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Improving neuronal adhesion on chip using a phagocytosis-like event

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Efficient integration of neuronal cells and electronic devices could result in hybrid bi-directional communication systems that would enable us to interact at fundamental levels with biological structures and gain insight in the mechanisms governing their functions. Such systems require a very tight coupling between the neuronal cell membrane and the surface of an electronic chip. In this paper we report an approach where the combination of specialized surface chemistry and the manipulation of biological processes, like a phagocytosis-like process, might improve this coupling. As a model, we used coated micro- and nano-sized beads and induced phagocytosis-like events by adding them to cultured cells. The development of the surface chemistry and the results obtained with beads functionalized with a laminin derived peptide are presented.

Keywords: Neuronal adhesion; Functionalized nanobeads; Surface chemistry

1. Introduction

Integration of nerve cells with microelectronics has been subjected to extensive research in the last few years [1–3]. Neuro-electronic hybrid devices are considered to become a useful tool for the research of neurodegenerative diseases like Alzheimer's disease or Parkinson's disease. They offer the possibility to perform long-term studies of the

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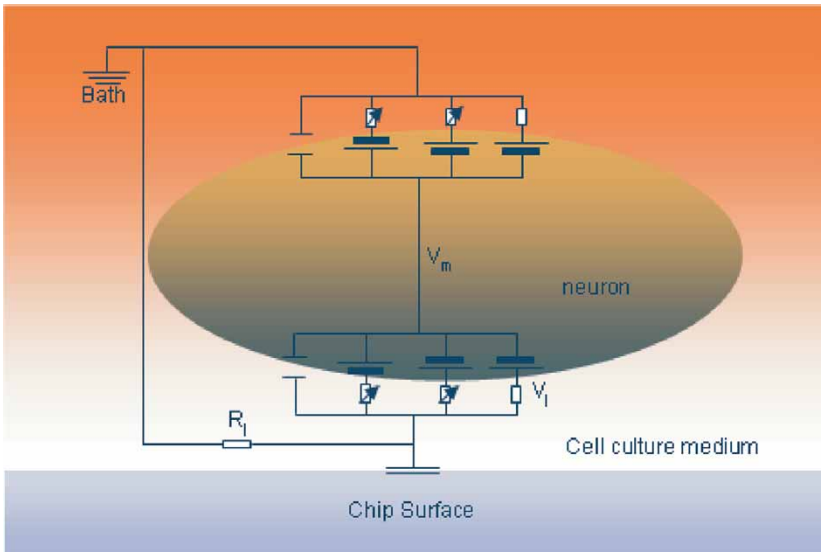

Figure 1. Electrical scheme of the coupling between a neuron and a field effect transistor (adapted from Schatzthauer and Fromherz [27]).

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electrical activity of each individual neuron in an entire predefined network of neurons in a non-invasive manner [4]. This is a great advantage compared to standard electrophysiological tools like the patch clamp technique. However, in order to record neuronal signals efficiently, tight coupling between the sensor and the cell membrane is one of the major requirements. When recording neuronal action potentials with field effect transistors, the coupling between a neuron and a sensor is capacitive, as depicted in figure 1 [1].

However, due to the presence of an ionic cleft between the cell membrane and the chip surface the signal to noise ratio of the recording can be poor [3, 5]. The easiest way to circumvent this problem is to bring the cell membrane closer to the chip surface. However this is a very complex interfacing problem that requires the merging of biological and electronic concepts at micro and nano-scale [6].

In this paper we describe a novel approach to achieve a tight membrane-surface coupling. A biological phenomenon, namely a phagocytosis-like process, is used to achieve tight coupling. Phagocytosis is a common process used by distinct cell types – although with a different magnitude – to take up large, micro-sized particles such as e.g. virusses, bacteria, etc [7]. The mechanism is different from other portals of entry commonly referred to as pinocytosis and occuring in all cell types [8]. Although phagocytosis is a primary route of entry in specialized cells such as macrophages, recent work has provided evidence that a similar mechanism is also occuring in neurons [9]. This knowledge can now be used to trigger phagocytosis-like events between the neuronal cell membrane and micron size nail (μ nail) structures decorating the active surface of a microelectronic device. The presence of the μ nails offers two major advantages:

Firstly, due to the presence of the μ nails, the surface of the active sensor area is increased. Therefore the signal to noise ratio will already increase. The presence of the

μ nails offers also a secondary advantage. If phagocytosis-like events could be induced around those μ nails, the coupling should improve because the distance between the sensor surface and the cell membrane in a phagocytic cup should be minimal.

However in order to acquire this, a specialized surface chemistry needs to be applied on the μ nails. The purpose of this surface chemistry is to present biomolecules (e.g. peptides, proteins, etc.) that bind specifically to cell membrane receptors. Poly-L-Lysine (PLL) is conventionally used for culturing neurons on plastic dishes or glass coverslips. However this type of coating is not interesting for our application since its interaction with the cell is purely electrostatic moreover the thickness of the PLL layer is non uniform and difficult to control. For these reasons, we turned to a small laminin derived peptide namely PA22-2. Laminin has diverse biological activities including promoting cell adhesion, migration, differentiation and growth of neurite extensions [10, 11]. Extensive research has been conducted in defining the active sequences in this protein that are responsible for all its functions. PA22-2 is situated in the A-chain of laminin and is 19 amino acids long. It is especially known to promote cell adhesion and stimulate neurite outgrowth [10–12]. Its small uniform size also makes it an attractive molecule for this application.

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Secondly, as a model to test the effect of the surface chemistry on the phagocytosis-like events, we used coated micro- and nano-sized beads which were added to cultured cells. The receptor that has been described to be involved in phagocytic-like events in hippocampal neurons is telencephalin (TLN). This protein is a member of the small family of Intercellular Cell Adhesion Molecules or ICAMs and is also referred to as ICAM-5. TLN is the sole member expressed in neurons and within neurons, its localization is restricted to the dendritic and somatic plasmamembrane [9, 13, 14]. TLN plays an important role in mediating the adhesion between two individual neurons via homophilic interactions and between a neuron and the extracellular matrix (ECM) via interactions with integrin $\alpha_L\beta_2$ [15, 16]. It was shown that TLN mediated a phagocytosis-like event using uncoated beads in hippocampal neurons [9]. Therefore TLN is likely to be an important receptor for our studies.

In this paper we describe the surface chemistry protocols, developed for the anchoring of the PA22-2 peptide on a gold surface and subsequently on mico- and nano-sized beads. These functionalized beads were subsequently used to study the phagocytosis-like events on cell lines and on primary neuron cultures.

2. Material and methods

2.1. *Peptide synthesis*

The PA22-2 peptide (sequence: CSRARKQAASIKVAVSADR) was custom made by Pepscan NV (Lelystad, The Netherlands).

2.2. *Surface chemistry*

The peptide is constructed in such way that it posseses a unique aminoterminal cysteine. This allows us to control the peptide orientation on the surface [17, 18]. The selected Self Assembled Monolayer (SAM) is a 100% 16-mercaptohexadecanoic acid

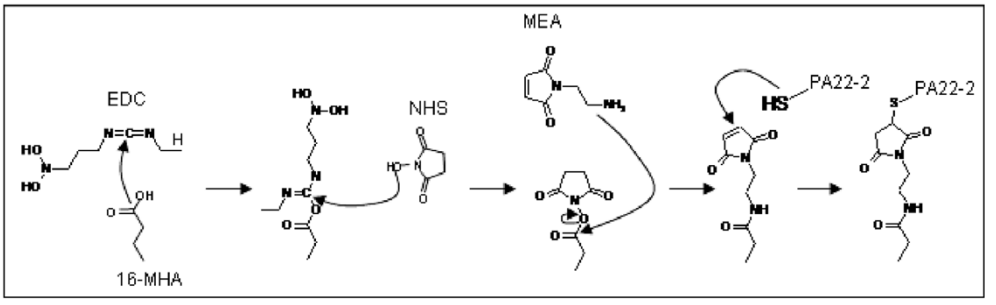

Figure 2. Schematical representation of the chemistry used to couple the PA22-2 peptide on the 16 MHA.

(16-MHA) (Sigma) [19]. The SAM is spontaneously formed by exposing the gold surface to the 16-MHA [20] during a 3 h incubation in a 10 mM 16-MHA solution. In a second step the resulting carboxyl surface was transformed into a maleimide surface. This modification was accomplished by treating the surface first with 0.1 M n-(3-diethylaminopropyl)-n-ethyl-carbodiimide (EDC) and 0.4 M n-hydroxysuccinimide (NHS) (both from Sigma). The activation of the carboxyl groups is necessary to couple in a second step the maleimide crosslinker, namely 2-maleimidoethylamine (MEA) (Molecular Biosciences). For this step a 50 mM MEA solution in 10 mM NaBorate buffer pH 8.5 was used. The active maleimide groups on the surface were used to couple the peptide covalently. A fresh peptide solution of 1 mg/ml was used for every experiment (figure 2).

The coupling was investigated qualitatively using Fourier Transform infrared spectroscopy (FTIR) on a Bruker IFS 66v/s system. Grazing Angle FTIR was used to study the immobilization of the peptide on gold surfaces. The test samples were gold plated silicon wafer pieces with the dimensions of 5×1 cm. After preparation of the required surface chemistry, they were dried in N_2 flow and immediately measured. To check the immobilization of the peptide on the beads, 75 µl bead solution was mixed with 150 mg KBr and dried for 48 h in a vacuum oven at 40°C to eliminate all the water. Thereafter they were compressed to transparent disks on which the FTIR analysis was performed. Peptide immobilization was studied quantitatively using the Surface Plasmon Resonance (SPR) technique in a Biacore 2000 system.

2.3. *Neuronal cell cultures*

Hippocampal neurons were isolated from E18 FVB strain mice according to established procedures [21]. Timed-pregnant mice were euthanized, and embryos were isolated. Hippocampi were dissected from both hemispheres in sterile Hanks' buffered saline solution (HBSS from Invitrogen) and incubated in 0.25% trypsin for 15 min in an incubator at 37°C and 5% CO_2 atmosphere. After trypsinization, cells were washed three times with HBSS and mechanically dissociated. The number of cells originating from half a hippocampus were seeded on the substrates (either 6 glass coverslips or 6 gold substrates) in Neurobasal medium containing 2% B27 supplement

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(both from Invitrogen) and 0.125% glutamate. After 4 h, most hippocampal cell bodies adhered to the surface and the subtrates were transferred upside down into dishes with a semi-confluent monolayer of glia cells, so that the neurons were facing the glia feeding layer. The substrates were suspended upside down above this feeding layer. After four days in co-culture, the media was changed from Neurobasal medium containing glutamate to Neurobasal medium without glutamate. The glia cells were cultured one week prior to the hippocampal culture from newborn FVB pups in the same manner as described for the hippocampal culture, except that these were seeded in 6 cm dishes coated with PLL (Sigma) and containing MEM medium (Invitrogen) supplemented with 10% horse serum. Four hours prior to adding the neuronal culture, medium was replaced by Neurobasal medium containing 2% B27. After 24 h in culture, arabinoside-C was added to prevent glial overgrowth.

2.4. *Cell lines*

HeLa cells were cultured in DMEM/F12 (1:1) supplemented with 10% fetal calf serum (FCS). Cells were seeded in 6-well plates at 150 000 cells/well and transiently transfected using Fugene 6 (Roche) with a plasmid encoding full length TLN (pSG5FLTLN). Stable TLN expressing HeLa cells were generated by cotransfecting pSG5FLTLN with a plasmid carrying the neomycin resistance gene and selected in the presence of 500 μg/ml G418 (Invitrogen).

Neuro-2a cells were cultured in DMEM/F12 (1:1) medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). To observe the effect of different surface chemistries on cell proliferation, the Alamar Blue Assay (Biosource) was used. Upon seeding of the cells at a density of 40 000 cells/ml, 10% Alamar blue was added to the cell culture medium and every hour 100 μl of this cell medium was transferred into a 96 well plate and the absorbance at 570 nm and 600 nm was immediately measured using a multi-well plate reader.

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2.5. *Bead experiments*

Yellow green fluorescent polystyrene beads of either 1 μm or 500 nm in diameter with a carboxyl coating (Invitrogen) were coated using the surface chemistry described above. After every step the beads were centrifuged at 4°C in a swinging bucket rotor at 1000 rpm for 5 min. The quality of the coating was observed using FTIR. Subsequently they were added to cultured cells at a density of 0.3×10^7 beads/ml medium and incubated for 24 h.

2.6. *Immunocytochemical stainings*

Cells were fixed using 4% paraformaldehyde and permeabilized in PBS containing 0.5% TX-100 for 5 min. After washing (3 times for 5 min in PBS), cells were blocked overnight at 4°C followed by a 1.5 h incubation in the same blocking solution containing a polyclonal anti-TLN antibody [22] at room temperature. After washing, immunoreactivity was revealed by incubation (1 h, room temperature) with Alexa 488 conjugated goat-anti-rabbit secondary antibodies (Invitrogen). To label actin filaments,

alexa 647 conjugated phalloidin was included in the incubation mix. Prior to mounting in moviol the cells were washed 3 times for 5 min with PBS and rinsed 3 times in water. 5 µl TOPRO-3 (Invitrogen) was also added to the moviol to stain the nucleus.

For labeling the plasma membrane of hippocampal neurons a lipophilic dye, $DilC_{12}$ (Invitrogen) was used immediately after fixation. The dye was incubated with the neurons at a final concentration of 1 µg/ml for 5 min and rinsed 3 times with PBS afterwards.

After staining cells were examined using an inverted microscope (Eclipse E800, Nikon) connected to a confocal microscope. (Radiance 2100; Zeiss) and images were acquired using LaserSharp 2000 software. Cultures on non-transparant gold substrates were examined using a CellR system (Olympus) equiped with an upright microscope (Olympus, BX 51-WI).

2.7. *Scanning electron microscopy (SEM)*

For SEM the neurons were fixed using 2.5% gluteraldehyde for 10 days at 4°C. Hereafter they were subjected to critical point drying. The cells were visualized using a Philips XL-30 electron microscope.

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3. Results

3.1. *Coupling of PA22-2*

Preconcentration experiments on SPR showed that the optimal pH value for coupling is 7.4 and that the coupling is significantly higher when using high salt concentrations. For this reason and the added advantage of being biocompatible, the best anchorage of the PA22-2 peptide is achieved using HBS buffer. The amount of peptide immobilized on the surface was 180 ng/cm^2 as monitored using SPR. By using an antibody against PA22-2 (kind gift of Prof. Dr. H. Kleinman), we were able to prove that the peptide is still recognized after coupling on the surface. Figure 3a and b shows the immobilization of PA22-2 as observed by Grazing Angle FTIR.

On the FTIR spectrum the binding of the maleimide group to the carboxyl group can be observed by the disappearance of the band around 1610 cm^{-1} and the appearance of the maleimide band around 1750 cm^{-1} (see also figure 3b showing an enlarged detail of the graph). Upon coupling of the peptide the carboxyl groups of the peptide cause the band around 1610 cm^{-1} to reappear and furthermore the amide band and the hydroxyl band between 3000 cm^{-1} and 3500 cm^{-1} become more pronounced. The bands at 2850 cm^{-1} and 2915 cm^{-1} are the typical bands of the $-CH_2$ groups in the alkane backbone of the 16 MHA.

3.2. *Biocompatibility*

One of the most critical parameters is the biocompatibility of the developed surfaces. Figure 4 shows the results obtained when hippocampal neurons were cultured on a surface coated with PA22-2 or PLL.

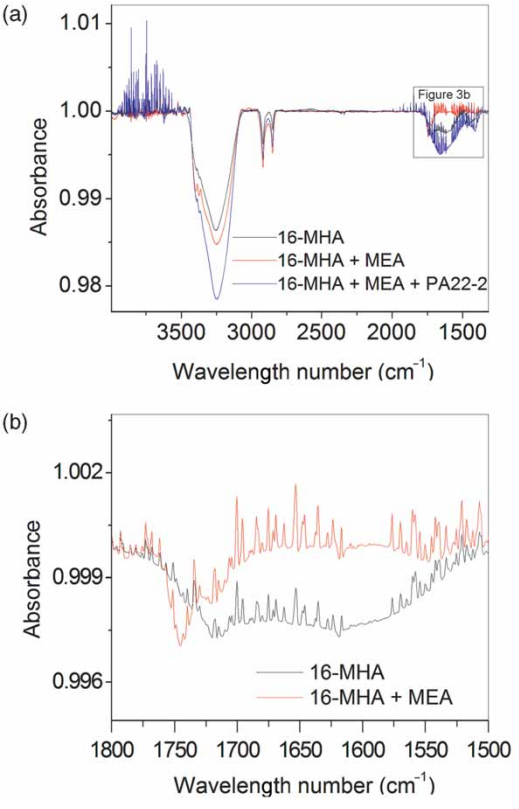

Figure 3. (a) Grazing Angle FTIR spectrum of PA22-2 immobilisation on a gold surface. Upon coupling of the peptide a large increase in the amide band and the hydroxyl band between 3000 cm^{-1} and 3500 cm^{-1} is observed as well as a reappearance of the carboxyl band at 1610 cm^{-1}, (b) Enlarged part of the Grazing Angle FTIR spectrum of PA22-2 immobilisation on a gold surface. The shift of the carboxyl band from 1610 cm^{-1} to the maleimide band at 1750 cm^{-1} upon coupling the MEA can be observed.

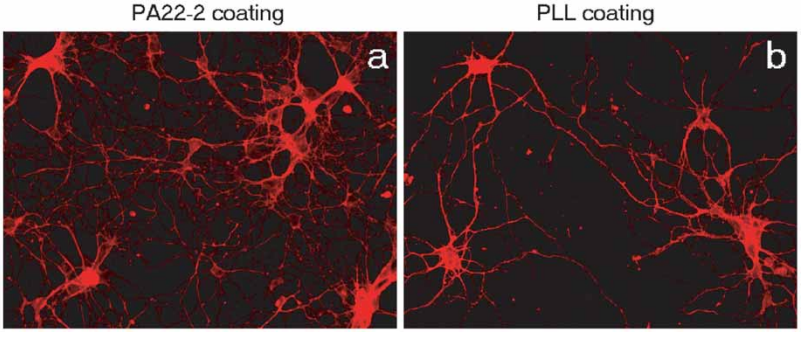

Figure 4. Comparable cultures of hippocampal neurons after 14 days in culture on PA22-2 (a) and PLL (b) coated gold substrates. More neurites are apparent on the PA22-2 coated surface.

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These experiments demonstrate that our surface chemistry results in comparable if not enhanced neuronal networks when compared to surfaces coated with PLL. In order to quantify this effect more in detail we have used the Alamar blue assay to study cell proliferation. However, this assay is toxic for the primary neuronal cultures and therefore the experiments were performed on a neuronal cell line, namely Neuro-2a. The results of the Alamar-blue assay (figure 5) show that proliferation over a period of 24 h on coated surfaces is similar for the PA22-2 coated substrates and the PLL coated substrates. On the other hand bare gold and a substrate coated with 16MHA are less benificial for the cellular proliferation.

3.3. *Phagocytosis experiments*

In order to study the occurance of phagocytosis-like phenomena, we used a model based on micro- and nano-sized beads.

At first the optimized surface chemistry, was transferred to the micro- and nano-sized beads. The peptide immobilization on these beads was investigated using FTIR spectroscopy. An FTIR spectrum of coated and uncoated beads is shown in figure 6.

Since the exact composition of the beads is not known, it is difficult to interpret the entire spectrum. Therefore it is best to look at the differences between the spectra of coated and uncoated beads. It can be observed that the uncoated beads lack the absorption band around $3500\,cm^{-1}$, which is a fingerprint for the amide bonds appearing upon coupling of the peptide.

Both the coated and uncoated beads were added to the cell culture medium and incubated for 24 h. The first experiments were conducted on HeLa cells and the results are represented in figure 7a and c.

These results clearly show that the uncoated beads are scattered in between the cells while the coated beads are far more frequently bound to the cells. This is also an indirect evidence of peptide presence onto the beads.

Since TLN was previously shown to be involved in phagocytosis-like events in neurons [9] and is not expressed in HeLa cells, we generated stable cell lines expressing TLN and studied the effects upon addition of uncoated and coated microbeads (figure 7b and d). As with HeLa cells, essentially no uncoated beads were found associated with TLN expressing cells. In contrast however the PA22-2 coated beads were highly clustered to TLN expressing cells. Figure 8a and b show in more detail the binding of the beads to HeLa cells that were transiently transfected with TLN.

This allows us to observe the effect of the beads in a mixed culture of TLN expressing and control (non-transfected) cells. The immunostainings show that the coated beads are enriched on the TLN expressing cells compared to control cells. In these cultures we find cells expressing various levels of TLN (figure 8b) and it appears that the higher the expression level of TLN, result in more associated coated beads.

After these initial experiments the beads were also added to the primary cultures of hippocampal neurons (figure 8c and d).

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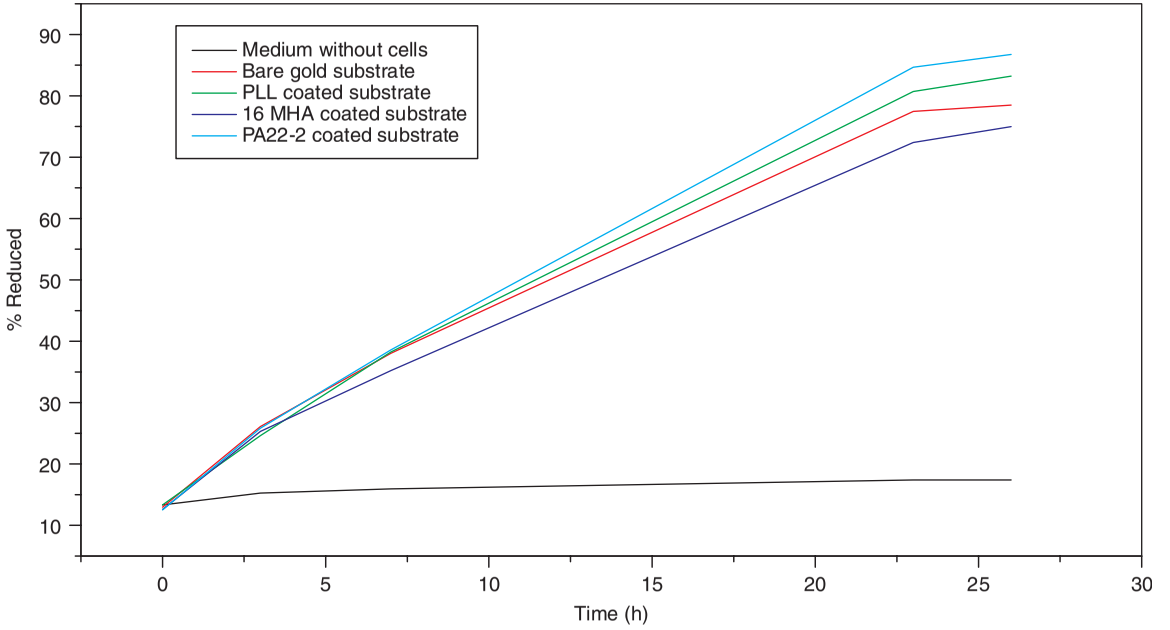

Figure 5. Proliferation results obtained with the Alamar Blue assay performed with Neuro-2a cells on differently coated substrate. The substrate coated with PA22-2 obtains similar results compared to PLL but is significantly better in sustaining the proliferation compared to bare gold substrates or 16 MHA coated substrates.

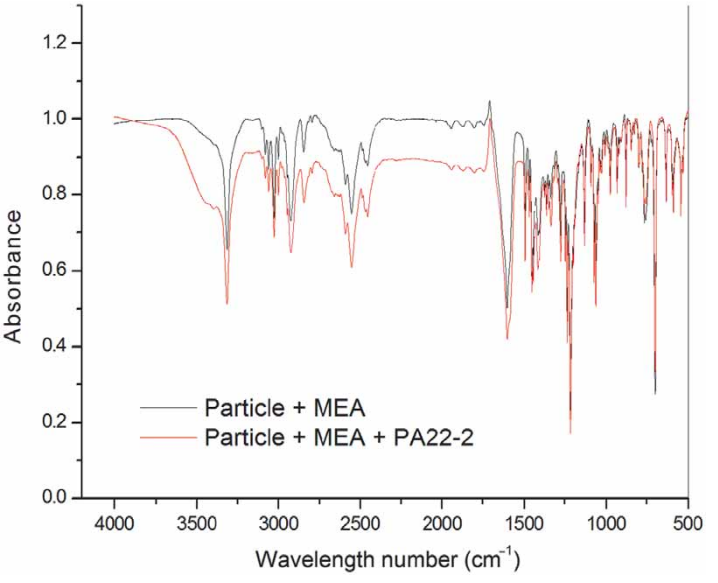

Figure 6. FTIR spectrum of microbeads coated with PA22-2 compared to control beads. The amide band appearing around 3500 cm^{-1} is typical for the presence of peptide on the beads.

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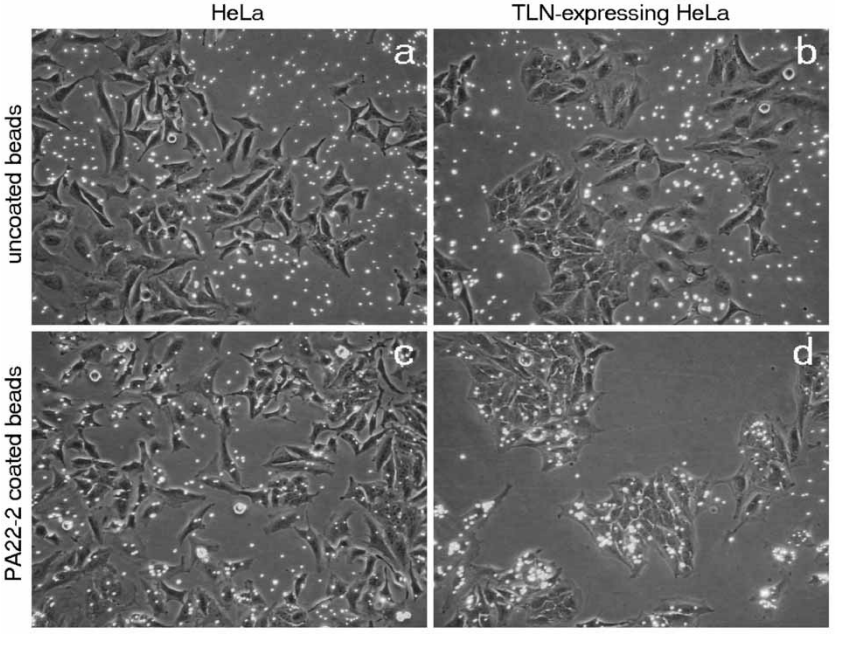

Figure 7. Interaction of HeLa cells with microbeads. (a) and (c) are control HeLa cells, (b) and (d) are HeLa cells stably transfected with full length TLN. The uncoated beads (a) and (b) are scattered in between the cells. The PA22-2 coated beads (c) and (d) bind to control HeLa cells and with more preference to the stable TLN expressing line.

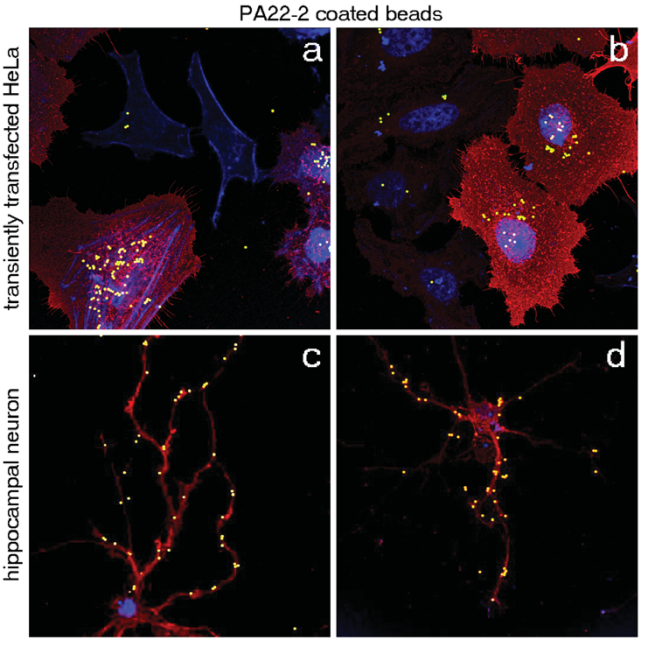

Figure 8. Higher magnification of the interaction between coated beads and transiently transfected HeLa Cells (a and b) and 7 days old primary culture of neurons (c and d). After incubation with the coated microbeads (green) and staining for TLN (red) and actin and nucleus (blue), it is shown that the beads are enriched on the TLN positive cells in panels a and b and that the coated beads redistribute along the neurites as observed in panel c and d.

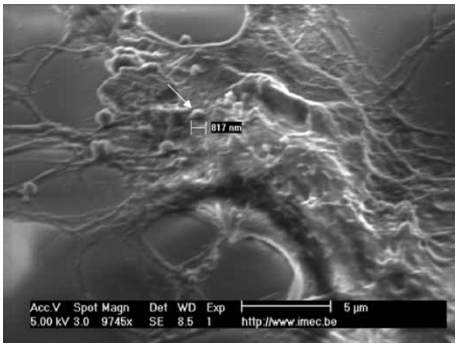

Figure 9. SEM picture of the neuronal cell membrane engulfing PA22-2 coated particles. The arrow points towards an apparently partial engulfed particle.

From these experiments we can deduce that the beads are preferentially bound to the dendrites. To observe the membrane topography, SEM analysis of the coated beads on neurons was conducted (figure 9).

These experiments demonstrated again the association of coated beads with neurites. In some cases beads even appeared to be engulfed by the neuron (arrow in figure 9).

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4. Conclusion

For the development of efficient neuron-electronic hybrid devices the coupling between the cell membrane and the sensor surface is a critical parameter. The strength of this coupling determines the signal to noise ratio of the recorded signals. Our approach to ameliorate the coupling consists of using small golden needles of micro-size that enlarge the sensor surface and create the possibility for the neurons to engulf those μ-nails in a phagocytosis-like process.

In order to obtain such events a specialized surface chemistry needs to be applied on the μ-nails. In this paper we describe the development of such surface chemistry together with its application in experiments involving a phagocytosis-like event.

Although PLL is commonly used as a coating to adhere primary hippocampal neurons, we decided not to use it because the thickness of the PLL layer is not controlable and the interaction with cells is based on an electrostatic interaction as opposed to a ligand-receptor interaction that occurs when using PA22-2. Therefore we turned towards small peptides such as PA22-2, a 19 amino acid long peptide that promotes cell adhesion and stimulates the neurite outgrowth and is derived from laminin.

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PA22-2 is known to bind to integrin $\alpha_6\beta_1$, the most widely expressed neuronal integrin [23]. Integrins are a family of receptors, comprised of an α and β subunit, which mediate interactions of cells with components of the extracellular matrix [24, 28]. Following adhesion and clustering, integrins recruit different cytoskeletal and cytoplasmic proteins, which anchor the newly formed complexes to the actin cytoskeleton [25, 26].

Our first challenge was to couple the peptide in a controlled way. We demonstrate that it can be immobilized in a controlled way on a gold surface and on micro- and nano-sized beads. This is realized by using an aminoteminal cysteine that allows us also to have directional control over the peptide orientation on the surface. The immobilization is qualitatively and quantitatively controlled by both SPR and FTIR. Using an Ab against PA22-2, we showed that the peptide is still recognized after coupling on the surface.

The biocompatibility of the coated surface was an next important issue. The growth and proliferation of Neuro-2a cells on our new substrate is indistinguishable from that of a reference substrate coated with PLL. The growth on bare gold or 16 MHA is, as expected, less pronounced compared to PA22-2 or PLL. This could be explained because the cell membrane is negatively charged just like the 16 MHA layer without peptide. Therefore there will be an electrostatic repulsion between the cell and the layer. On bare gold, proteins tend to denature and due to this denaturation the growth is most likely slowed down.

If hippocampal neurons are cultured on these substrates, apparently they form more dendrites compared to the ones cultured on PLL. However we were unable to quantify the biocompatibility of neurons on our substrates as the alamar blue assay was on its own toxic for the neurons.

Next, the phagocytosis-like events were monitored using micro- and nano-sized beads. The surface chemistry that we optimized in the first step was transferred to these beads and the effect of the beads on stable cell lines and primary cultures of

hippocampal neurons was observed by means of indirect immunofluorecence microscopy (confocal microscopy) and SEM. The peptide immobilization was confirmed by FTIR spectroscopy and was reminiscent of the immobilization on gold surfaces.

Using HeLa cells we can convincingly show that the PA22-2 peptide was required for the adherence of the microbeads to the cells. Interestingly when TLN is expressed (stably or transiently) in HeLa cells, a dramatic increase in the number of beads associated with transfected cells was observed. This is most likely due to the fact that TLN is involved in phagocytosis-like events [9]. By combining now the functionalized beads with expression of a cell adhesion molecule like TLN that functions in phagocytosis, we could therefore significantly increae the immobilization of the beads on the HeLa cells.

Similar results were obtained using hippocampal neurons. Immunostainings showed that the beads align perfectly along the dendrites and the soma of the neurons where endogeneous TLN is exclusively localized. SEM analysis also allowed us to study in more detail the membrane topography around the bead. It appears that some beads are partially engulfed by the cell membrane and suggests that there is a specific interaction between the beads and certain receptors during a phagocytosis-like event.

The next step is to transfer this surface chemistry on a chip with μ nails; to grow neurons on these chips and to start morphological and physiological studies on the behavior of these neurons. In a later stage the characterization of the capacitive coupling between the neurons and the chip will be required. In conclusion, the data we present here provide an exciting new avenue to generate a more intimate coupling between neurons and chips, a crucial element in the further development of neurons-on-chip.

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