Biosensors and Bioelectronics 23 (2008) 1900–1903

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Short communication

Nanoelectrode ensembles as recognition platform for electrochemical immunosensors

S. Pozzi Mucelli[b,d], M. Zamuner[a], M. Tormen[c], G. Stanta[b], P. Ugo[a,*]

[a] Department of Physical Chemistry, University of Venice, Santa Marta 2137, 30123 Venice, Italy
[b] Molecular Histopathology Laboratory, ICGEB, Padriciano 99, 34012 Trieste, Italy
[c] Laboratorio Nazionale TASC-INFM-CNR, S.S. 14, Km. 163.5, I-34012 Trieste, Italy
[d] European Center for the Sustainable Impact of Nanotechnologies, Viale Porta Adige 45, 45100 Rovigo, Italy

ARTICLE INFO

Article history:
Received 14 December 2007
Received in revised form 26 February 2008
Accepted 29 February 2008
Available online 8 March 2008

Keywords:
Nanoelectrode
Ensembles
Immunosensor
Voltammetry
Trastuzumab
HER2 receptor

ABSTRACT

In this study we demonstrate the possibility to prepare highly sensitive nanostructured electrochemical immunosensors by immobilizing biorecognition elements on nanoelectrode ensembles (NEEs) prepared in track-etch polycarbonate membranes. The gold nanodisk electrodes act as electrochemical transducers while the surrounding polycarbonate binds the antibody-based biorecognition layer. The interaction between target protein and antibody is detected by suitable secondary antibodies labelled with a redox enzyme. A redox mediator, added to the sample solution, shuttles electrons from the nanoelectrodes to the biorecognition layer, so generating an electrocatalytic signal. This allows one to fully exploit the highly improved signal-to-background current ratio, typical of NEEs. In particular, the receptor protein HER2 was studied as the target analyte. HER2 detection allows the identification of breast cancer that can be treated with the monoclonal antibody trastuzumab. NEEs were functionalized with trastuzumab which interacts specifically with HER2. The biorecognition process was completed by adding a primary antibody and a secondary antibody labelled with horseradish peroxidase. Hydrogen peroxide was added to modulate the label electroactivity; methylene blue was the redox mediator generating voltammetric signals. NEEs functionalized with trastuzumab were tested to detect small amounts of HER2 in diluted cell lysates and tumour lysates.

1. Introduction

Nanoelectrode ensembles (NEEs) are new nanoelectrochemical tools very useful for electroanalysis and sensors (Menon and Martin, 1995; Ugo et al., 2002). They are prepared by electroless deposition of gold within the pores of track-etch polycarbonate membranes. A NEE is made by a very large number of very small ultramicroelectrodes confined in a rather small space, with a density $\geq 10^8$ electrodes/cm^2. NEEs can exhibit distinct voltammetric response regimes depending on the scan rate or distance between the nanoelectrode elements. The total overlap regime is commonly observed at ensembles of nanodisk electrodes prepared from commercial track-etched membranes. Under these conditions, the faradaic current (signal) is proportional to the geometric area (A_{geom}; area of nanodisks and polycarbonate), while the double-layer capacitive current (background) depends on the active area (A_{act}; area of the nanodisks). Therefore, NEEs are characterized by detection limits two to three orders of magnitude lower than regular electrodes (Ugo et al., 1996; Brunetti et al., 2000).

In typical schemes used for electrochemical biosensors, a biorecognition layer is immobilized directly on the electrode surface and the signal is produced by exchange of electrons with the underlying electrode; this was applied also to arrays of nanoelectrodes (Lin et al., 2004; Li et al., 2003; Lapierre-Devlin et al., 2005; Delvaux et al., 2005). However, for extremely miniaturized electrodes, such as NEEs, the amount of biomolecule immobilized on the nanoelectrodes can be too small to furnish useful signals (De Leo et al., 2007a). In order to increase the electrode area available for the immobilization, the template membrane can be etched (Yu et al., 2003; Lapierre-Devlin et al., 2005; Krishnamoorthy and Zoski, 2005) to obtain ensembles of gold nanofibers. However, this causes the increase of capacitive current and lowering of signal-to-background current ratios (De Leo et al., 2007a).

This prompted us to explore a different approach, using the template membrane of the ensemble and not the nanoelectrodes to immobilize the biorecognition elements. In such a design no

* Corresponding author. Tel.: +39 041 2348503; fax: +39 041 2348594.
E-mail address: ugo@unive.it (P. Ugo).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2008.02.027

increase in A_{act} is required and voltammetric signals should be produced at the highest signal/background current ratio.

On the basis of its popularity in classical ELISA tests as well as in advanced electrochemical immunoassays (Yu et al., 2006) we chose horseradish peroxidase (HRP) as the enzyme label, using methylene blue (MB) as the redox mediator (in the solution phase) to shuttle electrons from the nanoelectrodes to the label. We tested this approach with an extremely actual issue that is the determination of the expression levels of the HER2 receptor and the binding activity of its agonist trastuzumab (or Herceptin®), a drug used in the adjuvant therapy of the breast cancer (Molina et al., 2001). The possibility to detect HER2 is extremely important for the identification of cancers that can be treated with Herceptin®, providing a good opportunity to define the so-called personalized therapies.

2. Materials and methods

2.1. Electrochemical apparatus

All electroanalytical measurements were carried out at room temperature (22 ± 1 °C) with a CH660A potentiostat, using a three-electrode single-compartment cell equipped with a platinum counter electrode and an Ag/AgCl (KCl-saturated) reference electrode.

2.2. Chemicals

All chemicals used were reagent grade and used without further purification. HRP type VI, 298 purpurogallin units/mg solid, was from Sigma. Purified water was obtained using a Milli-Ro plus Milli-Q (Millipore) system.

2.3. Sensors

NEEs were prepared by template gold electroless deposition (Ugo and Moretto, 2007), using recently described updates (De Leo et al., 2007a,b). Templating membranes were polycarbonate track-etched membranes (SPI-pore, 47 mm filter diameter, 6 μm thickness) with a nominal pore diameter of 30 nm, average pore density 6×10^8 pore/cm^2, coated by the producer with polyvinylpyrrolidone. The final NEEs were assembled as previously described (Ugo and Moretto, 2007). A hole punched in an insulating layer of plastics (Monokote by Topflite) determined the A_{geom}, typically 0.07 cm^2. The value of A_{act}, estimated by SEM analysis, was 0.001 cm^2.

The detection strategy is summarized as follows. At first, the specific antibody trastuzumab is immobilized on the polycarbonate. Then, it is incubated with the sample to capture the target protein HER2. Finally, the captured protein is reacted with the primary antibody (namely, monoclonal CB-11) and the secondary labelled antibody, i.e. anti-mouse HRP-conjugated antibody. Electrochemical signal is generated by MB added as soluble mediator which shuttles electrons from the nanoelectrode to HRP, when the latter reacts with its substrate, typically, 1.5 mM H_2O_2.

Trastuzumab functionalized NEEs (T-NEEs) were prepared by incubating on the NEE 2 μl of 0.5 μg/μl trastuzumab (Hercerptin®, Genentech, South San Francisco, CA) in carbonate buffer, for 2 h at 4 °C. Polycarbonate contains carbonyl groups which can react with amine groups of proteins (Rucker et al., 2005). After washing with phosphate buffer saline, added with 0.2% (v/v) Tween 20 (PBST), and final incubation for 10 min in $NaBH_4$ to block reactive groups remaining eventually on the membrane (Afanassiev et al., 2000), the T-NEE was ready to react with the target analyte. For capture tests 10 μl of four dilutions of a 10 μg/μl SKBR3 lysate (diluted 1:1, 1:5, 1:10, 1:50) were incubated on the T-NEE for 4 h at 4 °C. After washing with PBST, the T-NEE was incubated for 60 min with the monoclonal antibody CB-11, diluted 1:5000 in PBST-milk. After washing again with PBST, the T-NEE was treated for 60 min in 10 ml of a 1:10,000 dilution of an anti-mouse HRP-conjugated antibody in PBST-milk. After final washing, the T-NEE was dipped in a three-electrode electrochemical cell containing 0.1 mM MB, 10 mM phosphate buffer, pH 7, deaerated with nitrogen. The activity of the immobilized HRP label was measured by CVs recorded at 50 mV/s in the absence and presence of 1.5 mM H_2O_2. All measurements were made as duplicate; precision was within 5%.

T-NEEs used as negative controls were not incubated with the cell lysate, or with lysates not containing HER2, and after the immobilization of trastuzumab, they were incubated with the CB-11 antibody and the secondary labelled antibody.

2.4. Samples

HER-2 overexpressing (SKBR3) and HER2 negative (MCF7) cell lysates were prepared using a lysis buffer (50 mM Tris–Cl pH 8.0, 0.4 M NaCl, 1% v/v, NP40, protease inhibitors), total proteins concentration was calculated and adjusted to 10 μg/μl following a Bradford assay. HER2 concentration was estimated 2 ng/μl by a Western blot analysis using a CB11 antibody.

Breast cancer samples were kindly provided by the Department of Biomedical Sciences and Human Oncology of the University of Turin, Italy. Samples were homogenized using a mechanical system (UltraTurrax T25, Rose Scientific Ltd., Canada) in the same buffer of cells, and analysed by Western blot to visualize the expression of HER2. HER2 concentration was estimated 1 ng/μl.

3. Results and discussion

Preliminary tests in solution showed that the best mediator suitable to shuttle electron from NEE to HRP is MB.

The dotted line cyclic voltammetry (CV) in Fig. 1, shows a well-resolved reduction peak recorded at a NEE, relevant to the reversible reduction (Ye and Baldwin, 1988):

$$MB + 2e + H^+ \leftrightarrows LB \quad (1)$$

where LB is the leuco (reduced) form of MB.

MB signals are significantly better resolved from background currents at a NEE than at a conventional Au-electrode, under the same experimental conditions (not shown).

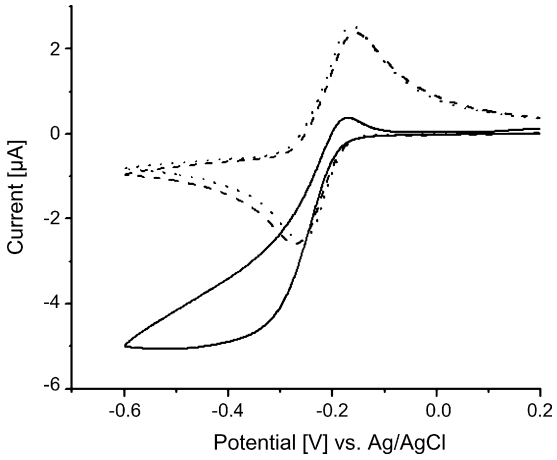

Fig. 1. Cyclic voltammograms recorded at a gold-NEE in 0.1 mM methylene blue before (dotted line) and after the addition of 1.2 mM H_2O_2 (broken line) and 0.18 mg/mL horseradish peroxidase (full line). Scan rate: 100 mV/s, supporting electrolyte 10 mM phosphate buffer, pH 7.

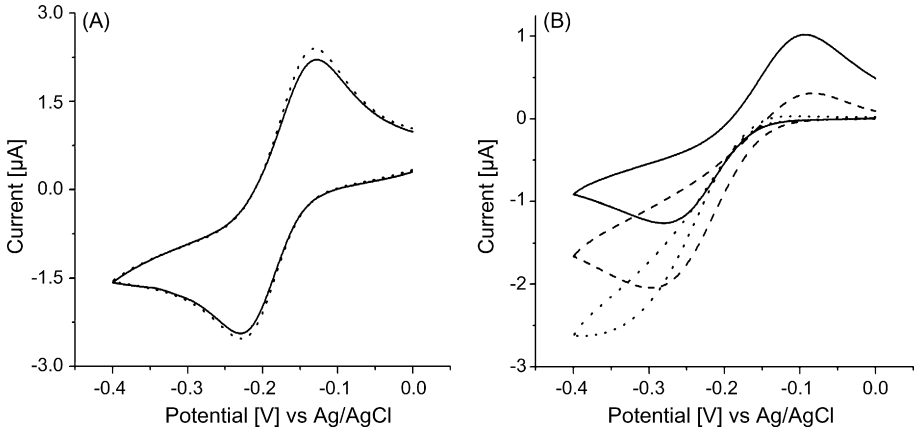

Fig. 2. Cyclic voltammograms recorded at 50 mV/s in 10 mM phosphate buffer, pH 7, at a NEE functionalized with trastuzumab. (A) Incubated in a 1:10 MCF7 cell lysate (HER2 negative) plus primary and secondary antibody with HRP label and dipped in solution containing 0.1 mM methylene blue without (full line) and with 1.5 mM H_2O_2 added (dotted line). (B) Incubated in a 1:10 cell lysate containing HER2 plus primary and secondary antibody with HRP label, in the presence of H_2O_2: 0 mM (full line), 0.5 mM (dashed line) and 1.5 mM (dotted line).

The broken line in Fig. 1, shows that, by adding only H_2O_2, no significant change in the CV is detected. Finally, when also the HRP is added (full line), dramatic changes become evident, namely:

- the reduction current increases and the voltammetric pattern tends to a sigmoid;
- the reoxidation peak tends to disappear.

Both effects increase by increasing H_2O_2 concentration (not shown). This agrees with the following reactions:

$$HRP_{red} + H_2O_2 + 2H^+ \rightarrow HRP_{ox} + 2H_2O \quad (2)$$

$$HRP_{ox} + LB \rightarrow HRP_{red} + MB \quad (3)$$

which, combined with reaction (1), give a complete electrocatalytic cycle.

In order to test the capability of trastuzumab to act as capture agent, preliminary immunoprecipitation assays were performed. As negative control, we immunoprecipitated our sample using an anti-PCNA (Proliferating cell nuclear antigen, Santa Cruz Biotechnology, CA) antibody, while as positive control, 70 μg of whole SKBR3 (Pasleau et al., 1993) lysate were loaded onto the gel. Western blot analysis performed after the immunoprecipitation, evidenced the bands at 185 and 95 kDa, both due to the HER2 protein, so confirming (Molina et al., 2001) the efficiency of trastuzumab as capture agent *in vitro*. This prompted us to undergo the study of trastuzumab functionalized NEEs.

Fig. 2A, shows the CV for MB at a NEE after the immobilization of trastuzumab, recorded in the presence of a MCF7 cell lysate (Her2 negative), primary and secondary labelled antibody, with (dotted line) and without (full line) added H_2O_2 (namely, 1.5 mM). The two curves are essentially overlapped, and compare well with the voltammetric signal recorded at bare NEEs. This confirms that no change in the voltammetric behavior of MB at the gold NEE is caused by functionalization with trastuzumab. This agrees with recent reports (Moulton et al., 2003) showing almost negligible adsorption on gold of proteins with high isoelectric point, such as trastuzumab, with a p*I* of 9.2 (Wiig et al., 2005). Moreover, no specific adsorption of primary and labelled antibody occurs if the target protein (HER2) is not present in the sample.

Fig. 2B shows the results after incubation in the cell lysates, containing the target protein HER2, and incubation with the primary antibody and secondary antibody with HRP label. The sample was diluted 1:10 (total protein concentration 1 μg/μl) and the three CVs were recorded at three different H_2O_2 concentrations (0, 0.5 and 1.5 mM). The CV with no added H_2O_2 (full line in Fig. 2B) is characterized by a larger peak-to-peak separation than those in Fig. 2A, however the peak current ratio is still close to unity. The immobilization of the all four-protein chain (specific antibody, target protein, primary antibody and secondary labelled antibody) hinders slightly, but does not block electron

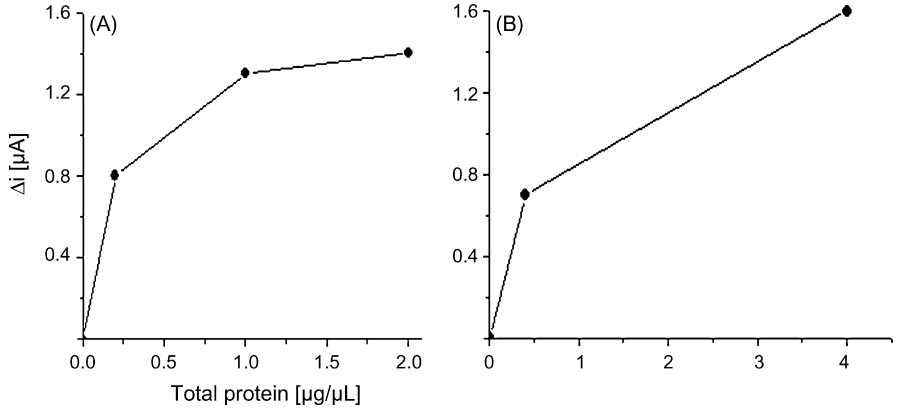

Fig. 3. Dependence of the electrocatalytic current increments on the total protein content (Δi) in 1.5 mM H_2O_2 for T-NEEs treated as in Fig. 2B: (A) samples diluted 1:5, 1:10, 1:50. (B) Lysate of a HER2 overexpressing cancer undiluted and diluted 1:10.

transfer at the nanoelectrodes. The voltammogram recorded in the presence of 1.5 mM H_2O_2 (dotted line in Fig. 2B) shows a typical electrocatalytic character. This H_2O_2 concentration gives the highest electrocatalytic increase in currents, without causing any undesired increase in background current, as observed for instance when 3 or 5 mM H_2O_2 was added to the sample. As shown by the plots in Fig. 3A, the electrocatalytic increase of reduction currents (Δi) scales inversely with the dilution of the lysate.

The results obtained with undiluted lysates put in evidence some distortion of the CVs (not shown); this was probably due to adsorption of proteins which are present at high concentration in undiluted samples.

Note that the sensitivity of classical immunochemical assays, such as Western blotting, is not high enough to detect the HER2 analyte in 1:10 or 1:50 diluted samples, while good electrocatalytic signals were detected with the T-NEE, specifically in the most diluted samples examined here, where the HER2 concentration was approximately 0.04 ng/μl. In principle, detection limits at NEEs can be further lowered by pulsed voltammetry (Moretto et al., 2004). These encouraging results prompted us to test a preliminary real application, that is the analysis of a breast cancer lysate.

To this aim, T-NEEs were incubated in 4 μg/μl lysates of a HER2 overexpressing cancer, both undiluted and diluted 1:10. Relevant voltammetric patterns are characterized by significant electrocatalytic effects both for the undiluted and diluted samples, with a trend in the increase in electrocatalytic current opposite to the dilution of the sample (Fig. 3B). Useful signals are obtained also for the 1:10 diluted sample, where HER2 concentration is approximately 0.1 ng/μl.

These results confirm, for the first time, the high potentialities of nanoelectrode ensembles as detection platforms for electrochemical immunosensors, where the recognition and detections elements are separated, albeit strictly integrated within a range of few nanometers.

4. Conclusions

This is, up to now, the first report demonstrating the usefulness of NEE as detection platforms for immunosensors where the high signal/background ratio typical of nanoelectrodes is preserved. This is particularly attractive for detection of trace proteins such as HER2. T-NEEs detect the target protein in diluted samples, where traditional immunochemical methods, such as Western blotting, fail. Specialized studies for quantifying the analytical performances of T-NEEs (detection limit, dynamic range, etc.) are in progress. The applicability of this approach to other antibodies was tested with preliminarily successful experiments (Pozzi Mucelli, 2007) with respect to the detection of single chain fragment variable (Sblattero and Bradbury, 2000). A final note concerns with the possible photoactivation of polycarbonate which could increase the loading of the capture antibody (Bora et al., 2006), so improving further the detection capabilities.

Acknowledgments

We thank Prof. Giuseppe Firrao (University of Udine) for helpful discussion and MUR (Rome), PRIN 2006, for financial support.

References

Afanassiev, V., Hanemann, V., Wolfl, S., 2000. Nucleic Acids Res. 28, E66.
Bora, U., Sharma, P., Kumar, S., Kannan, K., Nahar, P., 2006. Talanta 70, 624–629.
Brunetti, B., Ugo, P., Moretto, L.M., Martin, C.R., 2000. J. Electroanal. Chem. 491, 166–174.
De Leo, M., Kuhn, A., Ugo, P., 2007a. Electroanalysis 19, 227–236.
De Leo, M., Pereira, F.C., Moretto, L.M., Scopece, P., Polizzi, S., Ugo, P., 2007b. Chem. Mater. 19, 5955–5964.
Delvaux, M., Walcarius, A., Demoustier-Champagne, S., 2005. Biosens. Bioelectron. 20, 1587–1594.
Krishnamoorthy, K., Zoski, C.G., 2005. Anal. Chem. 77, 5068–5071.
Lapierre-Devlin, M.A., Asher, C., Taft, B., Gasparac, R., Roberts, M., Kelly, S., 2005. Nano Lett. 5, 1051–1055.
Li, J., Ng, H.T., Cassell, A., Fan, W., Chen, H., Ye, Q., Koehne, J., Han, J., Meyyappan, M., 2003. Nano Lett. 3, 597–602.
Lin, Y., Lu, F., Tu, Y., Ren, Z., 2004. Nano Lett. 4, 191–195.
Menon, V., Martin, C.R., 1995. Anal. Chem. 67, 1920–1928.
Molina, M.A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., Baselga, J., 2001. Cancer Res. 61, 4744–4749.
Moretto, L.M., Pepe, N., Ugo, P., 2004. Talanta 62, 1055–1060.
Moulton, S.E., Barisci, J.N., Bath, A., Stella, R., Wallace, G.G., 2003. J. Colloid Interface Sci. 261, 312–319.
Pasleau, F., Grooteclaes, M., Gol-Winkler, R., 1993. Oncogene 8, 849–854.
Pozzi Mucelli, S., 2007. An electrochemical array for the quantitative detection of specific proteins in a complex system. Ph.D. Thesis. Department of Clinical, Morphological and Technological Sciences, University of Trieste.
Rucker, V.C., Havenstrite, K.L., Simmons, B.A., Sickafoose, S.M., Herr, A.E., Shediac, R., 2005. Langmuir 21, 7621–7625.
Sblattero, D., Bradbury, A., 2000. Nat. Biotechnol. 18, 75–80.
Ugo, P., Moretto, L.M., 2007. In: Zoski, C. (Ed.), Handbook of Electrochemistry. Elsevier, Amsterdam, pp. 678–709.
Ugo, P., Moretto, L.M., Bellomi, S., Menon, V.P., Martin, C.R., 1996. Anal. Chem. 68, 4160–4165.
Ugo, P., Moretto, L.M., Vezzà, F., 2002. Chemphyschem 3, 917–925.
Ye, J., Baldwin, R.P., 1988. Anal. Chem. 60, 2263–2268.
Yu, S., Li, N., Wharton, J., Martin, C.R., 2003. Nano Lett. 3, 815–818.
Yu, X., Munge, B., Patel, V., Jensen, G., Bhirde, A., Gong, J.D., Kim, S.N., Gillespie, J., Gutkind, J.S., Papadimitrakopoulos, F., Rusling, J.F., 2006. J. Am. Chem. Soc. 128, 11199–11205.
Wiig, H., Gyenge, C.C., Tenstad, O., 2005. J. Physiol. 567, 557–567.