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Regeneration of surface plasmone resonance chips for multiple use

A. Sankiewicz*, A. Tokarzewicz, E. Gorodkiewicz

Department of Electrochemistry, Institute of Chemistry, University of Bialystok, Al.J.Pilsudskiego11/4, PL-15-443 Bialystok, Poland

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The aim of this work was to investigate the possibility for regeneration of used biosensors SPRI using a special cleaning mixture. The presented procedure regenerates the thiol surface without affecting its surface properties and reproducibility of determination. Surface chip control before and after regeneration demonstrates the efficiency of the presented regeneration method. What is new is the use of a known mixture of regeneration yielding clean chips with the appropriate structure (layers of photopolymer and various monolayer immobilized substance).

Keywords: regeneration, surface plasmon resonance, biosensor

INTRODUCTION

The Surface Plasmon Resonance Imaging (SPRI) technique operating jointly with specific biosensors is a very promising tool for medical diagnosis [1-5]. In many cases, SPRI provided a diagnostic opportunity which was previously unavailable. A significant factor for popularization of SPRI is the reduced cost of a single measurement [6,7]. Therefore, the aim of this work was to investigate the possibility for regeneration of used biosensors and evaluation of their analytical applicability.

In terms of transduction, SPRI biosensors are optical biosensors. Several optical techniques are based on the phenomenon of surface plasmon resonance (SPR) and its modified versions: SPRI and Multi-Parametric Surface Plasmon Resonance (MP-SPR) [8-11]. These techniques are used for "label-free" detection. The effect of molecular interactions may be analyzed directly on a surface. The effect of SPR occurs when polarized light hits a prism covered by a thin metal layer. Under certain conditions (wavelength, polarization and incidence angle), a thin layer of metal on a high refractive index glass surface can absorb laser light, producing electron waves (surface plasmons) on the metal surface [8]. A thin layer of gold is the most suitable one for SPR measurements. The SPR signal is directly proportional to mass changes on the metal surface. SPR has also been used to monitor the adsorption of biological molecules onto a chemically modified gold surface [8-10]. The SPR signal is converted into an image in the SPRI technique.

Biosensor design should be adapted to the use of a specific receptor-analyte response. Generally, a unmodified gold layer is not a suitable surface environment for biomolecular interaction. Therefore, gold on chips is modified with a thiol monolayer. The thiol monolayer is formed by the gold interaction with thiol sulphur. Interaction energy is of the order of chemical bond energy. A specific receptor for analyte molecules can be immobilized on the thiol surface. Various immobilization strategies can be used: adsorption, covalent bonds or hydrophobic interaction [12,13]. The most important parameters for biosensors are specificity, chemical stability, sensitivity, and reproducibility.

Two biosensors based on regenerated chips were investigated: (i) the biosensor for podoplanin determination, and (ii) the biosensor for proteasome 20S determination. The biosensor for podoplanin determination used immobilized podoplanin antibody as a receptor. The receptor was immobilized onto the chip surface by covalent bond through formation of an amide bond between a thiol with a terminal amino group (cysteamine (2-aminoetanotiol)) and an antibody, i.e. a molecule with an active carboxyl group. This system was used for AFM microscopic observation of surface changes on new and regenerated biosensors.

The determination of proteasome 20S with application of new and regenerated biosensors was an example of the effectiveness of such an approach in terms of quantitative determination. An immobilized PSI inhibitor (Z-Ile-Glu(OBut)-Ala-

* To whom all correspondence should be sent:
E-mail: ania@uwb.edu.pl

Leu-H) was used as a receptor. The latter was immobilized by covalent interaction between a thiol with a terminal amino group (cysteamine (2-aminoetanotiol) and PSI. The only opportunity considered in this work was the alkaline cleavage of the biosensor surface layer with regeneration of the gold surface covered with thiol. Such an approach is more universal. Different regenerated biosensors have the same state of surface (gold covered with thiol), provided that the same thiol was used as a linker. The opportunity to only remove an analyte layer during regeneration was not considered in this work. It was necessary to check that the procedure used for regeneration of different types of biosensors can be adapted to the regeneration of the above mentioned biosensors.

EXPERIMENTAL

Chemicals

The proteasome 20S (mammalian) of concentration 12 mg/ml was prepared in a solution of composition: 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM sodium azide (AFFINITI Research Products Ltd, Mamhead, United Kingdom). Z-Ile-Glu(OBut)-Ala-Leu-H (PSI) ($C_{32}H_{50}O_8N_4$, 618.77 Da) (BIOMOL, Lörrach, Germany), recombinant human podoplanin and sheep antibody (IgG) specific for podoplanin (R&D System. Inc.), 11-aminoundecanethiol hydrochloride (MUAM) (PROBIOR, München, Germany) cysteamine hydrochloride, N-ethyl-N'-(3-dimethyl aminopropyl) carbodiimide (EDC), HEPES sodium salt (all SIGMA, Steinheim, Germany), 1-octadecanethiol (ODM), N-hydroxysuccinimide (NHS) (ALDRICH, Munich, Germany), dichloroethane (FLUKA, Munich, Germany), absolute ethanol (POCh, Gliwice, Poland), photopolymer ELPEMER SD 2054, hydrophobic protective paint SD 2368 UV SG-DG (PETERS, Kempen, Germany) were used as received. Aqueous solutions were prepared with filtered milliQ water (Simplicity®MILLIPORE).

Biosensor preparation

The biosensor was manufactured in several stages. Initially, a 1nm Cr layer was deposited on BK7 glass slides and then a 50 nm thick gold layer was also deposited [14]. Next, in order to protect the surface, the chip was covered with photopolymer ELPEMER SD 2054. The photopolymer was dried at 70°C for 30 min and irradiated with UV light for 2 min. The photopolymer layer was covered with a hydrophobic photomask by application of the screen printing technique. The paint was irradiated with a UV lamp. Thus, the free gold surface of the obtained sensor was separated with a photopolymer and a hydrophobic photomask [3]. 9 places with 12 free gold surfaces were obtained. Chips were rinsed with ethanol and water and dried in an argon flow. A thiol monolayer was formed on the prepared chip. The kind of thiol used is dependent on the type of molecules used to capture the analyte from the sample. Generally, two thiol types were used: a thiol with a terminal amino group ('short' cysteamine (2-aminoetanothiol) and a 'long' MUAM (11-amino-undecane-1-thiol), as well as a thiol with a terminal alkyl group ODM (octadecyl thiol). The chips were then immersed in 20 mM cysteamine ethanolic solution or in 1 mM MUAM ethanolic solution for at least 2 h, or alternatively in 2.7 mM ODM ethanolic solution for at least 24 h. The chips were then rinsed with ethanol and water and dried in an argon flow. The inhibitor, or antibody or phospholipids were immobilized on the thiol monolayer under the suitable conditions [15,16]. Some inhibitors or phospholipids were immobilized on the ODM monolayer, while other inhibitors or antibodies were immobilized on the cysteamine or MUAM monolayer. Hydrophobic inhibitor or phospholipid solutions were placed on the thiol-modified surface, and incubated at room temperature for 24 h. For covalent attachment of antibodies or inhibitors, they were activated with NHS (250 mM) and EDC (250 mM), placed on the amine-modified surface and incubated at 37°C for 1 h. These molecules specifically react with the species-to-be-determined. The biosensor is then ready for analyte concentration measurement.

Chip regeneration procedure

Successful regeneration of SPRI chips and possibility of re-using the biosensor are important, especially when the latter is applied for diagnostic tests.

The cleaning procedure of the chip to a layer of gold has been previously studied [17]. We have used ethanol, Triton X-100 (1% solution), SDS (0.1 mol/l in water), chromic acid ($K_2Cr_2O_7$ saturated in concentrated H_2SO_4), dichloroethane/ethanol (pure/pure), Triton X-100/methanol (1% /pure).

The most commonly used conditions for antibody receptor regeneration included low pH (e.g. 10 mM glycine in HCl at pH 1.5-3) [18]. Although the pH range of inhibitor-protein interaction is very broad, usually the surfaces are regenerated satisfactorily by the use of base or acid hydrolysis.

A mixture of Triton X-100 (1%) containing NaOH (100 mM) was applied as the cleaning agent [19]. Used biosensors were rinsed extensively with this mixture. In this way, the deposited receptors (inhibitors or antibodies) and the analyte layer were removed from the sensor surface. The chips with the remaining thiol monolayer were washed by flushing with water for 5 min and put into water overnight. The last step was repeated 3-4 times. The rinsed chips were dried in an argon flow. In this manner, the prepared chips with the thiol layer were regenerated.

Atomic Force Microscopy (AFM) and Surface Plasmon Resonance Imaging (SPRI) measurements

AFM measurements were performed with a commercial Ntegra Prima scanning probe microscope (NT-MDT, Russia) using a tapping mode in air. In order to increase lateral resolution, high aspect ratio (5:1) ETALON probes (NT-MDT) were used. AFM measurements were performed to confirm the creation of subsequent layers (gold, thiol and protein layer) and to check the chip surface after regeneration. All measurements were done in ambient conditions.

SPRI measurements for protein array were performed as described in a previous paper [3]. On the basis of the registered images, the signal was measured twice - after immobilisation of the PSI inhibitor and after interaction with proteasome 20S. The SPRI signal, which is proportional to the mass of coupled biomolecules, was obtained by subtraction of the signal after and before interaction with a biomolecule, for each spot separately. Then the SPRI signal was integrated over the spot area. NIH Image J version 1.32 software was used to evaluate the SPRI images in 2D form.

Preparation of the standard curve

The response of the analytical SPRI signal for the proteasome 20S concentration was measured over the concentration range between 2 and 25 nM. The chip surface was covered by a monolayer of cysteamine and a layer of immobilized PSI inhibitor (Z-Ile-Glu(OBut)-Ala-Leu-H, $C_{32}H_{50}O_8N_4$) with concentration of 80 nM. The chip was then treated with the proteasome 20S solution. Time of interaction was 10 min. Experiments were performed at pH=7.4 [4].

Precision of the method for proteasome 20S determination

The precision of the method was tested under optimal conditions, i.e. pH=7.4 and inhibitor concentration at the chip preparation stage equal to 80 nM. The precision of proteasome 20S determination was tested for a concentration of 4 nM.

Determination of proteasome in biological samples

The procedure of biological samples (human plasma) preparation and the method for proteasome 20S determination were described in a former paper [4].

RESULTS AND DISCUSSION

The architecture of the applied chip is shown in Fig. 1. Nine different solutions can be simultaneously measured without mixing of the tested samples. Twelve single SPRI measurements can be performed from one solution.

Manufacturing and regeneration of the biosensor was controlled by observation of subsequent layers using Atomic Force Microscopy.

The presented AFM pictures confirm that the stages of the creation of each layer on the biosensor surface have really taken place. As indicated in Fig.1, thiol (Fig.1C) or protein (Fig.1D) immobilization significantly alters surface morphology. The receptor layer (podoplanin antibody 1,5 μg/ml) (Fig.1D) has globular domains. Fig. 1E shows a picture obtained after applying the regeneration procedure. The chip surface after regeneration has very similar morphology to that of the fresh thiol surface (see Fig. 1C).

Surface chip control before and after regeneration demonstrates the efficiency of the regeneration method. Only a thiol monolayer remains on the chip after the regeneration and the regenerated chip with the thiol layer can be reused. After immobilization of a new receptor (inhibitor, antibody, etc.), the biosensor is ready for analyte determination.

The measured SPRI signal before and after regeneration confirmed that the thiol layer remained on the chip surface.

Proteasome 20S determination with a PSI inhibitor (80 nM) as a receptor was used as an example for demonstrating the analytical applicability of the regenerated biosensors. In order to verify the analytical signal before and after regeneration, calibration curves of proteasome 20S with the application of the new and the re-used chip were prepared (Fig. 2A). The response of the analytical SPRI signal for proteasome 20S concentration was measured over the concentration range between 2 and 25 nM. Both calibration curves are of Langmuir's isotherm type. The roughly linear sections of these curves are within the range of 2 - 10 nM and this range is useful for

analytical purposes. The plateau of the curve corresponds to saturation of the active sites of the sensor. The analytical SPRI signal of proteasome 20S obtained with the regenerated biosensor is roughly the same as that obtained with the new biosensor, taking into account the precision error [4].

The number of successful regenerations of a single chip was also investigated. The determination of proteasome 20S (6 nM) with the biosensor with PSI inhibitor (80 nM) as the receptor was used as an example. The results are given in Fig. 2B. Generally, the regenerated chips exhibit a slightly lower and gradually decreasing signal. After the first regeneration the analytical signal is reduced to about 98% and after the fifth regeneration even to 85% of the initial signal. This loss can be compensated by construction of a calibration curve with a regenerated biosensor.

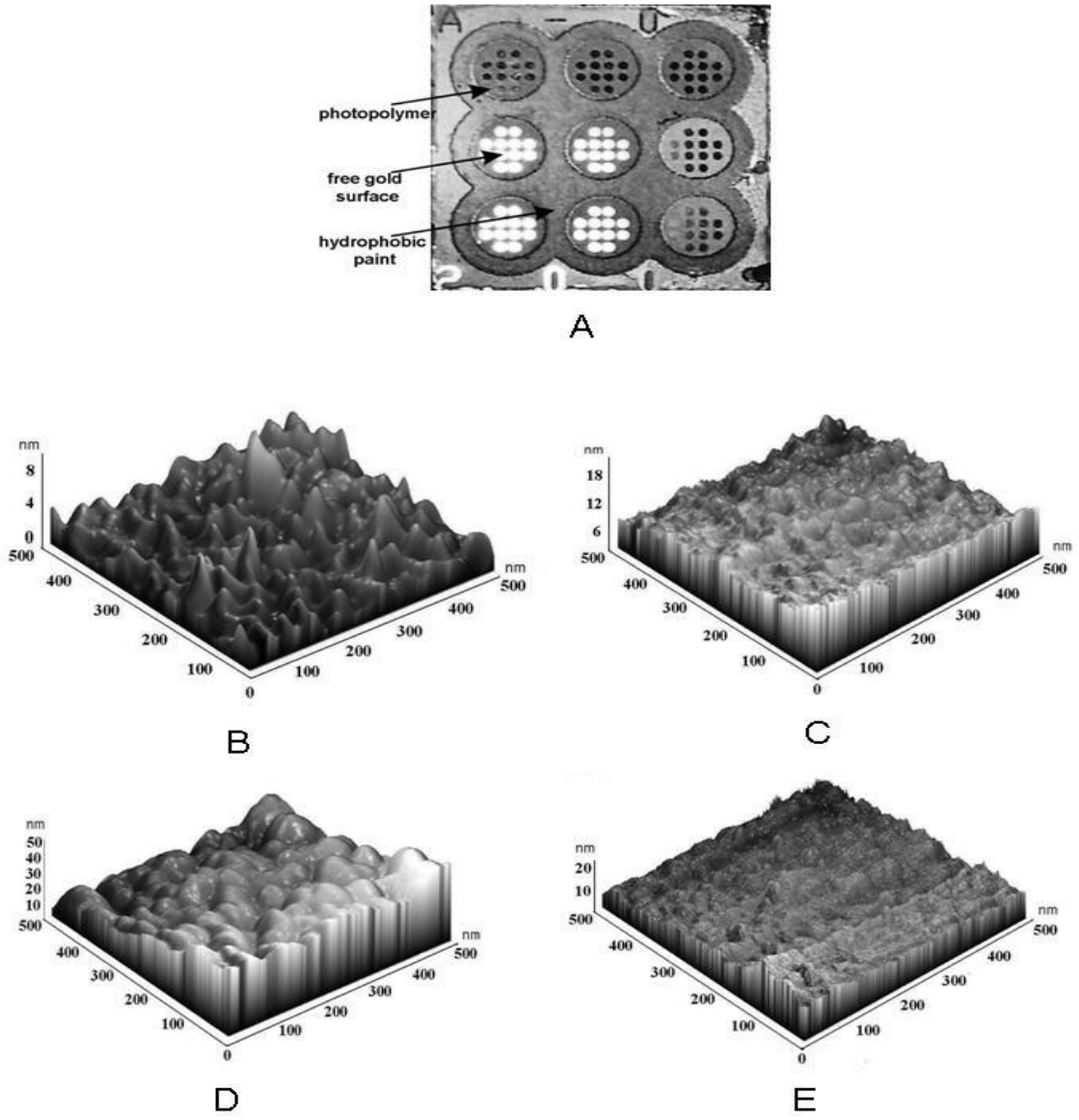

Fig. 1. Picture of chip (A) and AFM pictures of bare gold (B), thiol (cysteamine 20 mM) (C), podoplanin antibody (1.5 μg/ml) (D), thiol after regeneration (E). Initial antibody concentration: 1.5 μg/ml. Initial podoplanin concentration: 1 ng/ml.

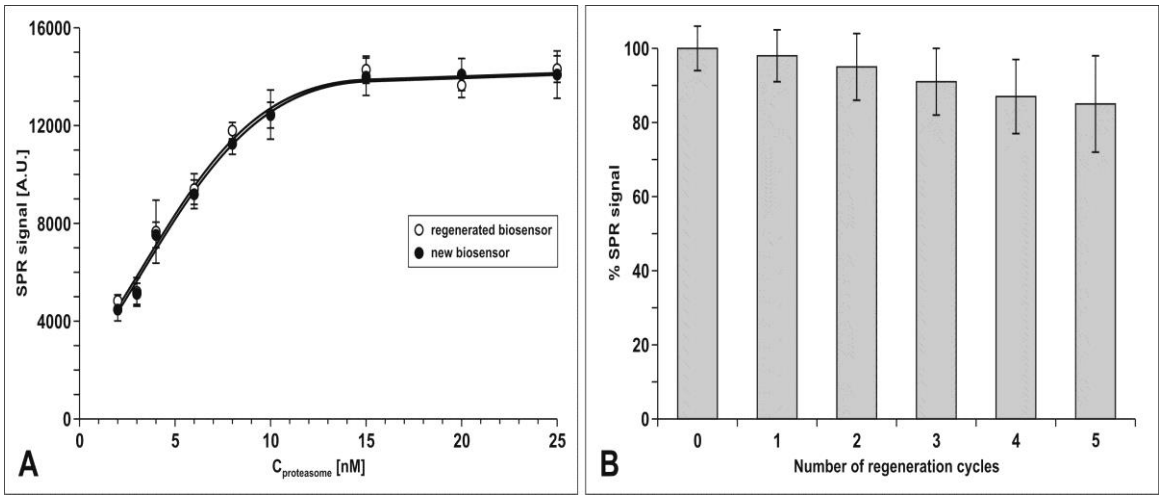

Fig. 2. (A) Dependence of SPRI signal (Arbitrary Units) on proteasome 20S concentration for the PSI inhibitor (80 nM) (B) Effect of repeated chip regeneration on the proteasome 20S SPRI signal on the PSI inhibitor. Concentration of proteasome 20S: 6 nM. pH value of proteasome solution: 7.4.

Table 1. Precision of the concentration measurement of proteasome 20S. The results were evaluated using calibration graphs constructed with biosensors having the same number of regenerations.

	Number of regeneration cycles	No of meas.	Added [nM]	Found [nM]	Recovery [%]	S.D. [nM]	Confidence limit (95%) [nM]
Proteasome 20S determination	0	24	4.00	4.10	102	0.41	0.27
	2	24	4.00	4,05	101	0.39	0.22
	5	24	4.00	3.97	99	0.40	0.23

Table 2. Proteasome 20S concentration in blood plasma of healthy volunteers. The results were evaluated using calibration graphs constructed with biosensors having the same number of regenerations.

	Proteasome 20S concentration in blood plasma [nM]		
Number of sample	New chip	Chip after two regeneration cycles	Chip after five regeneration cycles
1	5.73±0.43	5.80±0.54	5.69±0.81
2	17.12±2.93	16.99±1.76	17.01±2.42
3	11.93±4.12	11.76±2.45	11.99±3.05
4	8.27±2.50	7.97±3.13	8.03±2.76
5	19.40±6.25	18.70±4.15	19.04±3.97
6	14.91±1.78	14.66±2.87	14.97±3.51
7	15.50±5.86	15.42±4.34	15.60±3.39
8	12.62±2.49	11.99±2.24	12.33±3.07
9	14.07±2.02	14.39±3.11	13.97±4.48
Mean value	*13.91 ± 2.78*	*13.08 ± 2.56*	*13.12 ± 2.61*

The precision of proteasome 20S determination was tested for a new and a re-used chip. The results are shown in Table 1.

Standard deviation and confidence limit, at a confidence level of 95%, are very similar for all chips. Generally, the precision of a single measurement is poor, however, the precision of the average value, as well as the confidence limits are much better and acceptable. The spike recoveries on the new and regenerated biosensors are very good, due to the application of suitable calibration graphs.

The proteasome 20S determination in blood plasma from 9 healthy volunteers was performed using new and reused chips. The results are given in Table 2.

The proteasome 20S concentration range in blood plasma of healthy volunteers is: on the new chip 13.91 ± 2.78 nM, after second regeneration of chip 13.08± 2.56 nM and after fifth regeneration 13.12 ± 2.61 nM. Similar values are obtained in the cases of all 9 analysed samples. Thus, the regeneration process does not significantly affect the determination of the concentration of the analyte.

Generally, it was shown that the chip cleaning procedure allows a removal of the immobilized protein layers. Only the thiol layer remains on the chip surface because thiol sulfur forms a strong bond with gold [20]. In the case of a biosensor with thiol containing a terminal amino group, the amine bond of the receptor with the analyte is hydrolyzed by NaOH contained in the cleaning mixture. This process restores the $–NH_2$ groups of the immobilized thiol. Triton X-100, also contained in

the cleaning mixture, is a non-denaturing surfactant, which can disrupt a hydrophobic interaction and lead to the removal of hydrophobically linked molecules [21].

It was shown that the sensor with a thiol monolayer can be reused and the regenerated chip can be used up to five times for the creation of a biosensor. The regeneration process, to a certain degree, affects the surface properties, including the level of surface binding capacity. However, this destructive process occurs gradually and several regenerations do not significantly affect the determination. On the other hand, chip regeneration allows skipping some steps in the biosensor preparation and saves time and cost of single determination.

The developed regeneration procedure can be used for biosensors having cysteamine (2-aminoetanotiol), MUAM (11-amino-undecane-1-thiol)), or ODM (octadecyl thiol) (thiol with a terminal alkyl group) as a linker, as well as immobilized enzyme inhibitor, antibody or phospholipid as a receptor, which was experimentally checked in numerous examples.

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РЕГЕНЕРАЦИЯ НА ЧИПОВЕ С ПОВЪРХОСТЕН РЕЗОНАНС ЗА МНОГОКРАТНА УПОТРЕБА

А. Санкиевич*, А. Токажевич, Е. Городкевич

Департамент по електрохимия, Институт по химия, Университет в Бялисток, Полша

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(Резюме)

Целта на тази работа е изследването на регенерацията на употребявани биосензори SPRI използвайки специална течност за почистване. С нейна помощ се регенерира тиоловата повърхност без да се влияе на повърхностните свойства и възпроизводимостта на определяне. Контролът на чиповете преди и след регенерацията показва приложимостта на настоящия метод на генерация. Нова е употребата на известна смес за почистване на чиповете с подходяща структура (слоеве от фотополимери и различни имбилизирани вещества).