BIOELECTRONICAL NEURONAL NETWORKS

Towards chemical analysis by coupling neurobiological entities to capacitive or capacitive-optical transducers

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen
zur Erlangung des Grades eines Doktors
der Naturwissenschaften

1999

vorgelegt von

AXEL BLAU

Tag der mündlichen Prüfung:	4. August 1999
Dekan:	Prof. Dr. U. Nagel
1. Berichterstatterin:	PD Dr. Ch. Ziegler
2. Berichterstatter:	Prof. Dr. G. Gauglitz

Berichte aus der Biochemie

BIOELECTRONICAL NEURONAL NETWORKS

Towards chemical analysis by coupling neurobiological entities to capacitive or capacitive-optical transducers

Axel Blau

D 21 (Diss. Universität Tübingen)

Shaker Verlag
Aachen 1999

Die Deutsche Bibliothek - CIP-Einheitsaufnahme

Blau, Axel:
Bioelectronical Neuronal Networks : Towards chemical analysis by coupling neurobiological entities to capacitive or capacitive-optical transducers / Axel Blau. - Als Ms. gedr. -
Aachen : Shaker, 1999
(Berichte aus der Biochemie)
Zugl.: Tübingen, Univ., Diss., 1999
ISBN 3-8265-6523-1

Als Manuskript gedruckt. Printed in Germany.

ISBN 3-8265-6523-1
ISSN 1434-5536

Shaker Verlag GmbH • Postfach 1290 • 52013 Aachen
Telefon: 02407 / 95 96 - 0 • Telefax: 02407 / 95 96 - 9
Internet: www.shaker.de • eMail: info@shaker.de

In awe of nature and its marvelous secrets

ABSTRACT

This thesis depicts the development of an **autonomous *in vitro* recording system** for neuronal cell cultures. It is intended to be used in analytical chemistry and sensorics as a transportable system independent from laboratory facilites. As the sensing entity, a neuronal network is cultured in a perfusion chamber mounted on a passive 60-electrodes multi-microelectrode array. The response behavior of that network to analytes will be recorded, amplified, and analyzed mathematically in the sub-millisecond range.

Besides the portrayal of the mechanical and electronical design of a modular, temperature controlled perfusion chamber, of a life-maintaining support system, and of a 60-channel amplifier for signal recording, various secondary problems have been addressed and partly solved in this context. These include post-modification procedures of the recording electrodes with respect to the improvement of their electrical as well as adhesion mediating properties.

Neuronal cultures from embryonic chicken may survive in the **modular, temperature controlled perfusion chamber** for up to three weeks. Their development and differentiation cannot be distinguished from cultures grown on cell culturing trays in CO_2-incubators. Cell survival turned out to be strongly dependent on perfusion parameters. While a constant fast-volume flow (15 µl/hour, equivalent to approx. $1/5^{th}$ of the total chamber volume) of fresh medium through the chamber stressed the cultures noticably, timed perfusion of about 30 µl once or twice a day kept the cultures viable. Longevity was limited rather by mechanical and electronical problems with the perfusion chamber and the controlling unit than by principle faults in the concept.

A **60-channel amplifier** in a sandwich configuration (32 + 28 channels) with very good noise and speed characteristics may amplify neuronal signals by a factor of 990 or 1551, respectively. However, for not yet understood reasons, the offset of that system is pulled to the lower supply potential of –5 V. The amplifier is connected to a high-speed (333 ksamples per second) data acquisition board with a total of 64 A/D-channels. A **signal recording program** in a virtual-instruments programming environment (Testpoint™ by Keithley Instruments, Inc.) has been developed.

The electrical behavior of the recording (and stimulation) electrodes with respect to a high capacitance, a high charge delivery capacity, a low impedance, and biocompatibility, has been improved by **electrochemical iridium deposition** (and iridium oxide formation) onto platinum from an aqueous solution of H_2IrCl_6. It turned out that various iridium layers of different and decreasing stabilities with increasing thicknesses will form on the electrodes. To enhance mechanical stability of those deposits, iridium may be embedded in a polypyrrole matrix. The total impedance could be lowered by almost two orders of magnitude in the low frequency range below 1 kHz with respect to a plain platinum electrode while the increase of electrode capacitance by almost two orders of magnitude did not suffer from the matrix environment.

Neuronal cultures from embryonic chicken may be grown in **serum-free medium** and will survive in some cases for up to 6 months in a standard CO_2-incubator. Cell development in serum-free medium lags noticably behind that of cells grown in medium that contains 10% (v/v) fetal bovine serum within the first two weeks. However, if the density of seeded cells surpasses a certain threshold (at least complete surface coverage of the substrate), cell networks grown in different media cannot be distinguished thereafter.

New **adhesion strategies** on the base of electroactive phenol and pyrrole derivatives for the selective coverage of electrodes have been developed. Besides mimicking standard laminin related adhesion promoting procedures with the help of a peptide fragment from laminin (*SRARKQAASIKVAVSADR*) that is linked to an electroactive monomer (e.g. 3-hydroxyphenylacetic acid), electropolymers of 2-(3-hydroxyphenyl)-ethanol and of 3-hydroxybenzyl hydrazine have proven to promote cell adhesion (and neuron differentiation) in serum-free medium. Furthermore, it has been observed that laminin or a combination of laminin and polylysine will not promote cell adhesion and neuron differentiation in serum-free culture while they work perfectly well in medium with serum. This finding might hint at an indirect adhesion mechanism of laminin that depends on secondary 'mediating' factors found in the medium, while the adhesion promoting properties of polymeric films mentioned above might be based on a more direct cell-film interaction.

As an alternative to capacitive-electrical electrode arrays with limited areal resolution, especially when studying signal spread, principle characteristics of a new **capacitive optical recording array** based on potential sensitive fluorescent dyes have been investigated. To circumvent common problems with membrane-bound potential sensitive dyes, the dyes will be trapped in a polymeric film on a suitable electrode material instead and thus stay in direct contact with the neurons. It could be shown that this strategy is working in principle by exhibiting voltage dependent fluorescence quenching. However, probably due to polymer thicknesses in the range of micrometers, the potentials necessary for generating noticable signals are still beyond capacitively detectable neuronal signals by almost four orders of magnitude.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I sincerely thank my two dissertation advisors for their invaluable support and guidance. *Prof. Dr. Dr. h.c. mult. Wolfgang Göpel* kindly offered to me this great and refreshingly interdisciplinary challenge and provided all necesseary support for this research project, extra lab space, and generous funding. I am also very grateful for having had the opportunity to get to know and use a multitude of techniques and apparatuses during the past three years thanks to the excellent infrastructure at his institute. His openness, his wits and kindness will always stay alive in my memory. With great interest, helpful advice and ample encouragement *PD Dr. Christiane Ziegler* guided and accompanied this work right from the beginning. I am very thankful to her for her unquestioned support, for her confidence in its success, and for always granting unconditional freedom in approaching and realizing the whole project. I was also very glad about her offer to assist the practical course for biochemist in physical chemistry for it became a welcomed and stimulating experience. Many thanks also to *Prof. Dr. Günter Gauglitz* for offering to be a referee and advisor in the defense of the thesis.

I am also very indebted to many people who accompanied this work and contributed to its success in the one or other way. Special thanks are due to *Prof. Dr. Guenter Gross* and his crew, who taught me the fundamentals and tricks in cell culturing techniques and recording procedures, and whose enthusiasm set roots in my own work on this topic. In the same respect I would also like to thank *Dr. Andreas Offenhäusser* for sharing his experience in this field, especially for his encouraging and congenial simplistic and pragmatic, yet solid approaches and suggestions, which helped me a lot in finding the right solutions to many problems.

I greatly appreciate the work of *Christine Weinl*, who devoted her diploma thesis on the details of the different adhesion strategies and facilitated the accomplishment of the inflationary manifold of those experiments. I am also indebted to *Elke Nadler* for taking all those wonderful SEM pictures and for performing the EDX measurements. Many thanks to the people from the mechanical workshop, especially to *Johannes Seidel* and *Michael Jäger*, for their skills and interest in giving shape to sometimes so minute and theoretical entities. Some of the adhesion strategies would have stayed mere theory without the skills of *Dr. Stefan Kienle* and *Dr. Jürgen Mack* in organic and peptide chemistry. Thanks to the two of them for providing us with modified laminin fragments, for promptly synthesizing the ManLev, and for their very patience regarding the outcome of their efforts. The realization of most of the electronic gadgets would have taken at least another century without the help and the experience of *Dr. Gerd Noetzel* and *Andreas Krauß.* I very much appreciate their often demanded and ingenious help in mastering electronics with its so endlessly many traps and peculiarities (for a chemist).

Many thanks also to *Christian Bauer, Dr. Ralph Burckardt, Michael Kaspar, Reiner Krapf,* and *Dr. Jan Rickert*, with whom I shared the lab and office space, and to the present and past Ziegler-crew, to *Thomas Ertl, Stefan Lach, Matthias Mondon, Eckhard Müller, Dr. Jochen Murr, Dr. Berthold Reusch, Dr. Ralph-Michael Rummel, Frank Rupp, Hartmut Stadler, Dirk Then, Aleksandar Vidic*, and *Christine Weinl*. Their interest in this somewhat exotic work, their different opinions and perspectives, and the inspiring discussions helped so many times to reconsider the sheer endless possibilities, and to find the (hopefully) best approaches.

Thanks to *Prof. Dr. Dieter Oelkrug* and his coworkers for giving me the chance to use their fluorescence spectrometer. Thanks also to *Prof. Dr. Rolf Gebhardt* for providing his laboratory space and cell culturing infrastructure at the very beginning of the project. Many thanks also to *Dr. Peter Heiduschka* for teaching me some basics and first steps in laminin adhesion strategies.

I am very thankful to *Dr. Steve Potter* for sharing his knowledge and thoughts that are based on many more years of experience in this field. I also appreciate the help of *Frank Hofmann* at the NMI who provided one of his slice cultures to test the performance of the amplifier. Thanks also to *Dr. Marko Burghard* for spreading *LANGMUIR-BLODGETT* films onto ITO-glass slides.

Life is stuffed with daily obstacles. Therefore many thanks to *Dr. Tilo Weiss, Mr. Egon Merz* and *Ms. Karin Vanesse* for their quick and uncomplicated help in mastering those sometimes annoying distractions. Not to forget the computer crew with helping out so many times with network inconsistencies and other computer oddities. Thanks to all the people in our institute for their friendly, open, and helpful attitude, which made life and research a lot easier.

Finally my very thanks to my parents for their continuous and unquestioned support in every respect. My life has also been made immeasurably better by my very friends. Thanks to all of them for their good company over all these years.

CONSTANTS

Basic constants

$R = N_A \cdot k$	gas constant	8.31441	$J \cdot mol^{-1} \cdot K^{-1}$ $m^2 \cdot kg \cdot s^{-2} \cdot mol^{-1} \cdot K^{-1}$
$F = N_A \cdot e$	*FARADAY* constant	96484.56	$C \cdot mol^{-1}$ $A \cdot s \cdot mol^{-1}$
N_A	*AVOGADRO* or *LOSCHMIDT* constant: # of particles per mol	$6.022045 \cdot 10^{23}$	mol^{-1}
e	elementary charge: charge of one electron	$1.6021892 \cdot 10^{-19}$	C $A \cdot s^{-1}$
k	*BOLTZMANN* constant	$1.380662 \cdot 10^{-23}$	$J \cdot K^{-1}$ $m^2 \cdot kg \cdot s^{-2} \cdot K^{-1}$
T	Temperature		K 0°C = 273.16 K
h	*PLANCK* constant	$6.62608 \cdot 10^{-34}$	J·s $m^2 \cdot kg \cdot s^{-1}$
ε_0	permittivity of the vacuum	$8.854187817 \cdot 10^{-12}$	$F \cdot m^{-1}$ $C \cdot V^{-1} \cdot m^{-1}$ $C^2 \cdot J^{-1} \cdot m^{-1}$

ABBREVIATIONS

Amino acids

One-letter code and three-letter notation [KOO 95 · MAL 98]

codes		amino acid	molecular formula	FW	polarity	pK_a*
G	Gly	glycine	$C_2H_5NO_2$	75.1	+2.4	
A	Ala	alanine	$C_3H_7NO_2$	125.6	+1.9	
V	Val	valine	$C_5H_{11}NO_2$	117.2	+2.0	
L	Leu	leucine	$C_6H_{13}NO_2$	131.2	+2.3	
I	Ile	isoleucine	$C_6H_{13}NO_2$	131.2	+2.2	
C	Cys	cysteine	$C_3H_7NO_2S$	121.2	-1.2	8.3
M	Met	methionine	$C_5H_{11}NO_2S$	149.2	-1.5	
F	Phe	phenylalanine	$C_9H_{11}NO_2$	165.2	-0.8	10.1
Y	Tyr	tyrosine	$C_9H_{11}NO_3$	181.2	-6.1	
W	Trp	tryptophane	$C_{11}H_{12}N_2O_2$	204.2	-5.9	
P	Pro	proline	$C_5H_9NO_2$	115.1	-6.0	
S	Ser	serine	$C_3H_7NO_3$	105.1	-5.1	
T	Thr	threonine	$C_4H_9NO_3$	119.1	-4.9	
N	Asn/Asx	asparagine	$C_4H_8N_2O_3$	132.1	-9.7	
Q	Gln	glutamine	$C_5H_{10}N_2O_3$	146.1	-9.4	
D	Asp	aspartic acid	$C_6H_{14}N_4O_2$	174.2	-11.0	4.0
E	Glu/Glx	glutamic acid	$C_5H_9NO_4$	147.1	-10.2	4.3
H	His	histidine	$C_6H_9N_3O_2$	155.2	-10.3	6.0
K	Lys	lysine	$C_6H_{14}N_2O_2$	146.2	-15.0	10.8
R	Arg	arginine	$C_6H_{14}N_4O_2$	174.2	-20.0	12.5

**Note: The change in free enthalpie ΔG for the transfer of the side-chain from a rather non-polar solvent (ethanol) to a very polar solvent (water) is a measure for polarity of the side-chain. The more negative the value for ΔG the more polar is the side-chain.*

Other abbreviations

2(3HPE)	**2**-(**3**-**h**ydroxy**p**henyl)-**e**thanol
3HBA	**3**-**h**ydroxy**b**enzaldehyde
3HBH	**3**-**h**ydroxy**b**enzyl**h**ydrazine
3HPA	**3**-**h**ydroxy**p**henyl**a**cetic acid
3PPA	3-(**p**yrrole-1-yl)-**p**ropionic **a**cid
a	activity
AC / a.c.	**a**lternating **c**urrent
ACA	**ε**-**a**mino**c**apronic **a**cid
ADP	**a**denosine **di**phosphate
AIROF	(anodically) **a**ctivated **i**ridium **o**xide **f**ormation
APS	**a**ctive **p**ixel **s**ensors (CMOS-based digital camera chip)
ATG	**β**-**a**lanine-**t**etraethylene glycole-**g**lycine
ATP	**a**denosine **tri**phosphate
ATR-IR	**a**ttenuated **t**otal **r**eflection **i**nfra**r**ed (spectroscopy)
BDNF	**b**rain **d**erived **n**eurotrophic **f**actor
C	electrical capacity [C] = F = $C \cdot V^{-1}$
CAM	**c**ell **a**dhesion **m**olecule
CCD	**c**harge **c**oupled **d**evice
CDC	**c**harge **d**elivery **c**apacity [CDC] = $C \cdot cm^{-2}$
CE	**c**ounter **e**lectrode
CMOS	**c**omplementary **m**etal-**o**xide **s**emiconductor
CNS	**c**entral **n**ervous **s**ystem
CNTF	**c**iliary **n**euro**t**rophic **f**actor
CoA	**co**enzyme **A**
CV	**c**yclo**v**oltammetry
CVD	**c**hemical **v**apor **d**eposition
d	postnatal **d**ay
D	constant of diffusion [D] = $cm^2 \cdot s^{-1}$
Da	**Da**lton = MW
DC / d.c.	**d**irect **c**urrent
DCC	**dic**yclohexyl**c**arbodiimide
Di I	DilC18(3) or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
dic	**d**ays **i**n **c**ulture
DMAP	4-**dim**ethyl**a**mino**p**yridine
DMF	N,N'-**dim**ethyl**f**ormamide
E	**e**mbryonic day
EIS	**e**lectrochemical **i**mpedance **s**pectroscopy
EP	**e**lectro**p**lated
ES	**e**mbryonic **s**tem (cells)
F	farad [F] = $C \cdot V^{-1} = s \cdot \Omega^{-1}$
FET	**f**ield **e**ffect **t**ransistor
FIA	**f**low **i**njection **a**nalyser
GB	**gig**a**b**yte = $1 \cdot 10^9$ bytes = $8 \cdot 10^9$ bits
GC	**g**as **c**hromatography
HPLC	**h**igh **p**erformance **l**iquid **c**hromatography
HTS	**h**igh **t**hroughput **s**creening
Hz	***HERTZ*** [Hz] = s^{-1}
I	current [I] = A
i	imaginary unit: $i \equiv \sqrt{-1} \equiv e^{\frac{i\pi}{2}}$
IC	**i**ntegrated **c**ircuit
ID	**i**nner **d**iameter
IHP	**i**nner ***HELHOLTZ*** **p**lane
IME	**i**nterdigitated **m**icrostructured **e**lectrode
IS	**i**mpedance **s**pectroscopy
ISA	**i**ndustrial **s**tandard **a**daptor
ISFET	**i**on **s**ensitive **f**ield **e**ffect **t**ransistor
ITO	**i**ndium-doped **t**in **o**xide
J	*JOULE* [J] = $kg \cdot m^2 \cdot s^{-2}$
l	liter
L	inductance [L] = *HENRY* = $V \cdot s \cdot A^{-1}$
LAPS	**l**ight **a**ddressable **p**otentiometric **s**ensor
LSD	**l**ysergic **a**cid **d**iethylamide
M	molar concentration [M] = $mol \cdot l^{-1}$
m	meter
m (as prefix)	milli (10^{-3})
ManLev	N-laevulinic acid-D-mannosamide
MB	**meg**a**b**yte = $1 \cdot 10^6$ bytes = $8 \cdot 10^6$ bits

MEA	**m**icro **e**lectrode **a**rray
MEM	**m**inimum **e**ssential **m**edium
MMEP	**m**ulti **m**icro **e**lectrode **p**late
mol	**mol**e: number of particles in a sample: 1 mole of a substance contains as many particles as there are atoms in exactly 12 grams of carbon (^{12}C).
MOS	**m**etal **o**xide **s**emiconductor
MOSFET	**m**etal **o**xide **s**emiconductor **f**ield **e**ffect **t**ransistor
Mr	**r**elative **m**olar mass [Mr] = g·mol^{-1}
MW (FW)	**m**olecular **w**eight [MW] = g·mol^{-1}
n	nano (10^{-9})
NCAM	**n**euronal **c**ell **a**dhesion **m**olecule
NGF	**n**erve **g**rowth **f**actor
NHE	**n**ormal **h**ydrogen **e**lectrode (E^0 = +0.242V @ 25°C by definition)
OD	**o**uter **d**iameter
OHP	**o**uter ***HELHOLTZ*** **p**lane
opamp	**op**erational **amp**lifier
PC	**p**ersonal **c**omputer
PCA	**p**rinciple **c**omponent **a**nalysis
PCR	**p**rinciple **c**omponent **r**egression
PDL	**p**oly-**D**-**l**ysine
PEA	**p**lanar **e**lectrode **a**rray
PECVD	**p**lasma **e**nhanced **c**hemical **v**apor **d**eposition
PEG	**p**oly-**e**thylene **g**lycole
pH	**p**otentia **h**ydrogenii: -log [H^+]
PNS	**p**eripheral **n**ervous **s**ystem
PTFE	**p**oly**t**etra**f**luor**e**thylene (Teflon™, Fluoron™)
PVD	**p**lasma **v**apor **d**eposition
R	resistance [R] = Ω
RAM	**r**andom **a**ccess **m**emory
RCDC	**r**eversible **c**harge **d**elivery **c**apacity = RCIL = SCIL
RCIL	**r**eversible **c**harge **i**njection **l**imit = SCIL = RCDC
RE	**r**eference **e**lectrode
RH 421	potential sensitive dye; N-(4-sulfobutyl)-4-(4-(4-(dipentylamino)phenyl) butadienyl)pyridinium, inner salt
RIE	**r**eactive **i**on **e**tching
RIFS	**r**eflectometric **i**nter**f**erence **s**pectroscopy
s	second
S/N	**s**ignal-to-**n**oise ratio
SCE	**s**aturated **c**alomel **e**lectrode (E^0 = +0.242V @ 25°C)
SCIL	**s**ave **c**harge **i**njection **l**imit = RCDC
SEM	**s**canning **e**lectron **m**icroscopy
SFM	**s**canning **f**orce **m**icroscopy
SFM	**s**erum-**f**ree **m**edium
SMD	**s**urface **m**ounted **d**evice
SOT23	**s**mall **o**utline **t**ransistor: a standardized packaging size for CMOS devices
SP	**sp**uttering / **sp**utter deposition
T	absolute **t**emperature [T] = K, 1 K = 1°C
THF	**t**etra**h**ydro**f**uran
UV	**u**ltra-**v**iolet radiation
V	volt [V] = J·C^{-1} = W·A^{-1} = kg·m^2·s^{-3}·A^{-1}
VD	**v**apor **d**eposition
VIS	**vis**ible light
WE	**w**orking **e**lectrode
XPS	**X**-ray **p**hotoelectron **s**pectroscopy
z	valency of an ion
Z_C	ractance of a capacitor
Z_L	reactance of an inductance
Z_W	*WARBURG* impedance
BAEE	Nα-**b**enzoyl-L-**a**rginine **e**thyl **e**ster
ε_r	relative electrical permittivity = relative dielectric constant
φ	*GALVANI* potential
μ	micro (10^{-6})
ν	frequency [ν] = s^{-1}
ω	angle velocity [w] = s^{-1}