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REPORT

Three-Dimensional Carbon Nanotube Electrodes for Extracellular Recording of Cardiac Myocytes

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Abstract Low impedance at the interface between tissue and conducting electrodes is of utmost importance for the electrical recording or stimulation of heart and brain tissue. A common way to improve the cell–metal interface and thus the signal-to-noise ratio of recordings, as well as the charge transfer for stimulation applications, is to increase the electrochemically active electrode surface area. In this paper, we propose a method to decrease the impedance of microelectrodes by the introduction of carbon nanotubes (CNTs), offering an extremely rough surface. In a multi-stage process, an array of multiple microelectrodes covered with high quality, tightly bound CNTs was realized. It is shown by impedance spectroscopy and cardiac myocyte recordings that the transducer properties of the carbon nanotube electrodes are superior to conventional gold and titanium nitride electrodes. These findings will be favorable for any kind of implantable heart electrodes and electrophysiology in cardiac myocyte cultures.

Keywords Microelectrode array · MEA · Carbon nanotubes · Extracellular recording · Three dimensional electrodes · Cardiac myocytes

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1 Introduction

Microelectrode arrays (MEAs) have been applied for in vivo and in vitro recording and stimulating of electrogenic cells, namely neurons and cardiac myocytes. The extracellular recording technique with MEAs permits a minimum of adverse effects on the cells, making long-term applications such as brain or heart tissue implants possible.

Biocompatibility and good electrical coupling at the interface between the solid-state devices and cardiac tissue are evidently important issues for this kind of electrode. While biocompatibility is primarily influenced by the materials at the chip's surface, electrical coupling is strongly determined by the impedance at the interface. Low impedance allows for low noise, implicating an improved signal-to-noise ratio (SNR) essential for the detection of very small amplitudes of action potentials in a noisy background. This low impedance is obtained with a large surface area. With the constraint to conserve the lateral dimensions of the microelectrodes, conventional noble metal electrodes are often coated with rough titanium nitride (TiN) or platinum black to increase the surface roughness. Platinum black can be easily deposited onto electrode sites, but its long-term stability is low, making it unacceptable for implants [13]. Rough TiN is the state of the art coating for microelectrode array chips [5] with good adhesion properties.

A promising new approach to this issue is the application of nanomaterials such as gold nanorods [2], carbon nanofibers [18], and carbon nanotubes (CNTs). The latter are characterized by excellent chemical [24], mechanical [22], and electrical properties [21]; they also exhibit very high biocompatibility [3] and have intrinsically large surface areas.

One approach to manufacturing electrodes coated with CNTs is by depositing a solution of CNTs onto the electrodes [8, 9]. The advantages of this method are low process

temperatures and high flexibility in terms of diameter and type of nanotubes (single or multi walled). However, low adhesion between nanotubes and electrodes make this approach rather unattractive for implants. This disadvantage might be overcome by using adhesion promoters such as conducting polymers as a link between CNTs and the metallic electrodes as reported by us elsewhere [20].

For improved adhesion characteristics, Ben-Jacob and Hanein [1] directly synthesized CNTs with a diameter of about 82 nm onto TiN-microelectrodes through a chemical vapour deposition process with a Ni-catalyst layer. Wang et al. [25] synthesized vertically aligned CNTs with an average diameter of approximately 16 nm onto polysilicon electrodes. To avoid the high temperature steps during CNT growth, the combination of CNTs with a polymer has only recently been proposed for deposition by electro polymerization [11]. The motivation for all publications mentioned above is the improvement of the electrical interface between the metal electrode and the neuronal cells.

Cardiac tissue has been recorded and stimulated successfully in vivo for more than 100 years by cardiac pacemakers [15]. According to Mond and Proclemer [17], more than 1,000,000 patients worldwide had a cardiac pacemaker implanted in their body by December 2009, with the number growing annually. However, good electrical contact between cardiac cells and electrodes is not only vital for implants; electrophysiology with cardiac cell cultures has also recently become popular in the field of drug research. In the past few years, extracellular recording with MEAs has become a standard method in this field [16].

With this as motivation, we investigated, for the first time, the potential of CNT microelectrodes for the recording of cardiac myocytes. For this purpose, CNTs with a diameter of only 4 nm were deposited onto planar gold microelectrodes. The extremely low diameter of the CNTs resulted in a high density, and therefore a high active surface area of the electrodes. In vitro cardiac myocytes were cultured on an array of CNT microelectrodes and extracellular recordings were performed.

Carbon nanotubes are reported to have no toxic effects on cardiomyocytes [10] although, cells that are cultured on CNT-substrates seem to show different physiological behavior which may result in a different amplitude or shape of the action potential [7]. The causes for these effects are not yet clear and need further research [14].

2 Materials and Methods

2.1 Microelectrode Array Fabrication

Microelectrode arrays were manufactured using standard microsystem technology (Fig. 1). Photoresist (AZ701mir, MicroChemicals GmbH, Ulm, Germany) was spin coated onto a 100 mm quartz wafer and photo lithographically patterned (Fig. 1a). To structure the electrodes with a diameter of 30 μm and connection lines with a width of 10 μm; 20 nm chrome (Cr) and 150 nm gold (Au) were thermally evaporated for a subsequent lift-off process (Fig. 1b). The wafer was then diced into single chips sized 15 × 15 mm, and 500 nm silicon dioxide (SiO_2) was sputtered onto the surface to insulate the circuit paths (Fig. 1c). In a second photoresist step (AZ9260, MicroChemicals), electrodes and bond pads were opened again by a dry etch process (80 % CF_4/20 % O_2 25 W) in a reactive ion etch chamber (Diener electronic, Ebhausen, Germany) (Fig. 1d, e).

The latter mask was then used a second time as a lift-off mask for the evaporation of titanium and the catalysts, aluminum (Al) and iron (Fe), onto the electrode sites. Finally, the chips were cleaned carefully in acetone and isopropyl alcohol, dried in inert gas (nitrogen, N or argon, Ar), and transferred to a three-zone CVD oven for the synthesis of the CNTs (Fig. 1f).

For assembly chips were glued to a printed circuit board (PCB) by a conducting polymer (PC3000, Heraeus, Hanau, Germany), and cured at 150 °C for 20 min. For improved leveling and bond strength, additional underfill glue (Polytec EP 630, Epotek PT, Waldbronn, Germany) was deposited and cured at 150 °C for 30 min. Due to its non-biocompatibility, the FR4 PCB material was covered by a thin layer of Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning Corporation, Wiesbaden, Germany) and thermally treated at 150 °C for 60 min. For cell cultivation experiments, a glass liquid containment was finally adjusted onto the chip. Obviously, care must be taken in assembling the chip, since some of the used material is not biocompatible and must be sealed with other biocompatible materials, such as PDMS.

2.2 Synthesis of Carbon Nanotubes

CNTs were synthesized by a water-assisted chemical vapour deposition process, according to a slightly modified protocol by Joshi et al. [12]. This procedure typically gives doubled walls CNTs with a diameter of 7–10 nm. The CNTs are free of any metal catalysts which are necessary and used for the CVD growth process. In a typical CVD experiment, 500 nm of titanium was evaporated onto the Cr/Au microelectrodes to fill the previously etched cavities. Thereafter, the growth catalysts, Al (30 nm) and Fe (3 nm), were deposited onto the electrodes. The bond pads were covered by a stencil mask during the evaporation processes in order to prevent the growth of CNTs on them. After a photoresist strip in acetone, the chips were heated to 800 °C in a 30 mm quartz tube under a flow of hydrogen

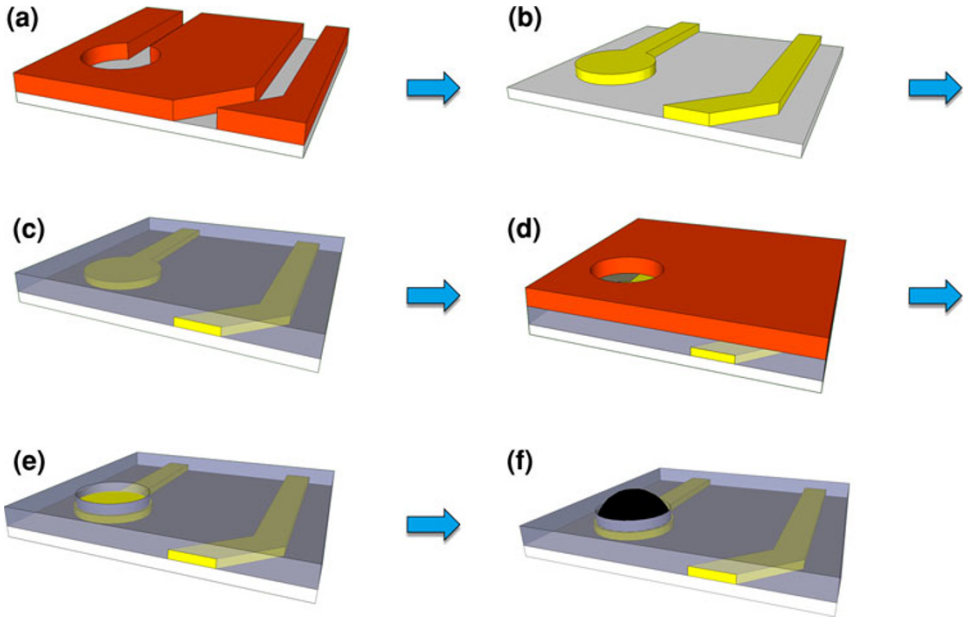

Fig. 1 Schematic representation of chip manufacturing. **a** Photoresist is spin coated onto substrate and photolithographically patterned. **b** Cr/Au electrodes and connecting lines are structured by a lift-off process. **c** SiO_2 is sputtered onto the electrodes. **d** Second resist mask is structured and **e** the isolation layer is opened for electrodes and bond pads. **f** Carbon nanotubes are synthesized by a chemical vapour deposition process [19]

(400 sccm) and argon (600 sccm) gases. When the growth temperature was reached, ethylene (100 sccm) precursor gas flow was initiated and a small amount of carrier gas was streamed through a water bubbler to carry a defined amount of water vapor along with it. The amount of water vapour was monitored by a water vapour sensor built in the line, and the gas flow was controlled by commercially available mass flow controllers (MKS instruments, Munich, Germany). After 20 min, the flow of all gases except argon was stopped and the oven were allowed to cool down to 30 °C before the electrode array was removed from the furnace for further assembling.

2.3 Cell Culture

The preparation of cardiac myocytes was carried out following a modified protocol of Daus and Thielemann [6]. Fertile chicken eggs (white Leghorn) were incubated at 37 °C in a surface breeder (Bruja 3000, Brutmaschinen-Janeschitz GmbH, Hammelburg, Germany). After 8 days, 8–12 chick embryos were removed, decapitated immediately, and transferred to an ice-cold Hank's buffered salt solution (HBSS, all chemicals: CCPro, Oberdorla, Germany, unless stated otherwise). The hearts were carefully removed and transferred to Ham's F12, where the tissue was chopped and then enzymatically dissociated with Trypsin/Ethylenediaminetetraacetic acid (EDTA, 0.05 %/0.02 %).

The chopped tissue was transferred into a centrifuge tube and then carefully washed with F12. After the tissue was settled, the supernatant was discarded and replaced by 8 ml Trypsin/EDTA solution. Again, after 10 min of incubation at 37 °C, the supernatant was discarded and replaced with 3.5 ml fresh Trypsin/EDTA solution. After 8 min at 37 °C, the supernatant was collected in 15 ml prewarmed cell culture medium Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal calf serum (FCS), 2 % chicken serum (CS), 0.5 mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin; once again, 3 ml fresh Trypsin/EDTA was then added to the tissue. This step was repeated 3–4 times, until the heart was completely dissociated. The resulting cell suspension was subsequently passed through a cell strainer (40 µm pore size) and centrifuged (900 rpm, 10 min). The pellet was resuspended in an aggregation medium (DMEM with 10 % FCS, 2 % CS, 4.5 g/l glucose, 0.5 mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin) and cells were cultivated in a culture flask in approximately 13 ml medium. After 60 min, the supernatant was harvested and the cell titer was counted in a Neubauer counting chamber. The suspension was set to 3.3×10^6 cells/ml.

75 µl of cell suspension with roughly 250,000 cells was cultured onto each electrode array and incubated in a humidified atmosphere (37 °C, 5 % carbon dioxide, CO_2) for 1.5 h. All array chips have been coated with fibronectin for 6 h (10 µg/ml in PBS) before cells were seeded. The containment rings of the MEAs were finally filled with about 1.5 ml culture medium, which was exchanged daily.

2.4 Recording and Signal Processing

Cellular signals (action potentials) were recorded with a sampling rate of 10 kHz using a preamplifier (Multi Channel Systems, Reutlingen, Germany) and custom-made Labview™ software. Further off-line signal processing was performed using a custom-made MATLAB™-based

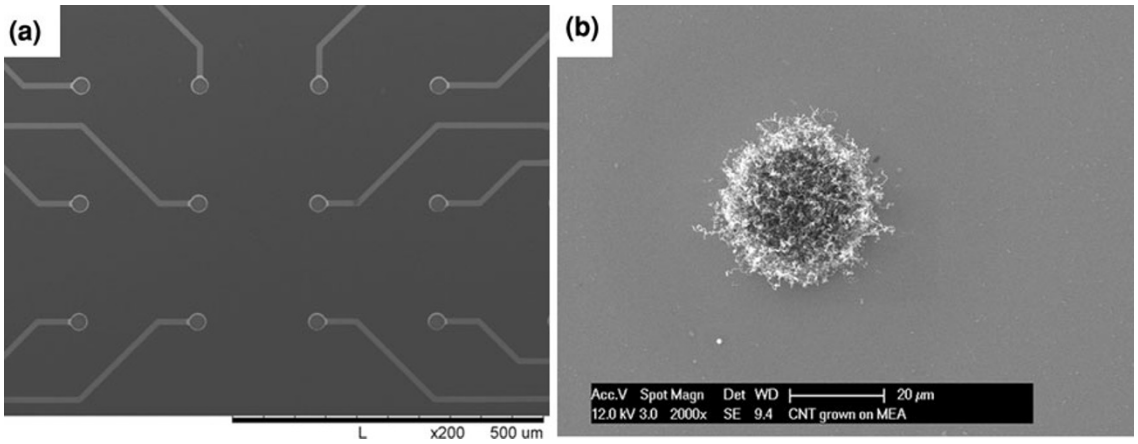

Fig. 2 CNT microelectrodes with a diameter of 30 µm. **a** Section of the electrode array including the circuit paths. **b** Single CNT-microelectrode on gold surface

software tool. For a reference potential, the bath was grounded with a silver/silver chloride reference electrode (Ag/AgCl electrode).

2.5 Characterization Methods

Scanning electron microscope (SEM) investigations were performed using a Philips XL 30 FEG microscope (now FEI, Hillsboro, USA) with an acceleration voltage of 20 kV. For all impedance measurements, a Solartron Modulab ModuLab-MTS system (Advanced Measurement Technology, Farnborough, United Kingdom) was used with a scanning range of 1 Hz–100 kHz and signal amplitude of 50 mV.

3 Results and Discussion

Carbon nanotubes were successfully synthesized onto the gold electrodes using a metal multilayer of Ti/Fe/Al as the growth substrate and catalyst.

For electrochemical characterization, impedance measurements were performed with an array of CNT-covered microelectrodes (Fig. 2a) in phosphate-buffered saline. The reference measurements were carried out with conventional planar gold microelectrodes, some of which were covered with rough TiN (Multichannelsystems, Reutlingen, Germany).

Measurements of the electrode impedances revealed an impedance sixteen times lower for the CNT electrodes at 1 kHz as compared to the state-of-the-art TiN electrodes, and an impedance 286 times lower than the planar gold electrodes with the same diameter. In numbers the impedance was measured at 1 kHz as 642.96 ± 221.1 kΩ for gold electrodes. It was 36.54 ± 1.7 kΩ for TiN electrodes and 2.25 ± 0.19 kΩ for CNT electrodes. These values lead to the improvement factor mentioned above.

The frequency of 1 kHz is within the range of biological signals (800–3,000 Hz) and therefore adequate for comparison of electrode properties.

The low impedance can be explained by the increase in the surface area. The CNTs lead to an extremely large surface that is accessible to the ions in the solution bath and thus can contribute to the interface impedance.

Finally, primary chicken cardiac myocytes were plated onto the new CNT microelectrodes and extracellularly recorded after 2 days in vitro. Again, reference measurements were carried out in the same manner, but with planar gold microelectrodes, some of which were covered with rough TiN. For cell culture experiments 15 chips where evaluated (5 × CNT, 5 × planar gold, 5 × rough TiN) with 59 measuring electrodes each. Cells formed a dense network on the electrode array no matter which electrode material was chosen, as shown in Fig. 3.

Cardiac cells adhered well to the CNTs and started to spontaneously contract after about 36 h. A recording result at a single electrode is shown in Fig. 4. Action potential amplitudes varied significantly over a single array, which is most likely due to variations in the cell adhesion properties. This let to high values of standard deviation. The average amplitudes were calculated to 196.66 ± 96.44 µV for CNT, 23.82 ± 4.05 µV for gold, and 65.92 ± 31.3 µV for TiN. In Fig. 4 action potential shapes are shown for each electrode type representing typical amplitudes.

Noise was characterized by measuring the standard deviation of action potential free time windows in the recorded signals. For TiN electrodes we measured a noise level of 4.58 ± 0.43 µV, for CNT electrodes 4.36 ± 0.46 µV, and for gold electrodes 7.31 ± 1.14 µV. Noise levels of CNT and TiN electrodes were comparable. This can be explained by the fact that other noise sources like amplifier and voltage source dominate the overall performance at low electrode impedances.

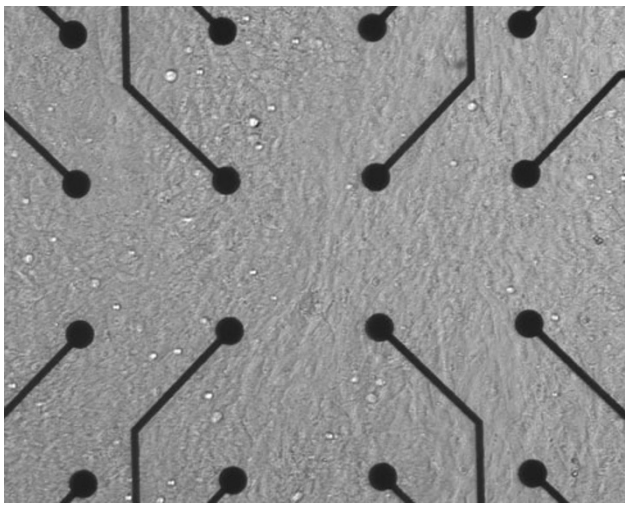

Fig. 3 Cardiomyocytes cultured on a TiN electrode array form a dense network and start to contract about 36 h after cultivation. Due to the formation of a tight cell layer, single cells are hard to distinguish. The distance between electrodes is 200 μm

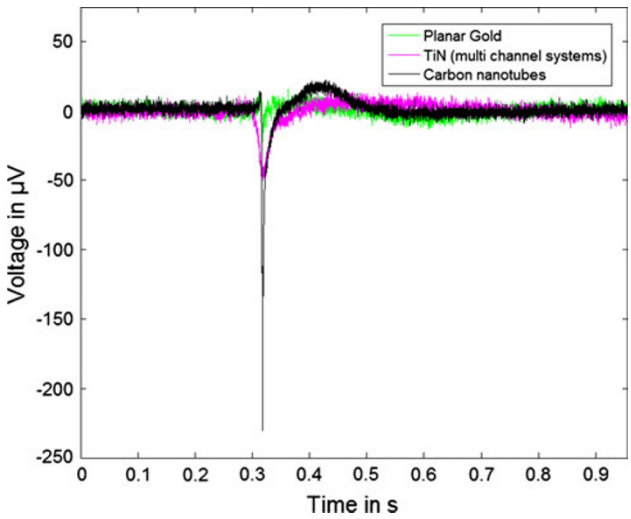

Fig. 4 Action potential of a cardiac myocyte recorded at 2 DIV with 30 μm diameter microelectrodes made of planar gold (*green*), rough TiN (*red*) and the custom-made carbon nanotubes (*black*)

Here, we define the SNR as the ratio of squared peak amplitude of the action potential to the variance of the noise SNR $= (v_{\text{peak}}/\sigma_{\text{noise}})^2$, which is similar to the definition in Choi et al. [4]. With the values given above SNRs of 10 for gold, 207 for TiN and 2,030 for CNT are calculated. The superiority of the CNT electrodes becomes obvious, when compared to the state-of-the-art TiN electrodes; the SNR is about tenfold higher for the CNT electrodes, and even 185 fold higher compared to planar gold electrodes. It should be mentioned that action potential measurements of gold electrodes were superimposed by high noise levels and only one chip (out of five) could be evaluated for this calculation. This implicates that the SNR of planar gold electrodes is even lower.

In order to study the biocompatibility of the CNT electrodes we cultured primary, embryonic, cortical neurons on the CNT islands and observed their behavior up to 25 days in vitro. The cells connect and form clusters at the CNT electrodes which is comparable to the observations made by [23]. Electrophysiological experiments with the neural cell culture systems will follow in the near future.

4 Conclusion

The goal of our research activities was to tailor surface properties in such a way that a favorable interaction of the material and a biological cell system was achieved. Here, we presented the integration of CNTs onto planar gold microelectrodes for improved electrical coupling properties to cardiac myocytes. In a multistage process, an array of multiple microelectrodes covered with high quality, tightly bound CNTs is realized. It is shown that the signal recording properties, i.e., the SNR, are superior to conventional gold and to state-of-the-art TiN electrodes. These findings will be favorable for any kind of implantable heart electrode. Further, and perhaps even more striking, experiments with two- and three-dimensional in vitro cardiac myocyte models will be easier to evaluate, if it comes to the interpretation of the action potential geometrical features. Further work will focus on the characterization of the mechanical stability of the interface between the CNTs and the electrode.

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References

1. Ben-Jacob E, Hanein Y (2008) J Mater Chem 18:5181–5186
2. Brüggemann D, Wolfrum B, Maybeck V, Mourzina Y, Jansen M, Offenhäusser A (2011) Nanotechnology 22:265104
3. Cellot G, Cilia E, Cipollone S, Rancic V, Sucapane A, Giordani S, Gambazzi L, Markram H, Grandolfo M, Scaini DFG, Casalis L, Prato M, Giugliano M, Ballerini L (2008) Nat Nanotechnol 4:126–133
4. Choi J, Jung H, Kim T (2006) IEEE Trans Biomed Eng 53:738–746
5. Cogan S (2008) Annu Rev Biomed Eng 10:275–309
6. Daus A, Thielemann C (2010) Eur J Cell Biol 89 (S1):67–68
7. Fung A, Tsiokos C, Paydar O, Chen L, Jin S, Wang Y, Judy J (2010) Nano Lett 10:4321–4327

8. Gabriel G, Gómez R, Bongard M, Benito N, Fernández E, Villa R (2009) Biosens Bioelectron 24:1942–1948
9. Gambazzi L, Toma F, Goff A, Fuchsberger K, Cipollone S, Stelzle M, Prato M, Markram H Giugliano M (2010) Bidirectional interfacing of carbon nanotube substrates to neuronal networks. 7th Meeting on substrate-integrated microelectrodes Reutlingen, Germany, p 234–35
10. Garibaldi S, Brunelli C, Bavastrello V, Ghigliotti G, Nicolini C (2006) Nanotechnology 17:391–397
11. Gerwig R, Fuchsberger K, Herrmann T Stelzle M (2011) CNT- and CNT/polymer-composite electrodes for neuronal diagnostics in MikroSystemTechnik Kongress (Darmstadt, Germany), VDE Verlag GmbH, Berlin, p 701–703
12. Joshi R, Schneider J, Yilmazoglu O, Pavlidis D (2010) J Mater Chem 20:1717–1721
13. Kovacs G (1994) Enabling technologies for cultured neural networks. Academic Press, New York, p 121–65
14. Martinelli V, Cellot G, Toma F, Long C, Caldwell J, Zentilin L, Giacca M, Turco A, Prato M, Ballerini L, Mestroni L (2012) Nano Lett 12:1831–1838
15. McWilliam J (1889) Br Med J 1:348–350
16. Meyer T, Boven K, Gunther E, Fejtl M (2004) Drug Saf 27:763–772
17. Mond H, Proclemer A (2011) Pacing Clin Electrophysiol 34:1013–1027
18. Nguyen-Vu B, Chen H, Cassell A, Andrews R, Meyyappan M, Li J (2007) IEEE Trans Biomed Eng 54:1121–1128
19. Nick C, Joshi R, Schlaak H, Schneider J Thielemann C (2011) Multi electrode array with carbon nanotube electrodes for the extracellular detection of action potentials in MikroSystem-Technik Kongress (Darmstadt, Germany), VDE Verlag GmbH, Berlin, p 720–23
20. Nick C, Joshi R, Schneider J, Thielemann C (2012) Int J Surf Sci Eng (in press)
21. Park J, Rosenblatt S, Yaish Y, Sazonova V, Üstünel H, Braig S, Arias T, Brouwer P, McEuen P (2004) Nano Lett 4:517–520
22. Saito R, Dresselhaus G, Dresselhaus M (2001) Physical properties of carbon nanotubes. Imperial College Press, London
23. Sorkin R, Gabay T, Blinder P, Baranes D, Ben-Jacob E, Hanein Y (2006) J Neural Eng 3:95–101
24. Tasis D, Tagmatarchis N, Bianco A, Prato M (2006) Chem Rev 106:1105–1136
25. Wang K, Dai H, Fishman H, Harris J (2005) Proceedings of SPIE-microfluidics, bioMEMS and medical microsystems III, San Jose, p 22–29