Biosensors and Bioelectronics 25 (2010) 2309–2312

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Conducting polymer nanowire-based chemiresistive biosensor for the detection of bacterial spores

Cristina García-Aljaro[a,1], Mangesh A. Bangar[b], Eva Baldrich[a], Francisco Javier Muñoz[a,*], Ashok Mulchandani[b]

[a] Institut de Microelectrónica de Barcelona (CNM-IMB), CSIC, Esfera UAB, Campus UAB, 08193 Bellaterra, Barcelona, Spain
[b] Department of Chemical and Environmental Engineering, University of California, Riverside, 92521 CA, USA

ARTICLE INFO

Article history:
Received 8 February 2010
Received in revised form 15 March 2010
Accepted 16 March 2010
Available online 20 March 2010

Keywords:
Polypyrrole
Nanowire
Spore detection
Bacillus sp.
Biosensor
Bioterrorism

ABSTRACT

Polypyrrole nanowires (Ppy) were assembled onto microfabricated gold interdigitated microelectrodes, to construct a chemiresistive biosensor for the detection of *Bacillus globigii*, used as simulant of the threatening bioterrorism agent *B. anthracis*. The fabricated biosensor showed good linear correlation ($r^2 = 0.992$) for low spore concentrations ranging from 1 to 100 CFU (colony forming units)/mL, a concentration that could be used in a bioterrorism attack, with a response time of 30 min, after which the sensor was saturated. The performance of the biosensor was also assessed in the absence of anti-*B. globigii* antibodies and in the presence of non-target bacterial cells (*Escherichia coli*) showing no significant non-specific interactions. We believe that Ppy nanowires are a good platform for the detection and also quantification of large molecules and biocomponents even at low concentrations.

1. Introduction

Rapid detection of hazardous microorganisms is of high importance, not only to prevent the occurrence of human diseases, but also to establish the appropriate diagnosis to start medical treatment as soon as possible. Infection routes are traditionally food or waterborne through the consumption of contaminated aliments or recreational activities. The use of microorganisms as biological weapons is another global concern that threatens the security of the populations worldwide. The recent bioterrorism attack occurred in the US in 2001 resulted in the death of 5 people after infection with *Bacillus anthracis* spores dispersed through envelopes.

B. anthracis is a Gram positive spore-forming bacterium causing the anthrax disease. Traditionally, the detection methods for this bacterium are based on ELISA tests and/or PCR-based methods that require expensive equipment and experienced personnel (see Herzog et al. (2009) for review of different detection techniques). Alternative techniques have been explored but because of the safety level requirements (level 3) for the manipulation of this bacterium and the fact that not all laboratories can meet these requirements, studies have been often been performed with simulant bacteria such as *B. globigii* (Jung et al., 2003; Rowe et al., 1999; Stratis-Cullum et al., 2003, 2008).

One-dimension structures (1D), including nanowires, nanotubes, nanobelts and nanosprings are attractive platforms for the fabrication of biosensors (Wanekaya et al., 2006). The use of 1-D instead of two-dimension structures (2-D) has gained interest in last years which is reflected by the increase in the number of publications because of their unique properties. Silicon nanowires (Zheng et al., 2005), gold nanowires (Gamby et al., 2006), carbon nanotubes (CNTs) (Kim et al., 2009), and conducting polymers based nanowires such as polypyrrol nanowires (Ppy) (Bangar et al., 2009) have been used as building blocks for the fabrication of biosensors. 1-D nanostructures, among other characteristics, are expected to provide a better performance of the biosensor in terms of sensitivity, since they have a larger specific surface area per volume unit. Theoretically, this property is translated into a lower number of target molecules that are necessary to elicit a response by the biosensor. Accordingly, they have been demonstrated useful for the detection of small proteins down to 50 fg/mL (Zheng et al., 2005) or nucleic acids at femtomolar concentrations (Hahm and Lieber, 2004). Gold nanowires, for example, have been proven useful for the detection of small

* Corresponding author. Tel.: +34 93 5947700x1305; fax: +34 93 5801496.
E-mail addresses: francescxavier.munoz@cnm.es, francescxavier.munoz@imb-cnm.csic.es (F.J. Muñoz).
[1] Current address: Departament de Microbiologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain.

0956-5663/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2010.03.021

molecules like the prostate specific antigen (Pampalakis and Kelley, 2009).

Conducting polymers are very promising materials used to construct nanowires as they show unique electrical, physical and optical properties together with the fact that their synthesis is relatively easy and inexpensive when compared with other 1-D structures such as CNTs (Wanekaya et al., 2006). These structures have been successfully integrated onto microelectrodes to construct chemiresistive or field-effect transistors for protein detection (Bangar et al., 2009). The objective of this study was to investigate the suitability of Ppy nanowires for the construction of cost-effective, sensitive and ease-to-use biosensors to be used as chemiresistive biosensor for the detection of bioterrorism threatening agents, like *B. anthracis* spores, using *B. globigii* spores as surrogate. In this case, Ppy nanowires were assembled onto interdigitated microelectrodes, taking advantage of the enhanced sensitivity generated by this kind of microfabricated devices (Laczka et al., 2008b). Covalent functionalization of Ppy nanowires with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride/N-hydrosuccinimide (EDC/NHS) chemistry was chosen for immobilization of antibodies.

2. Experimental

2.1. Bacterial strains

B. globigii spores were obtained from NAMSA (Northwood, OH, USA) at a concentration of 10^7 CFU/mL in phosphate buffer (PB). A 10-fold dilution series was prepared in PB to be used for sensing with the biosensor. *Escherichia coli* K12 was used as negative control to evaluate the performance of the biosensor in the presence of interfering bacteria. With this aim, a stock culture was prepared by overnight culture of the cells in Luria Bertani (LB) broth at 37 °C, followed by washing the cells by centrifugation and resuspension of the cells in PB for three times.

2.2. Biomolecules and chemical reagents

Monoclonal antibodies specific towards *B. globigii* spores (anti-*B. globigii* MAb) were purchased from Tetracore (Rockville, MD, USA) and stored at 4 °C at a concentration of 1 mg/mL. All the chemicals used were of analytical grade and were used as received without any further purification unless otherwise stated. The PB stock solution (0.1 M, pH 7.4) was prepared by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 until reaching the desired pH. A 10-fold dilution of this stock (10 mM PB) was sterilized by filtration though a 0.22 μm pore sterile filter (Millipore Corporate, Billerica, MA, USA) and was used as working solution. All the solutions were prepared in double distilled water unless otherwise stated.

2.3. Polymerization of Ppy nanowires

Pyrrole was obtained from Sigma–Aldrich (St. Louis, MO, USA), distilled and stored at −20 °C until used. The polymerization was performed as described elsewhere (Bangar et al., 2009) using a three-electrode configuration cell, composed by a working electrode containing a 200 nm diameter pore alumina template (Whatman International Ltd., Maidstone, England), a platinum counter electrode, and a reference electrode (Ag/AgCl, 3 M KCl). A solution containing 0.5 M pyrrole and 0.2 M $LiClO_4$ (pH 6.3) was used as electrolyte for the PPy electrodeposition. The solution was purged for 30 min with 99.99% nitrogen for 30 min before deposition. The polymerization of Ppy was performed potentiostatically at 0.9 V vs. Ag/AgCl for 15 min and the template was removed as described by Bangar et al. (2009).

2.4. Working electrodes

The fabrication of the gold interdigitated electrodes was made using standard photolithographic techniques as described elsewhere (Streeter et al., 2007). After fabrication, the wafers were diced into 3 mm × 3 mm individual chips and then wire bonded to suitable print circuit boards. The encapsulating resin used to protect the connection pads of the PCB and wire bonds was an Epotek thermo-curable polymer. These devices, which consisted in digits 10 μm wide separated by 10 μm gaps, had been extensively characterized as described by Laczka et al. (2008b).

2.5. Biosensor fabrication

The working electrodes were first cleaned with ethanol and then electrochemically activated in 0.1 M KCl by applying a series of potential pulses from 0 to −2 V vs. Ag/AgCl (3 M KCl) using a CHI750C bipotentiostat run by a Windows XP based PC. After this, 10 μl of a 1:100 dilution of the above described nanowire stock solution were deposited onto the gold microelectrodes and aligned using AC electrophoresis at 4 MHz and 3 V peak-to-peak for 1 min. The excess of solution was removed. Subsequent anchoring of the nanowires was performed as described previously (Bangar et al., 2009) by gold maskless deposition at −0.5 V and room temperature for 10 min using Technigold (Technic Inc., CA, USA) as the electrolyte. The three-electrode cell included the above described counter and reference electrodes, and the interdigitated electrode with the assembled nanowires as working electrode.

2.6. Biofunctionalization of Ppy nanowires and sensing procedure

Biofunctionalization of the anchored nanowires was performed as previously described (Dhand et al., 2007). Briefly, the nanowires were incubated with 0.1 mg/mL of anti-*B. globigii* MAb (5 μl) in the presence of 60 mM EDC and 2 mg/mL NHS. The reaction was allowed to proceed for 3 h at room temperature. EDC/NHS reacts first with the –COOH groups in the antibody, and then to the –NH group on the nanowire surface. For the negative control experiments, designed to evaluate the level of spore non-specific binding, the antibody solution was replaced by bovine serum albumin (BSA) (0.1 mg/mL). After completion of the reaction, the electrodes were washed three times with PB–Tween 20 (0.1%, V/V) and three more times with PB, followed by sensor blocking with BSA (1%, W/V) for 1 h at room temperature and two new series of washing steps as described above. The biosensor was then ready to be used for the sensing purposes. Target capture was performed by sensor incubation with increasing concentrations of the microorganisms to be detected for 30 min at room temperature, followed by washing of the biosensor as described before prior to the measurement.

2.7. Electrochemical measurements

Linear sweep voltammetry measurements were performed using a semiconductor parameter analyzer (model 4155A, Agilent Technologies Inc., CA) from −0.2 to 0.2 V. The slope of the current/voltage (I/V) plot between −0.1 and 0.1 V was calculated using linear regression analysis and the inverse of this value, the resistance (R), was used for comparison purposes. All measurements were carried out in 10 mM PB, pH 7.4. All results are expressed as the mean of at least three independent replicates with its corresponding standard deviation.

3. Results and discussion

The dimensions of the fabricated Ppy nanowires (∼200 nm in diameter by 15–20 μm in length) were in accordance with data

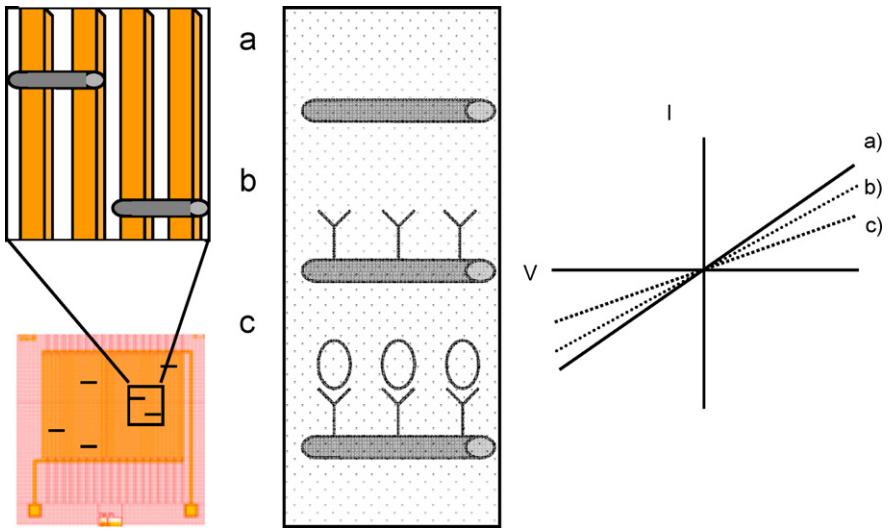

Fig. 1. Schematic representation of the biosensing platform after the assembly of PPy nanowires onto the gold interdigitated microelectrodes (left). Illustration of *I*/*V* plot (right) obtained at (a) bare nanowires, (b) Ab-functionalized nanowires, and (c) Ab-functionalized nanowires after spore immuno-capture. Nanowire modification contributes to increase its resistance (*R*).

reported previously for this electropolymerization method (Bangar et al., 2009). The stock solution of nanowires was diluted 100-fold in H_2O, deposited onto the electrodes' surface and subjected to electrophoretic alignment as described in Section 2 (Fig. 1). Assembly of the nanowires onto the interdigitated electrodes correlated with an initial change in resistance of around 100 kΩ (Fig. 1) that was highly reproducible. The electrodes were then kept at room temperature for 24 h to allow stabilization and were then subjected to biofunctionalization and biosensing.

Biofunctionalization of PPy nanowires with anti-*B. globigii* MAbs through EDC–NHS chemistry resulted in stable attachment of the Abs to the nanowires. An average increase of 60% in resistance was detected after EDC/NHS mediated Ab conjugation (Fig. 1) and was stable for at least 24 h (data not shown). The correct orientation of the Abs and their accessibility to their target is of outmost importance to produce a sensor with good sensitivity (Lu et al., 1996). While the covalent linkage method employed in this work can not guaranty the correct orientation of the Abs, it has some advantages over other methods usually employed for the biofunctionalization of conducting polymers (Cosnier, 2000) such as the physical entrapment (Minett et al., 2002) or direct adsorption (Khan and Wernet, 1997; Smith and Knowles, 1990). In the first case, the entrapment in the polymer can result in loss of activity of the biomolecules because of the harsh conditions used for the polymerization and therefore the suitability of the method will strongly depend on the characteristics of the biomolecule to be immobilized. On the other hand, direct adsorption of polyclonal Ab has been described to provide efficient and stable surfaces for the sensing of complex targets, such as bacteria (Laczka et al., 2008a), but usually generates poorer performance in the case of monoclonal Ab (Butler, 2000). Although covalent functionalization with EDC/NHS chemistry cannot guaranty the correct orientation of Abs, the fact that the nanowire structure has a high surface area/volume ratio may overcome this drawback by allowing exposure of enough Ab units attached to the surface. Also, the fact that as few as 5 μl of antibody solution is needed to perform the EDC–NHS reaction, it is a good immobilization method to be used with this kind of sensor. In fact, the results of a previous work comparing EDC/NHS chemistry to other covalent linking methods, such as glutaraldehyde coupling, suggested that the former generated more efficient immuno-functionalized nanowires for the production of chemiresistive sensors (Bangar et al., 2009).

The performance of the biosensor was assessed by monitoring the changes in the resistance of the system after successive incubations of increasing concentrations of bacterial spores. Using interdigitated microelectrodes was expected to provide enhanced system sensitivity compared to the use of a single pair of microelectrodes. The reason is that interdigitated electrodes of smaller features show significantly higher perimeter and smaller surface areas than single electrodes occupying a similar footprint. It has been demonstrated that the perturbation produced by the binding of big targets, such as bacteria, becomes more significant for electrodes of smaller active surface area (Laczka et al., 2008b). Additionally, the unique design and characteristics of interdigitated microelectrodes eliminates the need for a reference electrode, providing a simpler experimental setting than classical three or four electrode systems (Varshney and Li, 2009). As shown in Fig. 2, the fabricated biosensor showed a lower detection limit of 1 CFU/mL (3 times the standard deviation of the response obtained for blanks/buffer) and a linear dynamic range between 1 and 100 CFU/mL, with a response time of 30 min. The sensor response reached a plateau/saturation above 100 CFU/mL and no further increase in resistance was registered. Additionally, the biosensor responses were highly reproducible with small standard deviations. A possible explanation of the narrow dynamic range of the

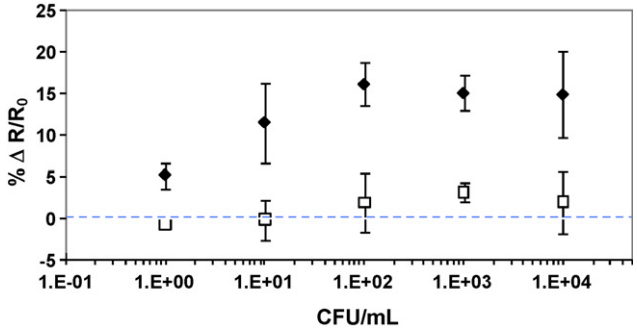

Fig. 2. Calibration plot for *B. globigii* spores biosensing at PPy nanowires covalently functionalized with anti-*B. globigii* Abs (♦) vs. PPy nanowires with no Abs, blocked with BSA (□). The differences in response confirm that spore detection is achieved through Ab-spore specific immuno-capture and not due to spore non-specific binding.

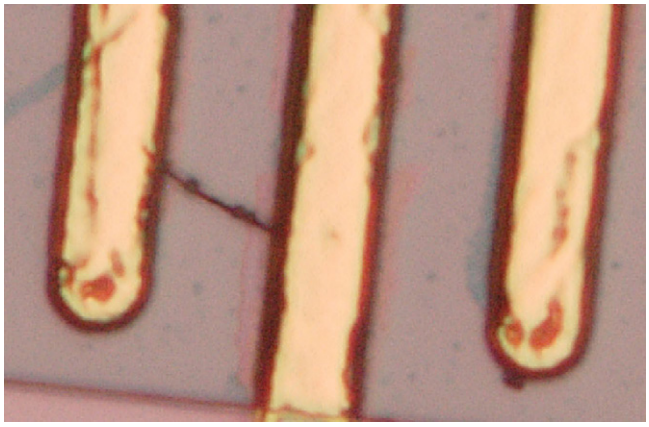

Fig. 3. Optical microscope image showing attachment of spores onto the PPy nanowires.

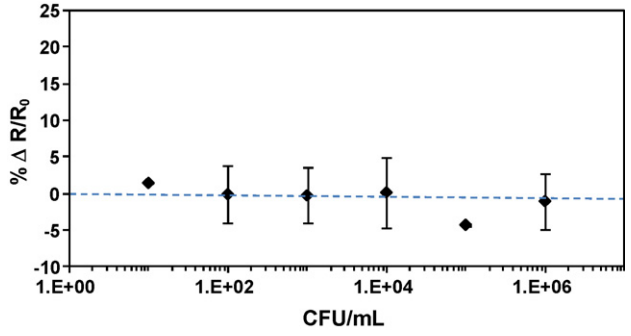

Fig. 4. Negative control experiment performed at PPy nanowires covalently functionalized with anti-*B. globigii* Abs in the presence of *E. coli*. The non-target bacterial cells generated undetectable levels of non-specific adsorption onto the sensing surface up to titers of 10^6 CFU/mL.

sensor is as follows. The isoelectric point of *B. globigii* spores ranges between 2 and 3.6 (Ahimou et al., 2001; Yamaha and Abe, 1934). Hence, at the pH used in the electrochemical measurements (pH 7.4), the spores display an overall negative charge. Taking into account that the average dimension (1.22 μm × 0.65 μm) of the spores (Carrera et al., 2007) is much greater than the Debye length characteristic of a semiconductor immersed in a 10 mM PB solution (Kim et al., 2009), only the negative charges on the section of the spore surface placed in proximity to the sensor surface contribute to the measured signal. Spore binding was additionally confirmed by optical microscopy (Fig. 3).

The specificity of the biosensor was also assessed by measuring the response in the absence of anti-*B. globigii* antibodies (negative control) (Fig. 2) and in the presence of non-target bacterial cells (*E. coli*) (positive control) (Fig. 4) in solution. None of these controls showed significant levels of non-specific interactions, even at very high concentrations of bacteria (10^6 CFU/mL) were used.

The use of nanowires for the fabrication of biodevices has attracted the interest of researchers for many applications including, among others, the detection of small proteins (Bangar et al., 2009) and DNA molecules. To our knowledge this is the first study reported using a chemiresistive nanowire-based sensor for the detection of bacterial spores. Pal et al. (2007) reported a direct electron transfer biosensor for the detection of *B. cereus* as model for *B. anthracis* based on sandwich assay format using two sets of polyclonal Ab coupled with polyaniline nanowires and capillary flow for the transport of reagents. The detection limit and dynamic range obtained by this millimeter size biosensor were similar to that obtained with our chemiresistive nanosensor. The results obtained in our work are very promising for the future development of low cost, sensitive, ease-to-use and portable bio-MEMs for the detection of spores using a straightforward methodology for the fabrication. Also, it is expected that the methodology developed in this study can be applied for the detection of other larger targets, such as bacteria and even yeasts, using an appropriate recognition molecule, since they all have isoelectric points below 7. Further, multiple targets can be analyzed simultaneously in very small sample and rapidly using an array with multiple sensors.

4. Conclusions

In summary, we have developed biosensor with excellent sensitivity, a detection limit of 1 CFU/mL and a dynamic range up to 100 CFU/mL in PB 10 mM. The results were highly reproducible. Besides, the ease of manipulation and the straightforward methodology needed for their synthesis makes them very promising structures to be used for the construction of label-free, cost-effective, ease-to-use, miniaturized biosensors for detection of different microorganisms.

.

Acknowledgments

This work was supported by the José Castillejo Program of the Ministry of Science and Innovation of Spain and grant CBET-0617240 from the United States NSF. Cristina García-Aljaro and Eva Baldrich are supported by the JAE Program of the Consejo Superior de Investigaciones Científicas (CSIC, Spain).

References

Ahimou, F., Paquot, M., Jacques, P., Thonart, P., Rouxhet, P.G., 2001. J. Microbiol. Methods 45 (2), 119–126.
Bangar, M.A., Shirale, D.J., Chen, W., Myung, N.V., Mulchandani, A., 2009. Anal. Chem. 81 (6), 2168–2175.
Butler, J.E., 2000. Methods 22 (1), 4–23.
Carrera, M., Zandomeni, R.O., Fitzgibbon, J., Sagripanti, J.L., 2007. J. Appl. Microbiol. 102 (2), 303–312.
Cosnier, S., 2000. Appl. Biochem. Biotechnol. 89, 127–138.
Dhand, C., Singh, S.P., Arya, S.K., Datta, B., Malhotra, B.D., 2007. Anal. Chim. Acta 602, 244–251.
Gamby, J., Abid, J.P., Abid, M., Ansermet, J.P., Girault, H.H., 2006. Anal. Chem. 78 (15), 5289–5295.
Hahm, J.I., Lieber, C.M., 2004. Nano Lett. 4, 51.
Herzog, A.B., McLennan, S.D., Pandey, A.K., Gerba, C.P., Haas, C.N., Rose, J.B., Hashsham, S.A., 2009. Appl. Environ. Microbiol. 75 (19), 6331–6339.
Jung, C.C., Saaski, E.W., McCrae, D.A., Lingerfelt, B.M., Anderson, G.P., 2003. IEEE Sens. J. 3 (4), 352–360.
Khan, G.F., Wernet, W., 1997. Thin Solid Films 300 (1–2), 265–271.
Kim, J.P., Lee, B.Y., Lee, J., Hong, S., Sim, S.J., 2009. Biosens. Bioelectron. 24 (11), 3372–3378.
Laczka, O., Baldrich, E., del Campo, F.J., Munoz, F.X., 2008a. Anal. Bioanal. Chem. 391 (8), 2825–2835.
Laczka, O., Baldrich, E., Munoz, F.X., del Campo, F.J., 2008b. Anal. Chem. 80 (19), 7239–7247.
Lu, B., Smyth, M.R., Okennedy, R., 1996. Analyst 121 (3), R29–R32.
Minett, A.I., Barisci, J.N., Wallace, G.G., 2002. React. Funct. Polym. 53 (2–3), 217–227.
Pal, S., Alocilja, E.C., Downes, F.P., 2007. Biosens. Bioelectron. 22 (9–10), 2329–2336.
Pampalakis, G., Kelley, S.O., 2009. Analyst 134 (3), 447–449.
Rowe, C.A., Tender, L.M., Feldstein, M.J., Golden, J.P., Scruggs, S.B., MacCraith, B.D., Cras, J.J., Ligler, F.S., 1999. Anal. Chem. 71 (17), 3846–3852.
Smith, A.B., Knowles, C.J., 1990. Biotechnol. Appl. Biochem. 12 (6), 661–669.
Stratis-Cullum, D.N., Griffin, G.D., Mobley, J., Vass, A.A., Vo-Dinh, T., 2003. Anal. Chem. 75 (2), 275–280.
Stratis-Cullum, D.N., Griffin, G.D., Mobley, J., Vo-Dinh, T., 2008. Anal. Bioanal. Chem. 391 (5), 1655–1660.
Streeter, I., Fietkau, N., Del Campo, J., Mas, R., Munoz, F.X., Compton, R.G., 2007. J. Phys. Chem. C 111 (32), 12058–12066.
Varshney, M., Li, Y., 2009. Biosens. Bioelectron. 24 (10), 2951–2960.
Wanekaya, A.K., Chen, W., Myung, N.V., Mulchandani, A., 2006. Electroanalysis 18 (6), 533–550.
Yamaha, G., Abe, S., 1934. Sci. Rep.: Tokyo Bunrika Daigaku Sect. B 1 (21), 221–229.
Zheng, G.F., Patolsky, F., Cui, Y., Wang, W.U., Lieber, C.M., 2005. Nat. Biotechnol. 23 (10), 1294–1301.