2 Methods* section. Examples of spectral curves recorded over time by the 2 micro transmission dip probes during one of the electrophoretic DNA manipulation experiments are presented in Fig. 13.

For a good understanding of the physical geometry of the experimental setup checking again Fig. 2 is recommended. As the baseline values of the graphs were identical at both probes, since after filling the reaction-cell the DNA solution must be homogeneous in t = 0 min., it can be clearly observed in the left side of Fig. 13 that DNA molecules were directed indeed in the upper part of the reaction-cell where the positive voltage was set, because the absorbance measured there increased gradually between 0-30 min. In the right side of Fig. 13 one can see that DNA molecules were directed indeed away from the lower part where the negative voltage was set, because the absorbance measured there decreased gradually between 0-30 min. These curves prove that our concept can provide whole spectral curves inside the biosensor reaction-cell with a certain spatial resolution, not just absorbance values at a certain wavelength. Furthermore, the biomolecules can be observed in their natural form, and no labelling is necessary.

Fig. 4. Schematics of the new modular biosensor reaction-cell development platform

stainless steel tube
reaction chamber
resilient inlet tube
resilient outlet tube
flange
PDMS (resilient)
substrate
(Si, glass, ceramic or PCB)
biosensor electrode

resilient, application specific PDMS sidewall element

bottom and cover plate elements
(can be the substrates of biosensor transducer structures themselves)

optical UV/VIS/NIR "Micro Transmission Dip Probe"

Fig. 5. Three dimensional exploded drawing of the main functional parts of the actual modular biosensor reaction-cell

According to the Beer-Lambert law, in a dilute solution the absorbance of the solution is directly proportional to the concentration of the solved molecules in the solution. The most practical method to measure the absorbance of a solution presents the result in Optical Density units (O.D.). O.D. is a normalised unit meaning that the optical path length to calculate with is 1 cm. Among others, suppliers of biomaterials provide the following data in the datasheet of the purchased material in the case of DNA oligos: total quantity (nmol), weight (μg), molecular weight (g/mol), absorbance at 260 nm if concentration is adjusted to 100 μM (O.D.), mass/absorbance ratio at 260 nm (μg/O.D.).

If the mass/absorbance ratio and the molecular weight or alternatively the O.D. value for a known concentration (e.g., 100 μM) is available, a line representing the relationship between the concentration of a specific biomolecule shown on the X axe and the measurable absorbance in O.D. shown on the Y axe can be easily calculated. This line has to go through the origo, and at 100 μM it has to cross the specified O.D. value. Fig. 14 shows such a line and the measured values of two experiments deploying a dilution line of DNA and different optical fibre transmission micro dip probes.

The correlation between the calculated and measured values seems to be appropriate in the majority of the presented concentration range, however, since the dynamic range of the photodetector of the spectrophotometer is limited to 16 000 arbitrary units (called "counts" in its software), it must be clear, that our measurement setup has a lower and upper limit of detection when a certain parameter setup is selected. The only adjustable software parameter influencing the upper limit is the "integration time" (in ms). In high light intensity conditions it is better, if the photodetector counts the photons just for shorter periods before sending out a certain voltage value to the signal processing circuitry in order to prevent overflow, whilst, in low light intensity conditions the "integration time" has to be set higher in order to accumulate enough energy from the counted photons in the photodetector before sending out the actual voltage value corresponding to the actual light intensity. But either way, if the "integration time" is set in an experiment the detector will overflow above a certain light intensity.

During a biomolecule manipulation experiment and with a fixed lightsource the highest light intensity condition happens when the solvent contains no biomolecules at all in the illuminated physical gap of the tip of the micro transmission dip probes. This light intensity condition can be realised by dipping the probe in the pure solvent and can be stored in the software as a "white reference" value. It is expedient to adjust the "integration time" so, that the "counts" are close to the maximum (16 000) at the wavelength of interest, before saving the "white reference". By doing this the system is prepared to monitor dilute samples without overflow.

However, above a certain level of DNA concentration, where almost all the photons travelling through the illuminated physical gap of the tip of the micro transmission dip probes are absorbed in dissolved DNA molecules during the "integration time", the calculated and displayed absorbance value will not increase anymore with the concentration of DNA, since the "photodetector" can report only, that all the 16 000 "counts" have
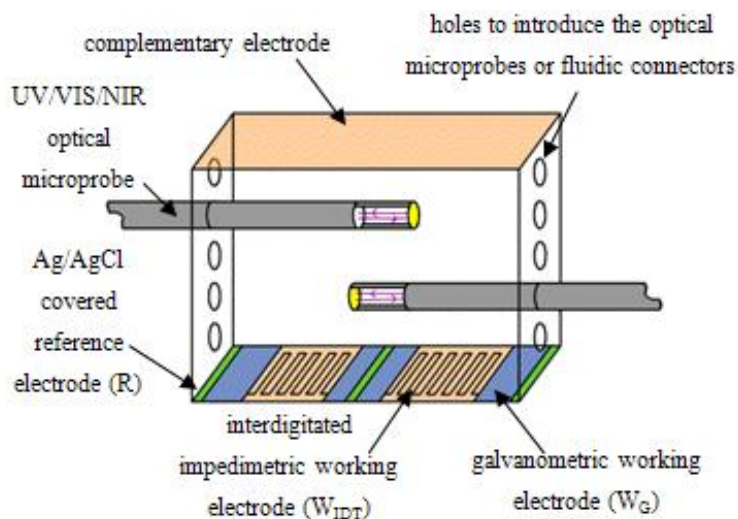
Fig. 6. Schematics of the internal space of the modular biosensor reaction-cell equipped with a combined biosensor electrode array in the bottom and a fully gold coated cover plate for counter electrode purposes at the top
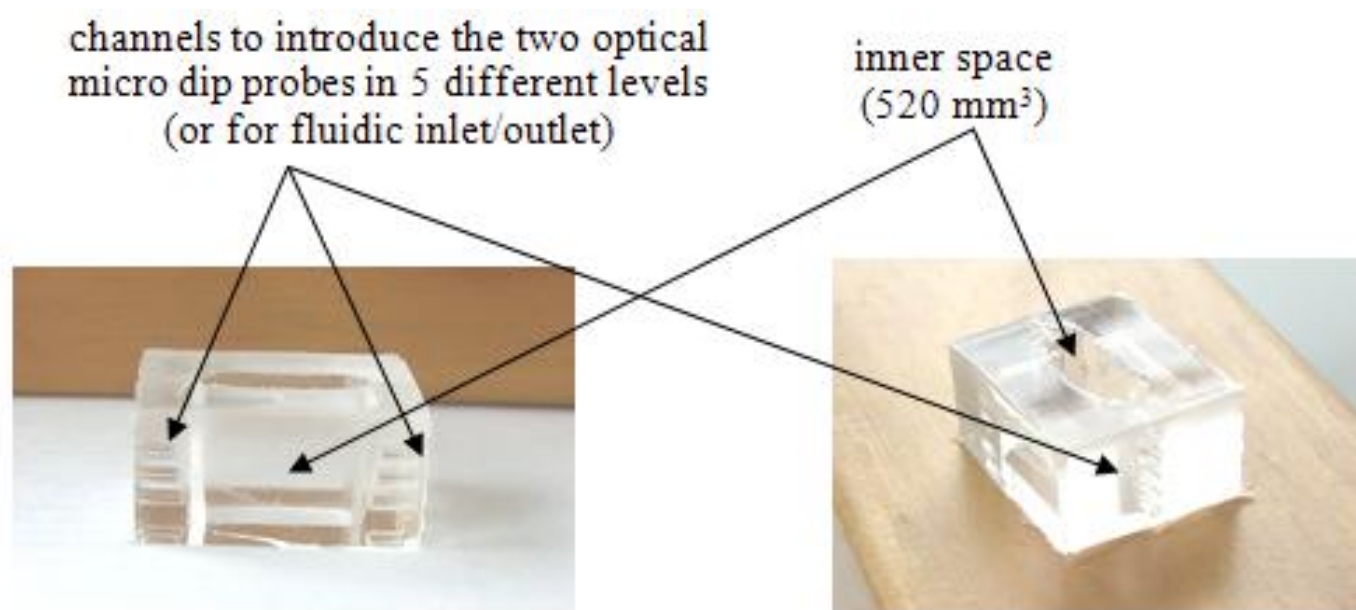
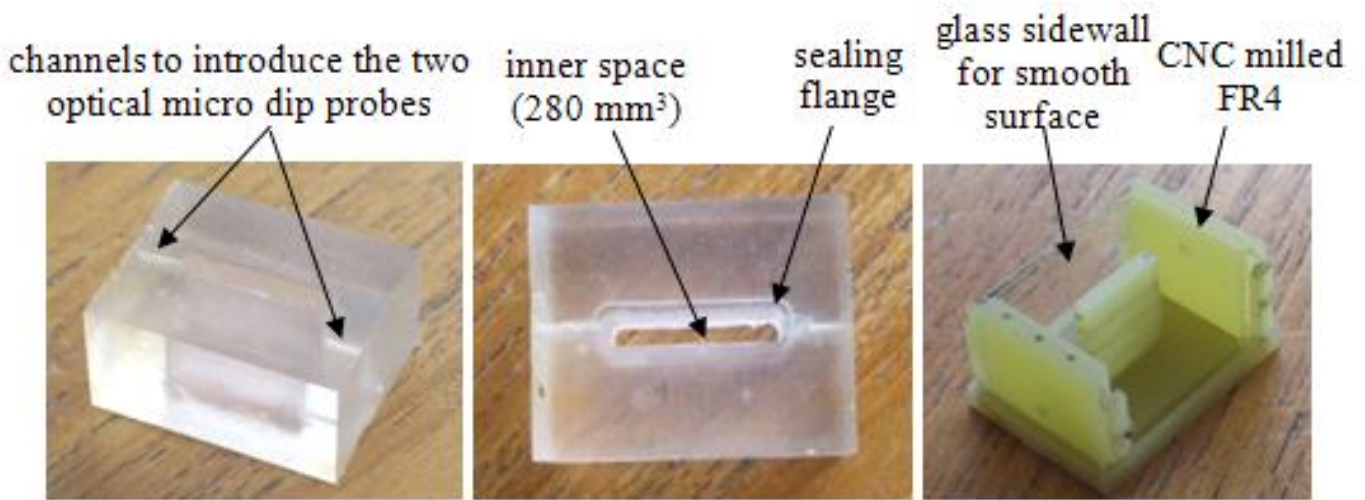**Fig. 7.** Photos of the 1st prototype of the sidewall element of the modular reaction-cell made of PDMS by silicone casting. Height: 10 mm, outer length: 21 mm, outer width: 16.5 mm, inner length: 13 mm, inner width 4.5 mm.

disappeared compared to the highest light intensity condition. This phenomenon is shown in Fig. 15.

It is also known from the Beer-Lambert law, that the measured absorbance is linearly proportional with the optical path length illuminated during the measurement. From this it is clear that with the same "integration time" setting during an experiment the micro transmission dip probes having 1.0 cm optical path length will transmit an undetectable lightintensity to the photodetector already at lower concentrations than the probes with 0.5 cm path length. In Fig. 15 the last measured dilutions fitting to the calculated value of absorbance are marked with red circles. The dashed circle indicates the highest measurable and experimentally verified concentration value of the 1.0 cm path length probes, which corresponds to 6.000 pmol/μl of the DNA used (1.103 O.D.), the continuous line circle indicates the highest measurable and experimentally verified concentration value
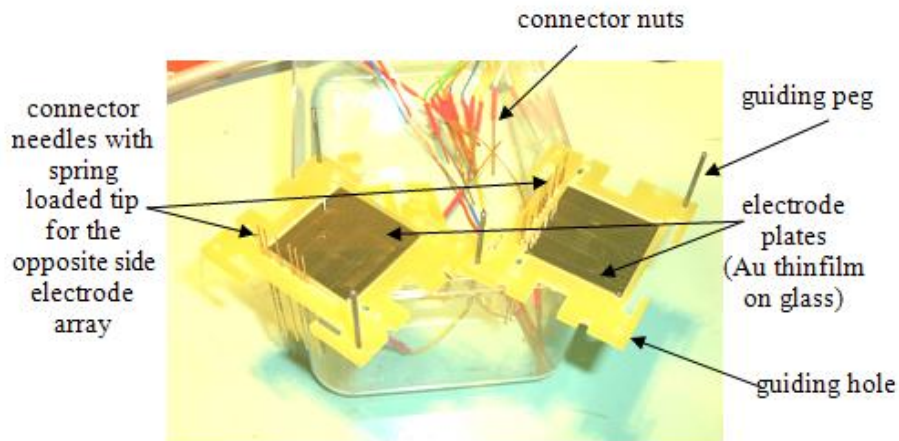
of the 0.5 cm path length probes, which corresponds to 12.916 pmol/μl of the DNA used (2.269 O.D.).

The lower detection border of the dynamic range, namely in the case of very dilute solutions, is caused by the thermally induced intrinsic charge carrier generation in the photodetector. This produces an offset value of voltage sent to the signal processing circuitry even when no photons are arriving to and accumulating energy in the photodetector. To deduct this offset value from the "real voltage value" induced by the incident photons a "dark reference" light intensity condition has to be made by turning off the lightsource of the spectrophotometer and a recording with the same "integration time" as in the case of the "white reference". MTo avoid the effect of small time-scale fluctuations, the user may adjust a certain number of "averaging" of measured values. The settings of the adjustable parameters used in our experiments are detailed in *2.2 Methods*.
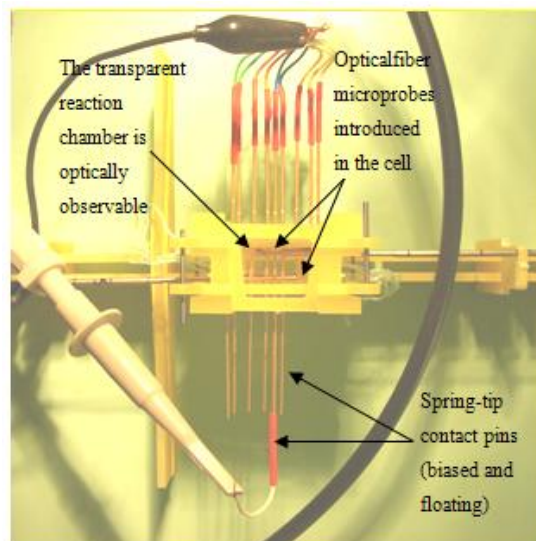
channels to introduce the two optical micro dip probes

inner space (280 mm³)

sealing flange

glass sidewall for smooth surface

CNC milled FR4

Fig. 8. Photos of the 2nd prototype of the PDMS sidewall element (Height: 10 mm, outer length: 21 mm, outer width: 16.5 mm, inner length: 13 mm, inner width 2.4 mm) having a sealing flange around its inner space and the mold structure on the right (one glass sidewall and the cover plate removed for better visibility) prepared by standard PCB manufacturing of FR4 and laser ablation of glass

connector nuts

connector needles with spring loaded tip for the opposite side electrode array

guiding peg

electrode plates (Au thinfilm on glass)

guiding hole

Fig. 9. The holder framework of the modular biosensor reaction-cell before assembling (the PDMS sidewall element is not shown)

The transparent reaction chamber is optically observable

Opticalfiber microprobes introduced in the cell

Spring-tip contact pins (biased and floating)

Fig. 10. The holder framework and the assembled modular biosensor reaction-cell in use (top view), the maximum number of connector pins is 2 x 13 (not all shown)
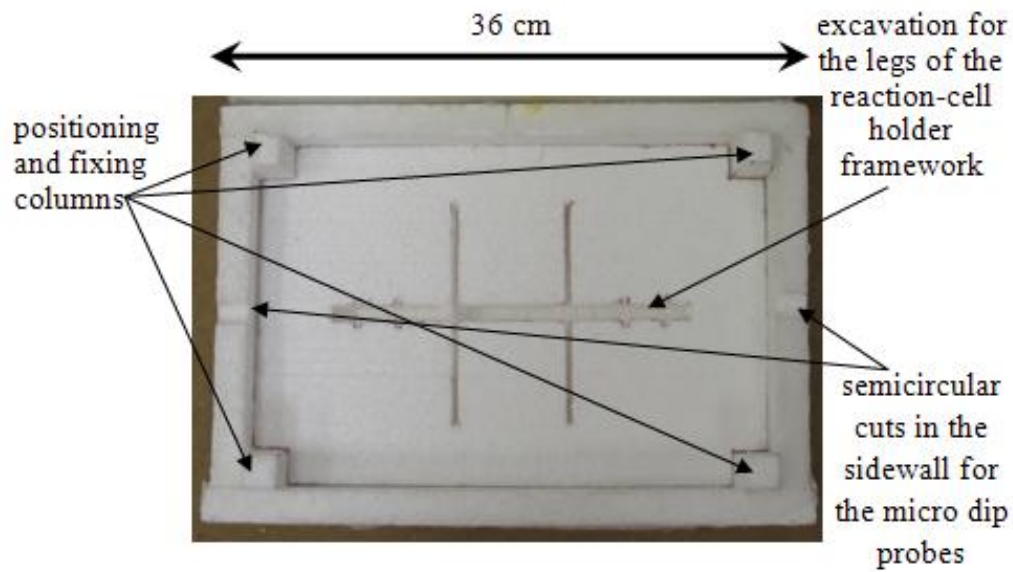
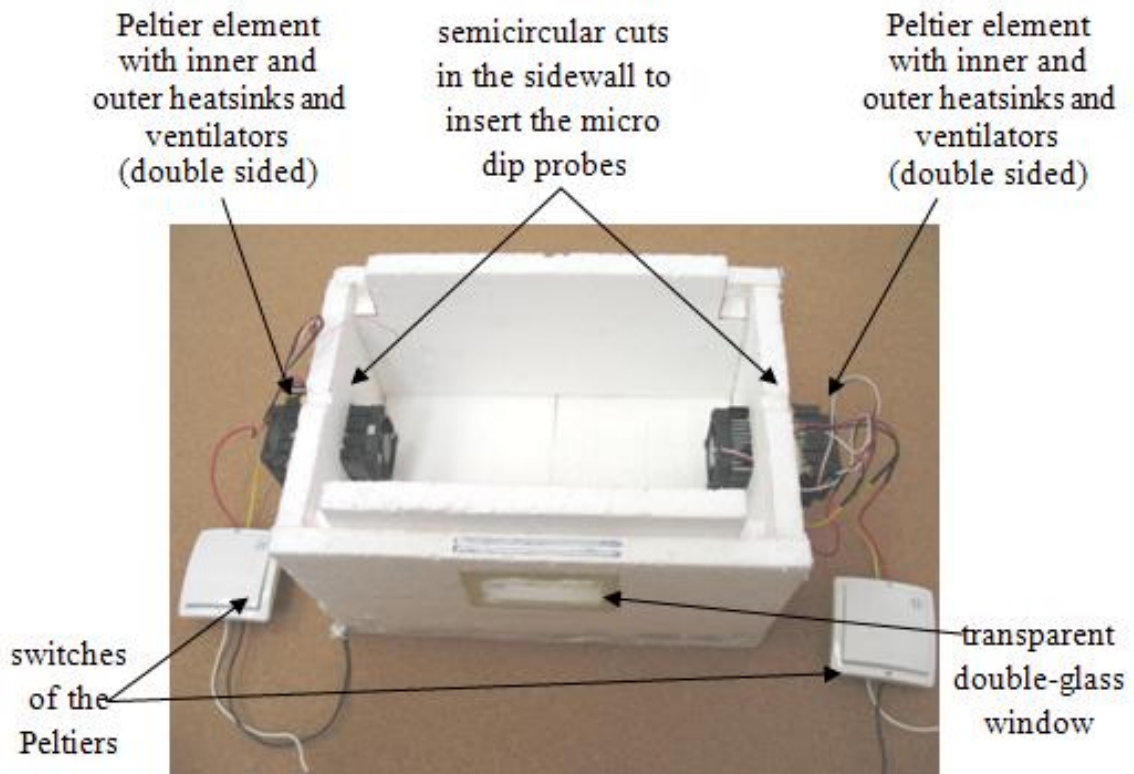**Fig. 11.** Base plate of the thermally controlled insulator box made of 2 cm thick polystirene sheets
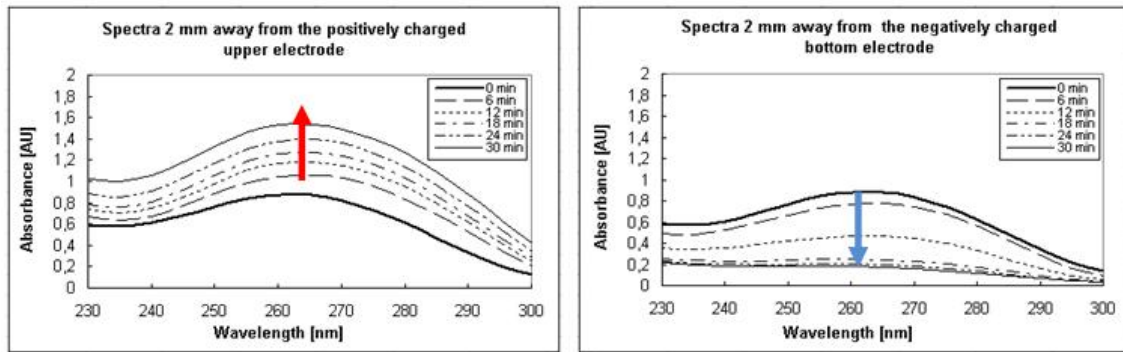

Fig. 12. Cover structure of the thermally controlled insulator box (shown upside down)

Fig. 13. Increase of absorbance on the left vs. decrease of absorbance on the right in function of time when the modular biosensor reaction-cell filled up with solution of DNA was biased to accumulate negatively charged DNA molecules at one electrode (close to the optical probe represented on the left) and distract DNA away from the opposite electrode (close to the optical probe represented on the right)
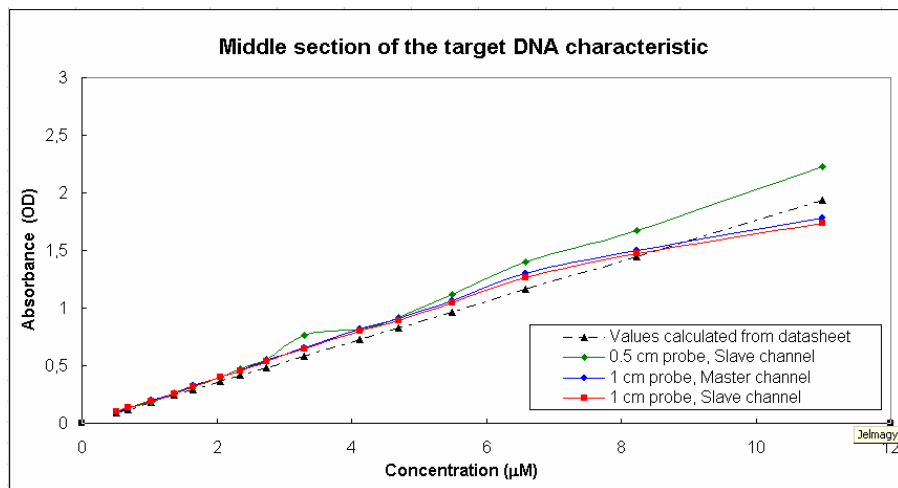

Fig. 14. Absorbance values at 260 nm vs. concentration of DNA. The calculated line and 2 experimentally measured values with in a dilution line of DNA
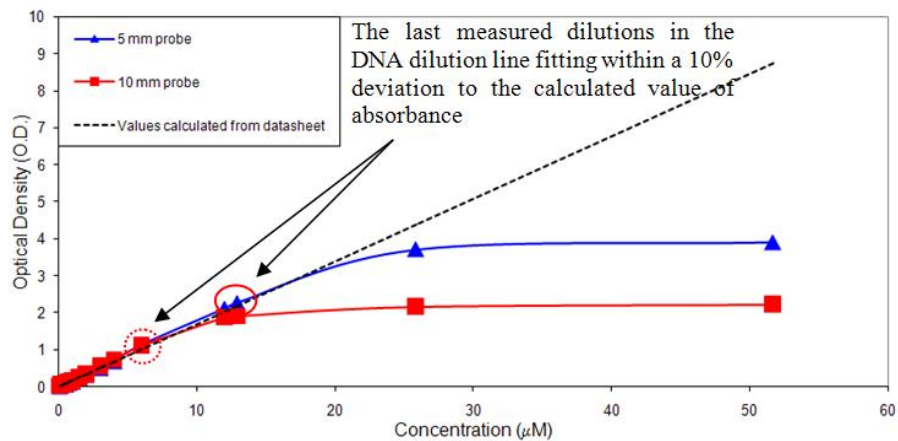

Fig. 15. Upper detection limits of DNA concentration with 0.5 or 1.0 cm probes
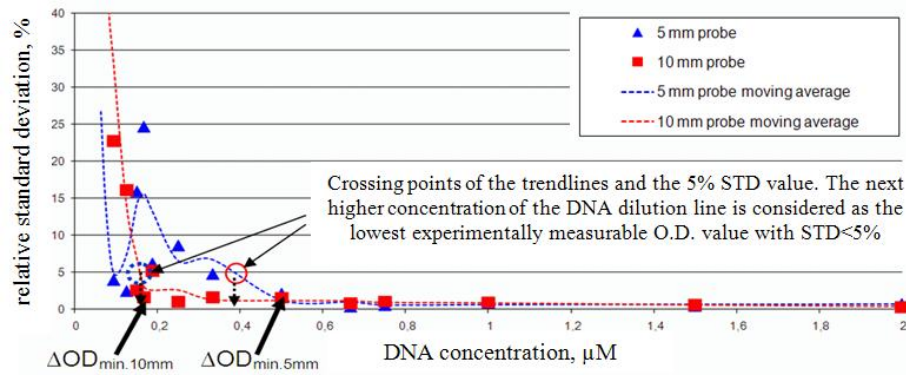
Fig. 16. Measured values and moving average (+/- 1 neighbour) of relative standard deviations (STD) O.D. values aquired with micro transmission dip probes of different path length in the different concentrations of DNA (based on 3 measurements)
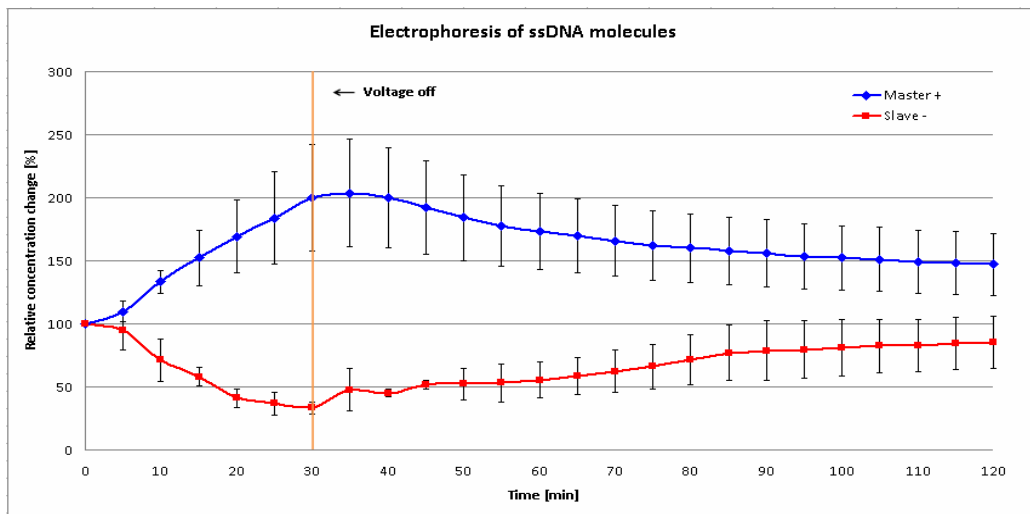
Fig. 17. Electrophoresis & self diffusion experiment of 17 base DNA in water
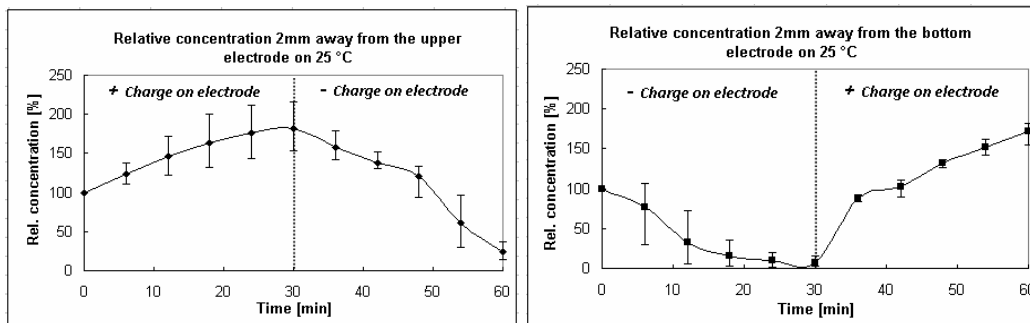
Fig. 18. Electrophoresis of DNA molecules at room temperature
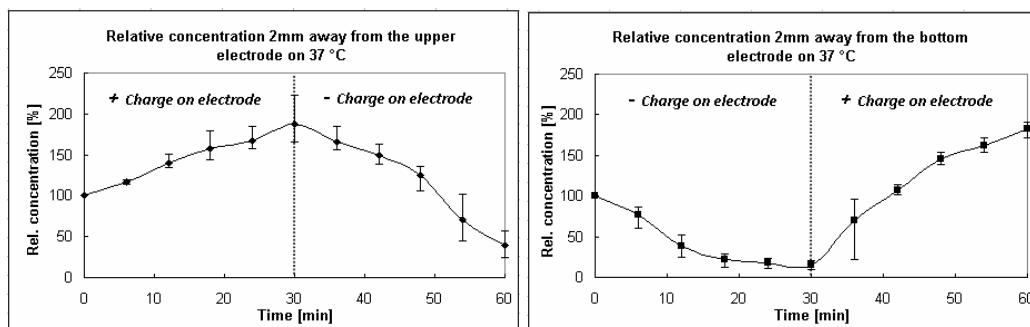
Fig. 19. Electrophoresis of DNA molecules at 37 ^{o}C
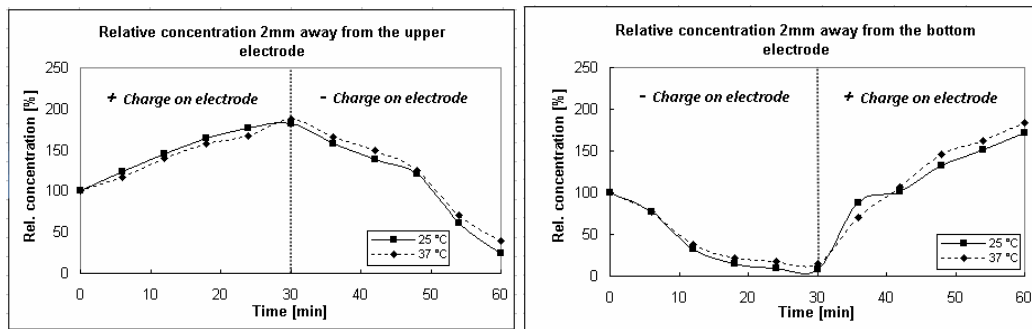
Fig. 20. Comparison of DNA manipulation by electrophoresis in 2 different temperatures (ca. 25 ^{o}C and 37 +/-1 ^{o}C)
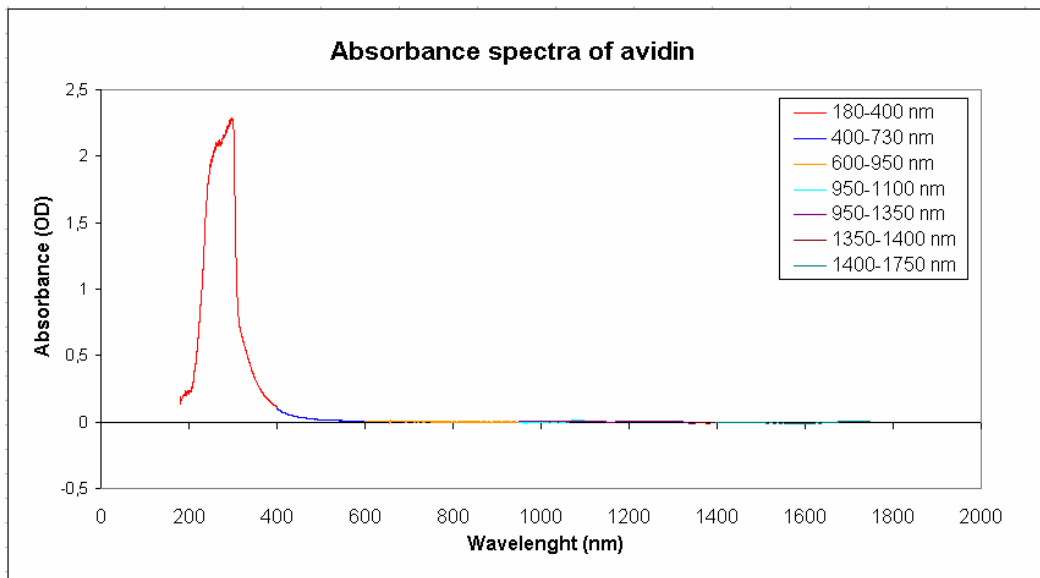

Fig. 21. UV/VIS/NIR spectrum of a 152 μM avidin solution measured in-house

To identify the point of lower detection limit, where the thermally induced offset of the photodetector begins to mask the very small decrease in photon counts caused by the small increase of concentration and becomes unacceptable we tried to define what is the smallest extent of change in the number of counts (or absorbance, if the integration time and other parameters are set) which can be reliably recognised.

The most straightforward approach in such a case is to monitor the variations between identical measurements. In 3 series of measurements the standard relative deviations (STD) of the measured O.D. values acquired with both the 0.5 cm and the 1.0 cm path length micro transmission dip probes in the different concentrations of DNA were calculated. The result is presented in Fig. 16. The black arrows pointing from down to upwards indicate the lowest measurable and experimentally verified concentration value of the 1.0 cm path length probe ($\Delta OD_{min.10mm}$), which corresponds to 0.167 pmol/μl of the DNA used (0.028 O.D.), and the lowest measurable and experimentally verified concentration value of the 0.5 cm path length probes ($\Delta OD_{min.5mm}$), which corresponds to 0.5 pmol/μl of the DNA used (0.084 O.D.).

Since the original concept and the first prototype of the modular biosensor reaction-cell allows the user to put one optical mi-

cro dip probe from the left and one from the right on the same level, the whole dynamic range, which can be monitored in a certain part of the inner space of the reaction-cell during one parameter setup can be considered as between 0.167 - 12.916 pmol/μl of the DNA used or between 0.028 – 2.269 O.D. defined in general, if one 1.0 cm path length micro dip probe is inserted together with an 0.5 cm one.

3.3 Biomolecule Manipulation Experiments

After the characterisation of the new biosensor reaction-cell and the connected spectrophotometer together with the described DNA oligomers, the optimal concentration of the DNA solution subjected to electrical biomolecule manipulation experiments was selected. The self diffusion experiments presented in Fig. 17 have revealed, that by means of a low voltage electrophoresis (2 V/cm) the concentration can be easily doubled compared to the initial value (100% in the graph) near the positive electrode in 30 minutes, while it can decrease to the third of the initial value in the neighbourhood of the negative electrode.

It is clear from the curves, that the self-diffusion cannot set back the homogeneous distribution in a subsequent 90 minutes, furthermore, there is an asymmetry between the upper and lower curves. The phenomena may be explained by the assumption

that DNA molecules are trapped for a certain time on the surface of the upper electrode after the 30 minutes continuous attraction, and serve as a source of DNA during the diffusion part of the experiment.

In an other series of experiments the second part of the measurement was also designed to deploy electrophoresis instead of diffusion, but with an opposite direction compared to the first 30 minutes. The results of these are presented in Figs. 18-20.

The observable changes of the realtive concentrations roughly follow the expectations, as the concentrated/deconcentrated part completely changes location during the second 30 minutes, but the results shown in Fig. 20 seem to be not consistent neither with the laws of nature nor among each other. Namely, we expect that at higher temperatures the higher diffusivity should hinder electrical mobility, and according to this in the first 3 halves of the graphs from left to right we see on the curves that the speed of DNA realloctaion at 37 ^{o}C is always behind the curve of the room temperature conditions, but the last 4 measuring points of the 4th half behave in the opposite way.

Up to now we have no explanation for this, but probably the series of 5 + 3 experiments at the two temperature levels and the small difference in the temperatures (ca. 12 ^{o}C) is not large enough, and future measurements may clarify if the phenomenon presented here really exists or not.

3.4 Synthesis of the Experimental Outcome in a Calculation Method

As the modular biosensor reaction-cell was proven to be docile and well deployable for optical spectroscopic monitoring of processes in the bulk of a sample solution, our interest was focused on its new utilisation possibilities with functionally active biosensing surfaces or biosensor transducers apart from the potential acceleration of operation of biosensors. There are 2 other types of experiments in which the independent *in situ* monitoring of the sample solution above a biosensor transducer can provide useable information.

In the first the kinetics of the capturing/binding process of an analyte onto its bioreceptor surface could be monitored, if the spectroscopic monitoring method is sensitive enough to detect the loss of molecules in the sample solution, as the analyte molecules captured by the biosensing layer will disappear from the sample solution. In the second type different chemical/nonchemical regeneration methods of affinity biosensor transducers could be monitored, if the spectroscopic monitoring method is sensitive enough to detect the appearance of the actual analyte molecules in the sample solution. In both cases there is a clear interdependency between the total number of molecules "sitting" on the total biosensor transducer area and the achievable change of concentration in the total volume of the sample solution/reaction-cell, hence, change of absorbance. One aim of this work is to provide a calculation method, which can predict from the readily available data of biomolecules and our measurement setup the conditions in which any series of experiments of these latter 2 types can be designed successfully.

As we learned already, that the lower detection limit of our spectrophotometer is 0.13 O.D. if the "white reference" is adjusted to the pure solvent (which has theoretically zero absorbance) and the "dark reference" to a level by switching off the light source, the so called 'OD' value of biomolecules can be used for the above mentioned purpose in a very simple manner, i.e. one of the typically available data of a biomolecule coming from the supplier is the 'OD' value, the absorbance at 260 nm for oligonucleotides or 280 nm for proteins measured in Absorbance Units (A.U.) if the concentration is adjusted to 100 μM value and the optical path length is 1 cm. The interdependency between the investigated biosensor surface capable to capture the analyte molecules and the volume of the reaction-cell has the following form:

$$\text{'OD'} : 100~\mu\text{M} = \text{'change of absorbance'} : (\text{'change of nr. of analyte molecules'} / V)$$

In order to focus on the achievable absorbance change and the dimensions and units the relationship can be formulated like below:

Achievable absorbance change in O.D. during capturing or regeneration =

$$(\mathbf{A}~[\text{cm}^2] \times \mathbf{cov}~[\text{cm}^{-2}] \times \mathbf{OD})/\mathbf{V}~[\text{cm}^3]/100~[\mu\text{mol/dm}^3] =$$

$$(\mathbf{A}~[\text{cm}^2] \times \mathbf{cov}~[\text{cm}^{-2}] \times \mathbf{OD})/\mathbf{V}~[\text{cm}^3]/100 \times 6 \times 10^{17} \times 10^{-3}~[\text{cm}^{-3}] =$$

$$\mathbf{A} \times \mathbf{cov} \times \mathbf{OD})/\mathbf{V}/\,6 \times 10^{16}$$

Where:

'A': is the total active area of the biosensor surface inside the reaction-cell (in cm^2)

'cov': is the achievable surface coverage value of a biosensor transducer available in the literature for the specific bioreceptor (in molecules/cm^2) or a theoretical maximum can be calculated from the 3D size of the related capture molecule

'OD': absorbance of the biomolecule (the analyte in this case) at 260 nm (in O.D.) if concentration is adjusted to 100 μM (1μM is ca. 6 $\times$ 10^{17} particles)

'V': volume of the reaction-cell (in cm^3)

By using this equation in another form of it, we can calculate what sort of customised modular biosensor reaction-cell with what 'A/V ratio' should be prepared to complement our spectrophotometer having a minimum detection limit of 0.13 O.D. in order to perform succesfully a certain series of experiments with a bioreceptor-analyte combination having certain 'cov' and 'OD' values:

$$\text{'}A/V~~ratio' = 0.13 \times 6 \times 10^{16} cov \times OD \qquad (2)$$

Similarly to the thinking above, and based on the same equation the problem and the solution can rise in an other form

too. If the 'A/V ratio' of an already existing modular biosensor reaction-cell and the 'minimum detection limit' of the spectrophotometer is given, what bioreceptor-analyte pairs having what 'cov × OD' number can be investigated in the existing experimental setup:

$$\text{`}cov \times OD\text{'} = \text{`minimum detection limit'} \times 6 \times 10^{16} \times V/A \quad (3)$$

The 'cov' values related to a certain immobilisation technique and a bioreceptor molecule can be found frequently in papers dealing with biosensors [14,16,19,20]. It must be noted, that for the final value of the 'cov × OD' number (measured in cm^{-2}) of a bioreceptor-analyte pair, exclusively the analyte alone defines the contribution of the 'OD' value, but the contribution of the 'cov' value up to its theoretical maximum depends on the size/diameter of the bigger part of the bioreceptor-analyte molecular combination, thus, both the bioreceptor or the anaylte can define that, because the steric hindrance between neighbouring biomolecules acts not just during the immobilisation of the bioreceptors, but also during the capturing of the analyte.

According to this we propose the usage of the 'cov × OD' number to characterize the known bioreceptor-analyte molecular combinations in order to assess their ability for spectroscopic monitoring in the bulk solution of a sample filled in the reaction-cell of a biosensor. Of course, if any of the 2 components of the 'cov × OD' number is the question separately, the multiplication between them can be easily broken up in the equation presented above.

3.5 Application of the Calculations to the Avidin-Biotin Combination

Beside the research work with DNA oligonucleotides and the DNA biosensor literature touched and presented here, the other large group of affinity biosensors, i.e., peptide based sensors/immunosensors were addressed to a limited extent too. As the completion of the characterisation of our new modular biosensor reaction-cell its applicability for the *in situ* monitoring of the behaviour of the biotin-avidin bioreceptor-analyte pair is discussed. Our primary aim is to calculate the adequate A/V ratio of a reaction-cell suitable for biosensor regeneration experiments with avidin as analyte and biotin as bioreceptor. Knowing the 'OD' value at least on one wavelength is inevitable for th eexperiments, and preferably the highest absorption peak should be used.

As it was not available, the UV/VIS/NIR absorbance spectrum of a known concentration of avidin was recorded in-house. The result is presented in Fig. 21. The maximal optical density value was found at 280 nm and was 1.603 O.D. in the case of our 19 μM avidin solution. The normalised 'OD' value for 100 μM is calculated as 8.44 O.D.

For the achievable coverage the literature search provided 3.57 × 10^{12} /cm^2[19]. The equation to be used is *A/V ratio* = 0.13 × 6 × 10^{16} / cov × 'OD'. The result is: *A/V ratio* = 258.87, which actually seems to be not manufacturable with our

technologies, thus, avidin-biotin based biosensor experiments focusing on monitoring of binding processes or regeneration of the biotin based biosensor surface will not be feasible.

4 Conclusion

A novel approach and an innovative modular biosensor reaction-cell has been designed and prepared which enables simultaneous monitoring of processes of a biosensor transducer surface by means of electrochemical methods and the bulk of the sample solution by means of optical spectrophotometric methods and their interaction in different buffers, at different temperatures and under different electrical conditions.

Both the electrochemical and optical spectrophotometric functionalities of the modular biosensor reaction-cell have been tested by means of several series of experiments of electrophoretic manipulation of unlabelled DNA molecules, and the results have validated the capabilities of the new reaction-cell.

As a part of the characterisation of the new experimental system composed of the modular biosensor reaction-cell and the optical fibre micro transmission dip probe based spectrophotometer the absorbance range which can be monitored with maximum ca. 5% standard deviation using a certain parameter setting during a certain measurement was determined for both the 5 mm and 10 mm optical path length probes. For the optical spectroscopic capabilities of the system 0.028 O.D., corresponding to 0.167 pmol/μl concentration of the DNA used was found to be the minimum, and 2.269 O.D., corresponding to 12.916 pmol/μl concentration of the DNA used was found to be the maximum measurable absorbance value having experimental validation. This dynamic range is more than 2 orders of magnitude.

Related to two types of biosensor experiments doable by the utlisation of the new modular reaction cell, namely, *in situ* spectrophotometric and electrochemical monitoring of regeneration methods and *in situ* spectrophotometric and electrochemical monitoring of capturing kinetics the introduction of the 'coverage × OD' number was proposed for the characterisation of any known bioreceptor-analyte molecular combinations in order to assess their compatibility for the *in situ* spectroscopic monitoring in the bulk solution of a sample filled in the reaction-cell of a biosensor. By calculating this number and the usage of the equation: *A/V ratio* = 0.13 × 6 × 10^{16} / 'coverage [molecules/cm^{-2}] × OD [n.a.]' one can calculate the adequate A/V ratio of a reaction-cell suitable for the 2 types of *in situ* biosensor experiments mentioned above.

All the presented experiments can be performed by leaving the studied molecules in their natural form without the attachment of any label. Main advantage of the presented technique is that both the PDMS part and the biosensor transducer/electrode array can be easily customized and adapted to already existing experimental setups depending on the actual needs and the actually available technologies. The leakage free operation, the ability to control and avoid bubbles, the possibility of manual

refilling and evacuation, the flexibility of applicable electrode structures are further proven advantages of our modular biosensor reaction-cell.

List of Abbreviations

A.U.   – Absorbance Unit
CE   – Capillary Electrophoresis
CNC   – Computer Numerical Control
DNA   – Deoxyribo Nucleic Acid
FR-4   – Flame Retardant 4
OD   – Optical Density
PCB   – Printed Circuit Board
PDMS   – PolyDiMethylSiloxane
PMMA   – PolyMethyl Methacrylate
RTD   – Research and Technical Development

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