Biosensors and Bioelectronics 24 (2008) 1027–1030

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Short communication

Engineering of the membrane of fibroblast cells with virus-specific antibodies: A novel biosensor tool for virus detection

Georgia Moschopoulou[a,f], Katerina Vitsa[a], Frederic Bem[b], Nikos Vassilakos[b], Antonis Perdikaris[a], Petros Blouhos[a], Costas Yialouris[c], Dimitris Frosyniotis[c], Ioannis Anthopoulos[c], Olga Mangana[d], Kiki Nomikou[d], Velichka Rodeva[e], Dimitrina Kostova[e], Stanislava Grozeva[e], Alexandros Michaelides[f], Alex Simonian[g], Spiridon Kintzios[a,*]

[a] *Laboratory of Plant Physiology, Faculty of Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece*
[b] *Benaki Phytopathological Institute, S. Delta 8, 145 61 Kifissia, Athens, Greece*
[c] *Agricultural University of Athens, Informatics Laboratory, Department of Science, Iera Odos 75, 11855 Athens, Greece*
[d] *Ministry of Agriculture, Centre of Athens Veterinary Institutions, Institute of Infectious and Parasitic Diseases, Virus Laboratory, Athens, Greece*
[e] *Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria*
[f] *EMBIO Diagnostics, Nicosia, Cyprus*
[g] *Department of Materials Engineering, Auburn University, AL, USA*

ARTICLE INFO

Article history:
Received 20 March 2008
Received in revised form 9 May 2008
Accepted 13 June 2008
Available online 4 July 2008

Keywords:
Bioelectric recognition assay
Cell biosensor
Cucumber green mottle mosaic virus
Tobacco rattle virus
Cell biosensor
Membrane-engineering

ABSTRACT

A novel concept for the assay of viral antigens is described. The methodological approach is based on a membrane-engineering process involving the electroinsertion of virus-specific antibodies in the membranes of fibroblast cells. As a representative example, Vero fibroblasts were engineered with antibodies against *Cucumber mosaic virus* (CMV) and used for the construction of an ultra-sensitive miniature cell biosensor system. The attachment of a homologous virus triggered specific changes to the cell membrane potential that were measured by appropriate microelectrodes, according to the principle of the bioelectric recognition assay (BERA). No change in the membrane potential was observed upon cell contact with the heterologous *cucumber green mottle mosaic virus* (CGMMV). Fluorescence microscopy observations showed that attachment of CMV particles to membrane-engineered cells was associated with membrane hyperpolarization and increased $[Ca^{2+}]_{cyt}$. In an additional field-based application, we were able to detect CMV-infected tobacco plants at an essentially 100% level of accuracy.

1. Introduction

The fast detection of viruses is still a challenging issue for the developers of biosensor-based diagnostic systems. Employment of cellular sensors is an attractive approach due their inherent similarity with cell culture, which is considered as the golden standard of the diagnostic community. However, several weeks may be required before a clearly defined symptom is observed in a test culture (Park et al., 2000). On the contrary, emerging cell sensor technologies require significantly less time (from a few moments to a day) in order to produce a reliable result. For example, Thach et al. (2003) investigated the interaction between the Sindbis virus (SV) and primary rat neural precursor cells or human peripheral blood mononuclear cells, as a first step towards the development of a cell-based biosensor for SV detection. Kintzios et al. (2004) reported that animal and plant cells immobilized in bioelectric recognition assay (BERA) sensors responded to different viruses primarily by changing their membrane potential. The response of immobilized cells against different viruses did not depend on the virus ability to penetrate the cell, but was modified after binding each virus to a virus-specific antibody or removal of its coat protein after treatment with a protease. Recently, Campbell et al. (2007) used electric cell-substrate impedance sensing in order to monitor the cytopathic effects caused by infectious pancreatic necrosis virus (IPNV) on chinook salmonid embryonic (CHSE-214) cells and the effects of infectious hematopoetic necrosis virus (IHNV) on epithelioma papulosum cyprini (EPC) carp cells.

One of the traits that make cell biosensors attractive as an analytical tool is their considerable sensitivity, which is assumed to enable, in some cases, the possibility to detect just a single target molecule (Kintzios, 2007). This ability, however, comes at a cost, or, rather, a compromise: because cells can react in roughly the same manner against an amazingly large number of different molecules, cell sensors can exhibit a very poor selectivity against a

* Corresponding author. Tel.: +30 210 529 4292; fax: +30 210 529 4286.
E-mail address: skin@aua.gr (S. Kintzios).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2008.06.039

particular infectious molecule (Van der Lelie et al., 1997; Riska et al., 1999).

In the present report we demonstrate a novel concept for cell-based detection of viruses. The method is based on the electroinsertion of virus-specific antibodies, at high density, on the surface of cultured mammalian cells, thus rendering the cell a very specific responder against homologous viruses binding to the membrane-bound antibodies. The working assumption of the method is that attachment of the viral particles to their corresponding antibodies will cause a change in the cell membrane structure, which is measurable as a change in the cell membrane potential. In order to prove this concept, Vero fibroblast cells were membrane-engineered with antibodies against the plant *Cucumber mosaic virus* (CMV). Engineered cells were immobilized in calcium alginate beads and their electrophysiological responses against the homologous virus (CMV) and the heterologous *Cucumber green mottle mosaic virus* (CGMMV) were measured according to the principles of the bioelectric recognition assay.

2. Experimental

2.1. Chemicals

Vero cell cultures were originally provided from LGC Promochem (Teddington, UK). Bovine serum albumin (BSA), L-glutamine propidium iodide and Fluo-3 were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Fluka (Buchs, Switzerland).

2.2. Cell culture

Monkey African green kidney (Vero) cells were cultured in Dulbecco's medium, as described previously (Kintzios et al., 2004). After cell detachment from the culture vessel by adding trypsine/EDTA for 10 min at 37 °C cells were concentrated by centrifugation (6 min, 1200 rpm, 25 °C), at a density of 2×10^6 ml^{-1}.

2.3. Electroinsertion of antibodies into the cell membrane

Membrane-engineered cells were created by electroinserting CMV polyclonal antibodies into the membrane of Vero cells following a modified protocol of Zeira et al. (1991). Briefly, the cells were centrifuged at $100 \times g$ for 6 min and then resuspended in Dulbecco's medium provided with 20% (v/v) fetal calf serum (FCS). Subsequently, cells were incubated together with the antibodies (0.5 μg ml^{-1}) for 20 min on ice. Then, the cells–antibodies mixture was transferred to appropriate electroporator (Thermo EC100, Waltham, MA) cuvettes. Electroinsertion was performed by applying two square electric pulses at 1800 V/cm. After electroinsertion, cells were incubated at 37 °C for 1 h. Finally, cells were centrifuged at $100 \times g$ for 6 min and resuspended in Dulbecco's medium provided with 20% (v/v) FCS. Following this procedure, $8–10 \times 10^3$ antibodies were incorporated on the surface of each membrane-engineered cell, as estimated by immunological assays (analytical data not shown).

2.4. Sensor fabrication

Engineered and non-engineered Vero cells (1 ml solution) were mixed with 3 ml of 2% (w/v) sodium alginate solution and then the mixture was added drop wise, by means of a 22G syringe, in 0.8 M $CaCl_2$. Each of the resulting calcium alginate beads had an approximate diameter of 2 mm and contained approximately 75×10^3 cells (as counted with the aid of a haemocytometer). Each bead was designated as a consumable BERA–CMV sensor.

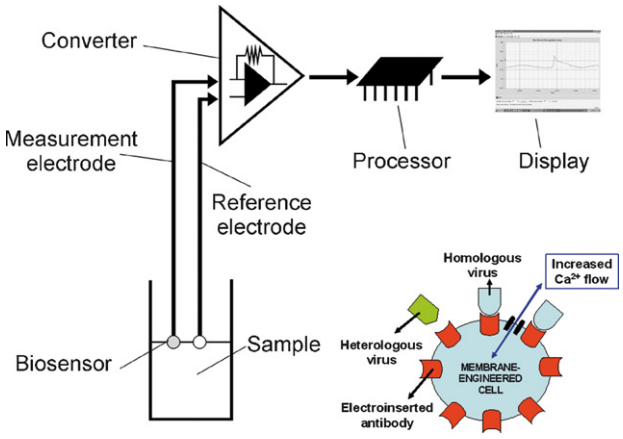

Fig. 1. Schematic representation of the immobilized cell biosensor. The reference electrode is inserted in a cell-free gel bead, while the measuring electrode is inserted in the immobilized cell-gel bead, which has an approximate diameter of 2 mm. Measurement and reference electrodes are connected via wiring to the PMD 1608-FS data converter. Cells are not shown in scale. *Insert figure*: Schematic representation of the molecular recognition of viruses by membrane-engineered cells.

2.5. Recording and data processing

Each cell-bearing bead (BERA–CMV sensor) was connected to an electrode made from 80% Cu, electrochemically coated with an Ag/AgCl layer and having a diameter of 0.75 mm. A cell free bead was attached to the reference electrode (Fig. 1). Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for the recording of the signal and processing of data was InstaCal (Measurement Computing).

For each assay, the sensor system, comprising of the beads attached to the working and the reference electrode, was immersed into the sample solution (200 μl). The response of each sensor was estimated by recording the absolute maximum change of the sensor potential from 0 to 330 s (achievement of stable response of the biosensor).

2.6. CMV and CGMMV detection assay

BERA–CMV sensors were used for assaying purified CMV virus preparations in PBS (1 mg ml^{-1}) as well as plants infected with CMV or CMV-free plants. Samples were prepared by diluting the virus particles to saline buffer 0.5% (w/v) according to the following concentrations: 0.0 ng ml^{-1} (control), 1.0, 5.0, 10.0 and 15.0 ng ml^{-1}. BERA–CMV sensors were also used for assaying purified CGMMV virus (the watermelon isolate PL104 of CGMMV) preparations in PBS at concentrations of concentrations: 0.0 ng ml^{-1} (control), 1.0, 5.0, 10.0, and 15.0 ng ml^{-1}. Virus isolates were derived from the stock collection of the Benaki Phytopathological Institute.

Plant extracts (crude sap extracts) were prepared from cucumber plants infected with CMV by homogenizing leaves in phosphate buffer (pH 7.2) (1 g leaf tissue: 10 ml buffer) containing 2% (w/v) polyvinylpyrrolidone 40 and centrifugation at 5000 rpm for 5 min. Infected leaves were removed from plants 11 days after inoculation. The extracts were assayed for CMV with F(ab')$_2$-ELISA and RT-PCR, as described previously (Ploeg et al., 1992; MacFarlane, 1999).

2.7. Assay of virus–cell interaction

Membrane potential changes in membrane-engineered Vero cells (Vero-CMV) were monitored before and after the addition of CMV at 10.0 ng ml^{-1}, by the cellular uptake of the lipophilic cationic

fluorochrome, 3,3-dipropylthiadicarbocyanide iodide, the distribution of which is affected by the potential difference across the cell membrane (Eddy, 1989). In another set of experiments, changes in cytoplasmic Ca^{2+} concentration in Vero-CMV cells before and after the addition of the virus were monitored by the uptake of the acetomethyl ester of Fluo3 (Whelan and Zare, 2003) After application of 5 μl of the dye, the fluorescence of the specimens was recorded for 5 min at 10 s intervals. Slides with stained cells were mounted on a Zeiss Axiolab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. 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.

2.8. Experimental design

Experiments were set-up in a completely randomized design and each experiment was repeated three times. In each application 100 different sample combinations (five virus concentrations or dilutions 20× samples each) for each plant virus were used.

The detection process was repeated on field samples (15 replications for each sample) prepared from plant extracts as described above (2.6). In fluorescent microscopy assays, the number of cells (fluorescent or not) was counted both manually and by using Doc-It® LS Image Acquisition and Analysis Software. For each microscopic observation, the average cell number and fluorescence assays were calculated from 10 different 7×10^3 μm^2 optical fields.

3. Results

3.1. Sensor response

The responses of BERA sensors based on Vero cells engineered with the CMV-specific antibodies against purified viruses and crude plant samples are presented in following.

3.1.1. Purified virus

The results of the assay of purified viral proteins at different dilutions with the BERA biosensors are shown in Fig. 2. Sensors containing Vero cells engineered with the CMV-specific antibody (Vero-CMV) responded to dilutions of the homologous virus (CMV) by considerable membrane hyperpolarization, as indicated by the negative increase of the sensor potential. The response was not concentration-depended, so that only qualitative detection of the virus was possible. On the other hand, sensors based on non-engineered Vero cells (i.e. bearing no CMV-specific antibodies) did not respond to the presence of the virus. In addition, sensors based on the membrane-engineered Vero-CMV cells failed to produce any essential response against the heterologous virus CGMMV.

When no virus was present in the sample (control sample), a constant sensor response of $-0{,}29 \pm 0.03$ mV was observed for Vero-CMV cells. The biosensor response CMV was quite reproducible, with 2.8–9.4% variation from the average in the response observed against 1–15 ng ml^{-1} CMV (variation increasing with virus concentration). On the contrary, a much higher, though statistically non-significant 11–16% variation was observed in the response against the heterologous CGMMV virus (at 1–10 ng ml^{-1} concentration range).

3.1.2. Plant samples

BERA–CMV sensors demonstrated a distinct and statistically significant higher change in response (74 ± 11) against extracts derived from plants infected with CMV than virus-free, control samples (0 ± 3 mV).

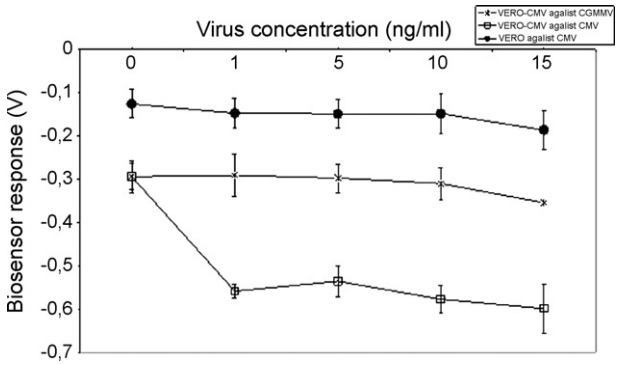

Fig. 2. Differential responses of sensor based on immobilized Vero or Vero-CMV cells to different plant viruses and viral concentrations. A BERA sensor containing non-engineered Vero cells (i.e. bearing no CMV-specific antibodies) (*closed circles*) essentially did not respond to the presence of the virus at increasing concentrations. On the contrary, BERA sensors containing membrane-engineered cells with antibodies against CMV ("Vero-CMV") responded to dilutions of the homologous virus (CMV) with considerable membrane hyperpolarization (*open squares*). Virtually no response was observed against the heterologous virus CGMMV (*stars*). Sensor response is expressed as a change in the membrane potential of immobilized cells ($n = 20$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration). Vero and "Vero-CMV"-based sensors show different sensor responses against control solutions (zero virus concentration), possibly due to altered permeability of the membrane-engineered cells.

3.2. Fluorescence microscopy assays of cell–virus interactions

Vero cells engineered with the CMV-specific antibody (Vero-CMV) incubated in 3,3-dipropylthiadicarbocyanide iodide emitted a bright red fluorescence, corresponding to their steady-state membrane potential under the applied experimental conditions. The intensity of fluorescence was changed upon contact with the homologous CMV virus (Fig. 3), a pattern indicating increased cell membrane hyperpolarization. In addition, the interaction between Vero-CMV cells and CMV was associated with a rapid increase of their cytoplasmic Ca^{2+} concentration (possibly due to calcium ion mobilization from other intracellular stores, e.g. mitochondria). On the contrary, no response was observed after the incubation of the membrane-engineered cells with control, virus-free solution.

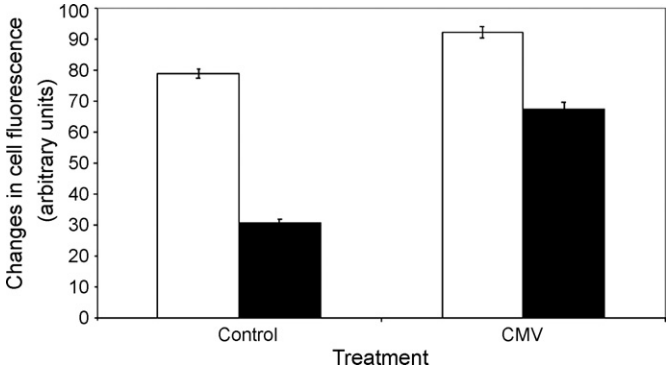

Fig. 3. Changes of the membrane potential (*open columns*) and of the intracellular Ca^{2+} concentration (*closed columns*) (both expressed as differences in fluorescence intensity) of Vero cells engineered with CMV antibodies before (*control*) and after interaction with CMV virus. After attachment of CMV particles, the membranes of Vero-CMV cells were hyperpolarized and $[Ca^{2+}]_{cyt}$ was increased.

4. Discussion

In earlier studies (Kintzios et al., 2001, 2004), we have reported the detection of plant viruses using BERA sensors based on tobacco protoplasts. The working principle of the sensor was based on the observation that the protoplast membrane potential instantly changed after the addition of a sample containing viral particles (such as CGMMV particles). Protoplasts were able not only to respond differently against different viruses, but also to recognize modifications of the viral surface, either by antibody binding, pronase treatment or heat inactivation. Using tobacco protoplasts, we have been able to detect either virus at concentrations as low as 25 ng ml^{-1}. However, we have not been able to discriminate the biosensor responses against different viruses when the viral concentration ranged from 25 ng ml^{-1} (detection limit) to 500 ng ml^{-1}.

Therefore, we feel that the present report is an considerable extension of the original cell biosensor principle, because: (a) the detection limit of the novel sensor is fivefold lower (1 ng ml^{-1}), therefore the sensitivity of the assay is increased; (b) the selectivity of the assay for CMV is increased, at least as far as discrimination from CGMMV is concerned at the 1–15 ng ml^{-1} concentration range. It is also the first time that a biosensor based on animal cells was used for the detection of a plant virus.

We assumed that, interaction of viruses with antibodies densely embedded on the surface of membrane-engineered cells caused an even stronger change of the cell membrane potential. The considerable cell membrane hyperpolarization and increase of cytoplasmic Ca^{2+} concentration after treating membrane-engineered Vero cells with CMV, but not with control samples (buffer), provides a supporting indication for this hypothesis. Whelan and Zare (2003) have previously shown that receptor-like interactions between molecules on the cell surface and target analytes resulted in a detectable change in the concentration of cytosolic Ca^{2+}. In this way, cells with electroinserted antibodies serve as recognition platforms for homologous viruses, whereas the translation of the recognition reaction into potential changes makes the assay much more rapid (and, in some cases, more sensitive) than conventional immunoanalytical methods. Based on the results of the present study, the biosensor was able to produce only a qualitative response against either the homologous (CMV) or the heterologous (CGMMV) virus. In fact, we have not observed any change of the cell membrane potential when viral concentrations were increased to 20 or 50 ng ml^{-1} (analytical results not shown). One way to explain the observed qualitative response is to assume that molecular recognition of virus particles by membrane-engineered cells is based on a non-titrimetric reaction between the viral particles and the cell membrane bearing anti-CMV antibodies. This may involve an electromechanical stress at the cite of antibody location on the membrane, leading to changes in membrane porosity and, in consequence, membrane conductivity. In other words, there may be a limit to the change of cell membrane potential caused by the aforementioned interaction, even if the electroinserted antibodies are not saturated with virus particles. This hypothesis is currently under investigation.

Membrane-engineering is also a promising alternative to the use of genetically engineered cells in cellular biosensors, such as B cell-based CANARY sensors used for the identification of *Yersinia pestis* (Rider et al., 2003). Nevertheless, the selectivity of membrane-engineered cell sensors strongly depends on the specificity of the embedded antibodies. Therefore, the present methodological approach shares some of the limitations present in enzyme immunoassays. On the other hand, the sensor response is independent from factors narrowing the applicability of plate-trapped antigen (PTA) immunoassays, such as the duration of incubation time (Mowat and Dawson, 1987).

The development of the novel sensor concept is still at a preliminary stage: for example, further experiments are required in order to define the linear range of the response and the possible interference of other viruses on the selectivity of the sensor.

5. Conclusions

The novel biosensor has a number of distinct performance characteristics that could make it an attractive future technology for routine, field-based viral assays. BERA-based virus assays have a comparable cost with available enzyme immunoassays but are considerably faster (with a total assay time less than 10 min). In addition, BERA viral assays are one-step procedures, requiring no reagent addition/washing, therefore facilitating scale-up of the assay process. Further increase in the sensor sensitivity could arise by increasing the concentration of immobilized cells per sensor or the concentration of electroinserted antibodies onto the cell surface. Both approaches are being currently tested by our research group.

Acknowledgements

The research project was co-funded by the EMBIO Project of the Cypriot Ministry of Industry, Commerce and Tourism, the European Social Fund and National Resources—O.P. "Education" II and the Greek-Bulgarian Joint Research and Technology Programme 4.3.6.1.

References

Campbell, C.E., Laane, M.M., Haugarvoll, E., Giaever, I., 2007. Biosens. Bioelectron. 23, 536–542.
Eddy, A., 1989. Methods Enzymol. 172, 95–101.
Kintzios, S., Pistola, E., Konstas, J., Bem, F., Matakiadis, T., Alexandropoulos, N., Biselis, I., Levin, R., 2001. Biosens. Bioelectron. 16, 467–480.
Kintzios, S., Bem, F., Mangana, O., Nomikou, K., Markoulatos, P., Alexandropoulos, N., Fasseas, C., Arakelyan, V., Petrou, A.-L., Soukouli, K., Moschopoulou, G., Yialouris, C., Simonian, A., 2004. Biosens. Bioelectron. 20, 907–916.
Kintzios, S., 2007. Mini-Rev. Med. Chem. 7, 1019–1026.
MacFarlane, S.A., 1999. J. Gen. Virol. 80, 2799–2807.
Mowat, W.P., Dawson, S., 1987. J. Virol. Methods 15, 233–247.
Park, J.C., Hwang, Y.S., Suh, H., 2000. Yonsei Med. J. 41, 836–844.
Ploeg, A.T., Brown, D.J.F., Robinson, D.J., 1992. Ann. Appl. Biol. 121, 619–630.
Rider, T.H., Petrovick, M.S., Nargi, F.E., Harper, J.D., Schwoebel, E.D., Mathews, R.H., Blanchard, D.J., Bortolin, L.T., Young, A.M., Chen, J., Hollis, M.A., 2003. Science 301, 213–215.
Riska, P.F., Su, Y., Bardarov, S., Freulich, L., Sarkis, G., Hatful, G., Carriére, C., Kumar, V., Chan, J., Jacobs Jr., W.R., 1999. J. Clin. Microbiol. 37, 1144–1449.
Thach, D.C., Shaffer, K.M., Ma, W., Stenger, D.A., 2003. Biosens. Bioelectron. 18, 1065–1072.
Van der Lelie, D., Regniers, L., Borremans, B., Provoost, A., Verschaeve, L., 1997. Mutat. Res. 389, 279–290.
Whelan, R.J., Zare, R.N., 2003. Biosens. Bioelectron. 18, 1065–1072.
Zeira, M., Tosi, P.F., Mouneimne, Y., Lazarte, J., Sneed, L., Volsky, D.L., Nikolau, C., 1991. Proc. Natl. Acad. Sci. U.S.A. 88, 4409–4411.