Research Article

Journal of Molecular Recognition

Received: 24 May 2013, Revised: 17 July 2013, Accepted: 31 July 2013, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2304

Visualization of the membrane engineering concept: evidence for the specific orientation of electroinserted antibodies and selective binding of target analytes

Anna Kokla[a], Petros Blouchos[a], Evangelia Livaniou[b], Christos Zikos[b], Sotiris E. Kakabakos[b], Panagiota S. Petrou[b] and Spyridon Kintzios[a]*

Membrane engineering is a generic methodology for increasing the selectivity of a cell biosensor against a target molecule, by electroinserting target-specific receptor-like molecules on the cell surface. Previous studies have elucidated the biochemical aspects of the interaction between various analytes (including viruses) and their homologous membrane-engineered cells. In the present study, purified anti-biotin antibodies from a rabbit antiserum along with in-house prepared biotinylated bovine serum albumin (BSA) were used as a model antibody-antigen pair of molecules for facilitating membrane engineering experiments. It was proven, with the aid of fluorescence microscopy, that (i) membrane-engineered cells incorporated the specific antibodies in the correct orientation and that (ii) the inserted antibodies are selectively interacting with the homologous target molecules. This is the first time the actual working concept of membrane engineering has been visualized, thus providing a final proof of the concept behind this innovative process. In addition, the fluorescence microscopy measurements were highly correlated with bioelectric measurements done with the aid of a bioelectric recognition assay. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: bioelectric recognition assay; biotinylated BSA; cell biosensors; cell membrane engineering; electroinsertion; membrane potential

INTRODUCTION

Membrane engineering is a generic methodology of artificially inserting (usually by electroinsertion) tens of thousands of receptor-like molecules on the cell surface, thus rendering the cell a selective responder against analytes binding to the inserted receptors. Receptor molecules can vary from antibodies to enzymes. The working assumption of the method is that attachment of the target molecule to its respective receptor causes a change in the cell membrane structure, which is measurable as a change in the cell membrane potential. This technology is an alternative method to cell transfection for increasing cell specificity, a requirement dictated by the usually poor selectivity of cell-based assay and sensor systems, because of the uniform response of cells against a vastly large number of different molecules.

Membrane-engineered cells have been developed and used as biorecognition elements in a plethora of applications, including the detection of superoxide (Moschopoulou and Kintzios, 2006; Moschopoulou *et al*., 2007, 2012), human viruses (Hepatitis B) (Perdikaris *et al*., 2009), plant viruses (Gramberg *et al*., 2012; Moschopoulou *et al*., 2008; Perdikaris *et al*., 2011), organic compounds such as 2,4,6-trichloroanisole (Varelas *et al*., 2010), aflatoxins (Larou *et al*., 2012), metabolic diseases, prions, foot-and-mouth disease virus, and blue-tongue virus (Kintzios, 2008).

It has been previously demonstrated that the specific interaction between membrane-engineered cells and homologous target analytes is associated with changes in intracellular Ca^{2+} traffic, which in turn affect cell membrane potential, as revealed by the selective inhibition of calcium ion pumps (Moschopoulou *et al*., 2012). In the same study, it was shown that cells, which were membrane-engineered with enzymes (superoxide dismutase [SOD]) as receptor-like molecules, acted as fully functional catalytic units, whereas the electroinserted SOD molecules retained their characteristic properties, as demonstrated by selective inhibition assays. However, similar studies have not been conducted with antibodies, which are more frequently used as recognition elements in membrane engineering experiments.

In the present study, purified anti-biotin antibodies from a rabbit antiserum, along with in-house prepared biotinylated bovine

* *Correspondence to: S. Kintzios, Department of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece. E-mail: skin@aua.gr*

a *A. Kokla, P. Blouchos, S. Kintzios*
Department of Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece

b *E. Livaniou, C. Zikos, S. E. Kakabakos, P. S. Petrou*
Institute of Nuclear and Radiological Sciences & Technology, Energy and Safety, NCSR Demokritos, 153 10 Athens, Greece

Abbreviations: *BERA, bioelectric recognition assay.; PBS, phosphate buffer saline.*

serum albumin (BSA) were used as a model antibody-antigen pair of molecules for facilitating membrane engineering experiments. It was proven, with the aid of fluorescence microscopy, that (i) membrane-engineered cells incorporated the specific antibodies in the correct orientation and that (ii) the inserted antibodies are selectively interacting with the homologous target molecules. This is the first time the actual working concept has been visualized, thus providing a final, determining proof of the concept behind this innovative process. In addition, the fluorescence microscopy measurements were highly correlated with bioelectric measurements done with the aid of a BERA.

EXPERIMENTAL

Chemicals

All solvents and chemicals used were of analytical quality. Water was double distilled. Vero (African green monkey kidney) cell cultures were originally provided from LGC Promochem (Teddington, UK). The vitamin biotin (Sigma-Aldrich, Taufkirchen/Munich, Germany) was chosen as the target molecule against which membrane-engineered cells would be created (as it will described in the following text).

Streptavidin/Alexa Fluor 546, anti-rabbit IgG/Alexa Fluor 546, and anti-mouse IgG/Alexa Fluor 546 were purchased from Invitrogen (Carlsbad, California, USA). All other reagents were purchased from Fluka (Buchs, Switzerland).

Isolation of anti-biotin rabbit IgG

Anti-biotin rabbit IgG was isolated from a rabbit antiserum raised against a synthetic biotin derivative conjugated to bovine thyroglobulin, as previously described (Papasarantos *et al*., 2010). Pooled antiserum, obtained from four consecutive bleedings, with a high enzyme-linked immunosorbent assay — titer value (1:50 000) was used as starting material. Isolation of the IgG fraction was performed through sequential precipitation with caprylic acid and ammonium sulfate (Perosa *et al*., 1990). Purity of the isolated IgGs was evaluated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentration was determined with the well-established bicinchoninic acid method (Sorensen and Brodbeck, 1986).

Preparation of biotinylated BSA

The protein BSA (MW: ~60 kDa) was biotinylated according to a previously published method (Zavali *et al*., 2007). Biotinylated BSA was purified with extensive dialysis against carbonate buffer 0.1 M, pH 8.5; protein concentration was determined with the bicinchoninic acid method (Sorensen and Brodbeck, 1986).

Cell culture

Monkey African green kidney (Vero) cells were cultured in Dulbecco's medium. After cell detachment from the culture vessel by adding trypsin/ethylenediaminetetraacetic acid for 3 min at 37°C, cells were concentrated by centrifugation (2 min, 1200 rpm, 25°C) at a density of $2{,}5 \times 10^6$ ml^{-1}.

Creation of membrane-engineered Vero cells

Membrane-engineered cells were created by electroinserting anti-biotin rabbit IgG (10 mg ml^{-1} per $2{,}5 \times 10^6$ cells) into the membrane of Vero cells following a modified protocol of Moschopoulou and Kintzios (Moschopoulou and Kintzios, 2006). Briefly, the cells were centrifuged at 1000 rpm for 6 min and then resuspended in PBS (pH 7.4). Subsequently, cells were incubated together with anti-biotin rabbit IgG for 20 min on ice. Then, the cells-antibody mixture was transferred to appropriate electroporator (Thermo EC100, Waltham, MA) cuvettes. Electroinsertion was performed by applying an electric field (two pulses) at 400, 1000, 1800, or 2400 Vcm^{-1}. After electroinsertion, cells were incubated at 37°C for 1 h. Finally, cells were centrifuged at 1000 rpm for 6 min and resuspended in PBS (pH 7.4). This step was repeated twice.

Functionality of the cell membrane electroinserted antibodies

Capability of the electroinserted anti-biotin rabbit antibodies to react with biotinylated BSA and fluorescently labeled streptavidin

The antibody-bearing, engineered cells were treated with biotinylated BSA (10 μg ml^{-1}) for 1 h and then with fluorescence-labeled streptavidin (streptavidin/Alexa Fluor 546) (10 μg ml^{-1}) for 1 hour (Figure 1A). Afterward, the cells were observed under a fluorescence microscope as described in 2.7.

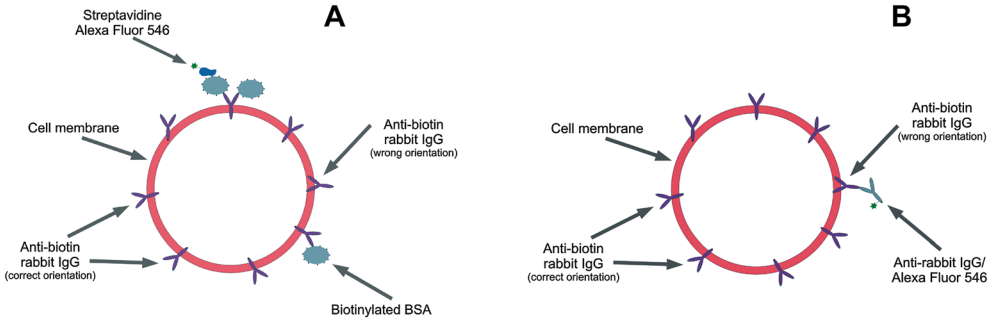

Figure 1. (A) Schematic representation of the first approach followed for evaluating the functionality of the cell membrane — electroinserted anti-biotin rabbit antibodies: Correct orientation (i.e. exposure of the biotin-binding sites outwards) would lead to the formation of fluorescent "sandwiches" comprised of anti-biotin rabbit antibodies immunocomplexed with biotinylated BSA and, through the latter, with fluorescently labeled streptavidin. (B) Schematic representation of the second approach followed for evaluating the functionality of the cell membrane — electroinserted anti-biotin rabbit antibodies: "Wrong" (inverted) orientation (i.e. exposure of the species-specific region outwards) would lead to the formation of fluorescent immunocomplexes between these antibodies and the Alexa Fluor 546-labeled anti-rabbit IgG molecules.

Journal of Molecular Recognition

Capability of the electroinserted anti-biotin rabbit antibodies to react with fluorescently labeled anti-rabbit secondary antibody

The antibody-bearing, engineered cells were incubated for one hour with a fluorescence-labeled anti-rabbit antibody or secondary antibody (anti-rabbit IgG/Alexa Fluor 546, 10 μg ml^{-1}, diluted 1:400) (Figure 1B). Afterward, the cells were observed under a fluorescence microscope as described in Fluorescence microscopy section. For comparison reasons, a fluorescence-labeled anti-mouse antibody (anti-mouse IgG/Alexa Fluor 546, 2 mg ml^{-1}, diluted 1:200) was used in a second series of experiment, instead of the fluorescence-labeled anti-rabbit antibody (negative control experiments).

Fluorescence microscopy

After 1–3 h of incubation, the fluorescent cells bearing the (anti-biotin antibody/biotinylated BSA/fluorescence-labeled streptavidin or anti-biotin antibody/fluorescence-labeled anti-antibody) complexes were observed under a fluorescence microscope (Axiolab ZEISS HBO50/AC equipped with an excitation filter G546, a chromatic beam splitter FT580 and a barrier filter LP590). A digital camera (SONY S75 digital still camera) was attached to the microscope with adjustable BP-546/FT-580 or G365/FT 395 excitation filter/chromatic beam splitter combinations. In order to control photobleaching, we kept specimen exposure times at a minimum level. No significant alteration of the intensity of the fluorescence was observed during the observation of the specimens.

Bioelectric measurements

Next, we measured changes in the antibody-engineered membrane potential of Vero cells bearing the anti-biotin rabbit IgG (as described in Creation of membrane-engineered Vero cells section) after the addition of biotinylated BSA. Potentiometric measurements were carried out according to the principles of the BERA and using a customized variation of the portable PG581 potentiostat (Uniscan, Buxton, UK). The reaction mixture, composed of membrane-engineered cells (40 μl ≃ 50×10^3 cells) and biotinylated BSA (10 mg l^{-1}), was placed on the top of a carbon screen-printed electrode contained in a disposable sensor strip, the latter comprising a 0.5-mm thick ceramic substrate with three screen-printed electrodes (working – WE, reference – RE, and counter – CE). The device had a replaceable guide bearing eight pairs of electrodes connecting on the underside. The system provided a connection interface to insert electrode strips directly into the instrument, utilizing one electrode strip per channel. Changes in the membrane potential of the cellular biorecognition elements, as a result of the response of the membrane-engineered cells to the presence of biotinylated BSA, were recorded as a time-series of potentiometric measurements (in Volts). The duration of each measurement was 180 s and 360 values/sample were recorded at a sampling rate of 2 Hz.

RESULTS AND DISCUSSION

Membrane-engineered cells bear antibodies with correct orientation and intact recognition properties

As schematically shown in Figure 1A, if, after electroinsertion, the biotin-binding sites of the majority of the antibody molecules were exposed outside the cell membrane and were, therefore, freely accessible to the corresponding antigen, then the regions of electroinserted anti-biotin antibodies complexed with biotinylated BSA and fluorescently labeled streptavidin should appear as fluorescent spots on the cell membrane. This was indeed the case, as shown with fluorescence microscopy (Figure 2): the membrane-engineered cells were proved to carry antibodies inserted at the correct orientation, i.e. pointing their antigen-binding sites outwards, in their major percentage (over 90%); because of their appropriate orientation, the antibodies retained their normal antigen-recognition properties. It should be mentioned here that biotinylated BSA was used as the model-antigen, instead of biotin, due to the much larger molecular weight of the biotinylated protein compared with that of biotin (MW: 244.31 Da), which allowed binding of streptavidin/Alexa Fluor 546 to the anti-biotin antibody/biotinylated BSA immunoconjugates through the formation of a "sandwich". Moreover, as noticed, the strength of the electric field had no effect on the observed results.

On the other hand, if the anti-biotin rabbit antibodies were electroinserted in the "wrong" (for our purposes) orientation, i.e. with an outward exposure of their species-specific, constant region, which is recognized by the secondary, anti-species antibodies, fluorescence would appear after their incubation with the fluorescence-labeled anti-rabbit IgG, as schematically outlined in Figure 1B. However, as shown in Figure 3, much less fluorescence was observed on membrane-engineered cells incubated with anti-rabbit IgG/Alexa Fluor 546, which indicates that probably fewer antibodies (less than 10%, as roughly estimated by the differences in fluorescence intensity) were electroinserted with the non-functional orientation (in comparison with those inserted keeping intact their ligand-binding characteristics. Moreover, almost lack of any fluorescence spots was obtained when the engineered cells were incubated with a fluorescently labeled anti-mouse IgG (Figure 4). Once again, results were independent of the strength of the applied electric field.

The present study is a further improvement of our knowledge of the novel technology of membrane engineering for the construction of cell biosensors with specific target-recognition properties. Having demonstrated that electroinserted antibodies are functionally intact and can interact with target antigens as expected obviates the need for chemical modification (e.g.

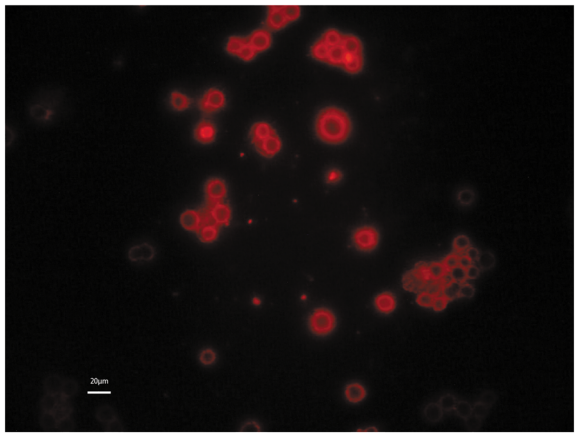

Figure 2. Vero cells were membrane-engineered with anti-biotin rabbit IgG antibodies, then incubated with biotinylated BSA and with streptavidin/Alexa Fluor 546, and finally observed under a fluorescence microscope. The clear and bright fluorescence pattern shown in the figure indicates that the antibodies were electroinserted mostly in the correct orientation (i.e. exposing the antigen-binding sites outwards).

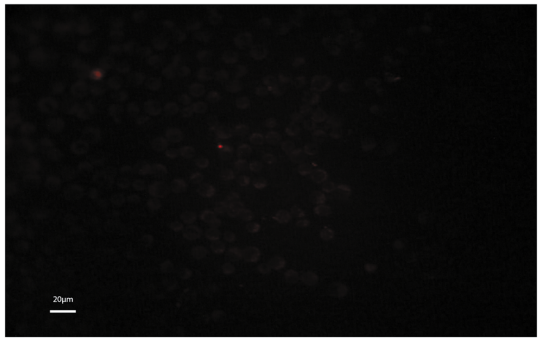

Figure 3. Vero cells were membrane-engineered with anti-biotin rabbit IgG antibodies, then incubated with anti-rabbit IgG/Alexa Fluor 546 and finally observed under a fluorescence microscope. Much less fluorescence can be shown in the figure, which indicates that rather low percentage of the anti-biotin antibodies were electroinserted in the "wrong" orientation (i.e. exposing the species-specific, constant region outwards). "Ghost" cell images in the background are due to differences in the microscope observation focus level.

Figure 4. Vero cells were membrane-engineered with anti-biotin rabbit IgG antibodies, then incubated with anti-mouse IgG/Alexa Fluor 546 and finally observed under a fluorescence microscope. Practically, no fluorescent spots can be seen in this figure (background signal).

farnesylation) of the antibody molecules, in order to improve their anchorage to the membrane structure.

The incorporation of water-soluble antibody molecules into the membrane of Vero cells could be explained by membrane lipid rearrangement due to the exposure of cell membranes to electric fields and resulting to the creation of conductive membrane pathways, known as "hydrophilic" or "conductive" pores (Baron *et al.*, 2000, Mohaved and Li, 2012; Neumann *et al.*, 1982). Of course, both the incorporation of antibodies into the cell membrane and their affinity to target antigens are largely dependent on the membrane lipid composition. This has been documented in numerous studies regarding the affinity of natural, membrane-bound receptors for their various agonists. For example, changing the percentage of cholesterol in the membranes of CHO cells altered the affinity of M2 muscarinic acetylcholine receptors for (^{3}H)-N-methylscopolamine ((^{3}H)-NMS) and the consecutive signaling through both preferential and non-preferential G-proteins (Michal *et al.*, 2009). Similar effects have been recently reported for the human serotonin$_{1A}$ receptor (Saxena and Chattopadhyay, 2012). In addition, the membrane lipid composition may affect pore radius and conductance after electroporation (Koronkiewicz and Kalinowski, 2004).

Changes in the antibody-engineered cell membrane potential depend on the concentration of biotinylated BSA

Even though the strength of the applied electric field did not affect the insertion of antibodies into the cell membrane and their antigen-recognition properties, it did have an effect on the pattern of membrane potential changes after the binding of the antigen molecules to the electroinserted antibodies. When electroinsertion was facilitated at a low intensity field (400 V cm^{-1}), membrane-engineered cells bearing the anti-biotin antibodies responded marginally to increasing biotinylated BSA concentrations by decreasing their depolarization potential relative to control and up to the concentration of 10 μg ml^{-1} (Figure 5A). When the strength of the electric field was increased to 1000 V cm^{-1}, this relative decrease was observed against all

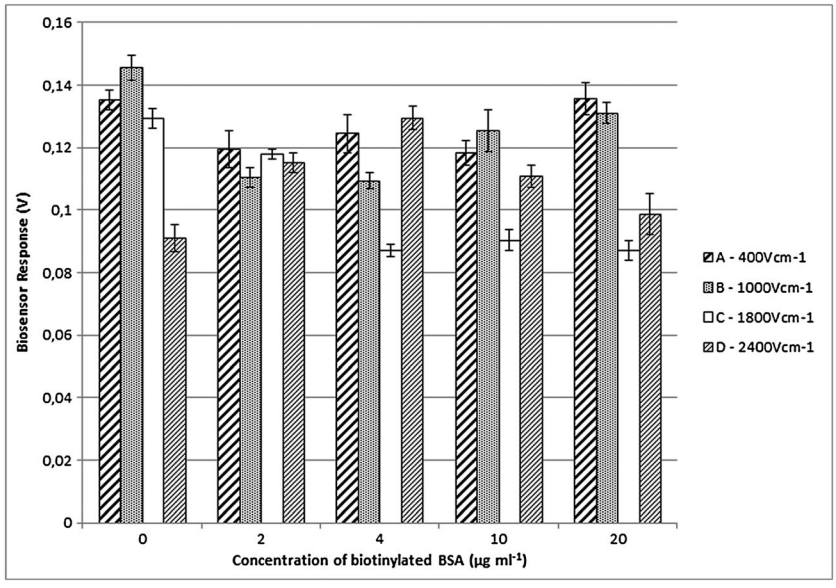

Figure 5. Bioelectric response (expressed as changes of the membrane potential) of Vero cells against different concentrations of biotinylated BSA. Cell membranes have been modified by the electroinsertion of anti-biotin rabbit IgG at an electric field of 400, 1000, 1800, or 2400 V cm^{-1}. An improved resolution of the concentration-dependent response is observed at 1800 V cm^{-1} (details in text).

tested antigen concentrations and was more apparent for the lowest ones (2–4 $\mu g\,ml^{-1}$) (Figure 5B). At an even higher electric field intensity (1800 $V\,cm^{-1}$), the resolution of the response between each of the tested concentrations of biotinylated BSA and the zero concentration was improved to the degree that the observed changes in cell membrane potential were positively correlated with antigen concentration ($r^2 = 0,8$) (Figure 5C). Finally, at the highest electric field intensity (2400 $V\,cm^{-1}$), a clear difference was observed in the response to control compared with that of biotinylated BSA (Figure 5D). In this case, however, membrane-engineered cells bearing the anti-biotin antibodies responded to biotinylated BSA by increasing their membrane potential in a concentration-dependent fashion (up to 4 $\mu g\,ml^{-1}$), thus demonstrating a membrane hyperpolarization process not observed at lower electric field strengths. It seems that for each specific application, a preliminary optimization step concerning antibody electroinsertion will certainly help best analytical operation of the immunosensor. In the specific application presented in this work, i.e. for analyzing biotinylated BSA, best analytical results were obtained with an electric field intensity equal to 1800 $V\,cm^{-1}$. Thus, as shown in Figure 5C, under the experimental conditions used, a clear analytical response (expressed as decrease in cell membrane potential) was obtained for antigen concentration up to 4 $\mu g\,ml^{-1}$, whereas no further response was shown at higher antigen concentrations. It is apparent that the conditions applied during electroinsertion may affect the mode of interaction between the electroinserted antibodies and the target antigens; at least as far as the pattern of the translation of this interaction in measurable changes of the cell membrane potential is concerned. Quite recently, Moschopoulou *et al*. (Moschopoulou *et al*., 2012) reported on the effect of the intensity of the electric field during electroinsertion of SOD molecules into Vero cells.

Therefore, a direct link was established between the fluorescence microscopy-based observations and the actual biosensor-based measurements, thus conclusively demonstrating the applicability of the membrane engineering process for the development of target-specific cellular biorecognition elements.

CONCLUSIONS

Although the principle of membrane engineering has been used in several detection applications since originally reported (Moschopoulou and Kintzios, 2006), it is the first time that the actual proof-of-concept is demonstrated in a direct, visualized way.

There are considerable advantages in using cellular biosensors containing membrane-engineered cells as the biorecognition elements and employing various non-optical signal-sensing systems. These include the very high speed of the assay (with a duration of just a few minutes), very high sensitivity (down to 0,1 ppt), high selectivity, high reproducibility, ease of handling, and minimum requirement of sample preparation. In addition, the current electrode-based instrumentation for measuring changes in the membrane-engineered potential is much cheaper than the corresponding optical one. The present results are quite encouraging in the sense that they show an increased perspective for the broader application of the membrane engineering technology in the development of cell-based biosensors with a selective response against a diversified group of analytes. We currently investigate additional effects on the efficiency of the technology, including the chemical constitution of the cell membrane as well as the cell line used as a carrier. In this way, the process can be optimized for each individual application and the assay system used.

Acknowledgements

The research was financially supported by the European Union FP7 Research for SMEs Project FOODSCAN "Development of an automated, novel biosensor platform for pesticide and chemical residue detection" (No 286442).

REFERENCES

Baron S, Poast J, Rizzo D, McFarland E, Kieff E. 2000. Electroporation of antibodies, DNA, and other macromolecules into cells: a highly efficient method. *J. Immunol. Methods* **242**:115–126.

Gramberg B, Kintzios S, Schmidt U, Mewis I, Ulrichs Ch. 2012. A basic approach towards the development of bioelectric bacterial biosensors for the detection of plant viruses. *J. Phytopathol.* **160**:106–111.

Kintzios S. 2008. Molecular Identification through Membrane Engineering (MIME): State-of-the-art biosensor technology for instant, ultra-specific and ultra-sensitive detection of infectious disease agents at global scale. In Commercial and Pre-Commercial Cell Detection Technologies for Defense against Bioterror, Lechuga LM, Milanovich FP, Skladal P, Ignatov O, Austin TR (eds). IOS Press: Amsterdam, The Netherlands; 115–124.

Koronkiewicz S, Kalinowski S. 2004. Influence of cholesterol on electroporation of bilayer lipid membrane: chronopotentiometric studies. *Biochim. Biophys. Acta* **1661**:196–203.

Larou E, Yiakoumettis I, Kaltsas G, Petropoulos A, Skandamis P, Kintzios S. 2012. High throughput cellular biosensor for the ultra-sensitive, ultra-rapid detection of aflatoxin M1. *Food Control* **29**:208–212.

Michal P, Rudajev V, El-Fakahany EE, Doležal V. 2009. Membrane cholesterol content influences binding properties of muscarinic M2 receptors and differentially impacts activation of second messenger pathways. *Europ. J. Pharmacol.* **606**:50–60. DOI: 10.1016/j.ejphar.2009.01.028

Mohaved S, Li D. 2012. Electrokinetic transport through the nanopores in cell membrane during electroporation. *J. Colloid Interface Sci.* **369**:442–452. DOI: 10.1016/j.jcis.2011.12.039

Moschopoulou G, Kintzios S. 2006. Application of "membrane-engineering" to bioelectric recognition cell sensors for the detection of picomole concentrations of superoxide radical: a novel biosensor principle. *Anal. Chim. Acta* **573-574**:90–96.

Moschopoulou G, Papanastasiou I, Makri O, Lambrou N, Economou G, Soukouli K, Kintzios S. 2007. Cellular redox-status is associated with regulation of frond division in *Spirodela polyrrhiza*. *Plant Cell Rep.* **26**:2063–2069.

Moschopoulou G, Vitsa K, Bem F, Vassilakos N, Perdikaris A, Blouhos P, Yialouris C, Frossiniotis D, Anthopoulos I, Maggana O, Nomikou K, Rodeva V, Kostova D, Grozeva S, Michaelides A, Simonian A, Kintzios S. 2008. Engineering of the membrane of fibroblast cells with virus-specific antibodies: a novel biosensor tool for virus detection. *Biosensors Bioelectron.* **24**:1033–1036. DOI: 10.1016/j.bios.2008.06.039

Moschopoulou G, Valero T, Kintzios S. 2012. Superoxide determination using membrane-engineered cells: An example of a novel concept for the construction of cell sensors with customized target recognition properties. *Sens. Actuat. B* **175**:78–84.

Neumann MS-R, Wang EY, Hofschneider PH. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**:841–845.

Papasarantos I, Klimentzou P, Koutrafouri V, Anagnostouli M, Zikos C, Paravatou-Petsotas M, Livaniou E. 2010. Solid-phase synthesis of a biotin derivative and its application to the development of anti-biotin antibodies. *Appl. Biochem. Biotechnol.* **162**:221–232. DOI: 10.1007/s12010-009-8870-y

Perdikaris A, Alexandropoulos N, Kintzios S. 2009. Development of a novel, ultra-rapid biosensor for the qualitative detection of Hepatitis B virus-associated antigens and anti-HBV, based on "membrane-engineered" fibroblast cells with virus-specific antibodies and antigens. *Sensors* **9**:2176–2186. DOI: 10.3390/s90302176.

Perdikaris A, Vassilakos N, Yiakoumettis I, Kektsidou O, Kintzios S. 2011. Development of a portable, high throughput biosensor system for rapid plant virus detection. *J. Virol. Methods* **177**:94–99. DOI: 10.1016/j.jviromet.2011.06.024.

Perosa F, Carbone R, Ferrone S, Dammacco F. 1990. Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. *J. Immunol. Methods* **128**:9–16.

Saxena R, Chattopadhyay A. 2012. Membrabe cholesterol stabilizes the human serotonin(1A) receptor. *Biochim. Biophys. Acta* **1818**:2936–2942. DOI: 10.1016/j.bbamem.2012.07.032.

Sorensen K, Brodbeck U. 1986. Assessment of coating-efficiency in ELISA plates by direct protein determination. *J. Immunol. Methods* **95**:291–293.

Varelas V, Sanvicens N, Marco MP, Kintzios S. 2010. Development of a cellular biosensor for the detection of 2, 4, 6- trichloroanisole (TCA). *Talanta* **84**:936–940. DOI: 10.1016/j.talanta.2011.02.029

Zavali M, Petrou PS, Kakabakos SE, Kitsara M, Rapis I, Beltsios K, Misiakos K. 2007. Label-free kinetic study of biomolecular interactions by white light reflectance spectroscopy. *Micro Nano Lett.* **1**:94–98.