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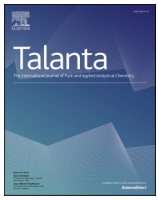

Detection of erythropoietin in blood plasma through an SPRi-based biosensor

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ABSTRACT

A new analytical method based on an SPRi biosensor has been established to determine erythropoietin (EPO) in biological fluids. This protein is involved in the growth of erythroid precursor cells and acts as a therapeutic agent in several diseases. It also appears to have increased biological activity in patients with Alzheimer's disease (AD). A validation process was carried out to assess the optimal ligand-antibody concentration by plotting a calibration curve of EPO. The low limit of detection (LOD) and limit of quantitation (LOQ) values indicate that the developed method can detect very low concentrations in the order of pg/mL. The LOD for EPO was 0.03 pg/mL. The LOQ was 0.10 pg/mL. In addition, determining the appropriate validation parameters confirmed that the designed biosensor has high precision, accuracy, and sensitivity. To prove the usefulness of the biosensor in practice, determinations were performed on blood plasma samples from a control group and a study group consisting of patients with AD. The preliminary results indicate that this biosensor can detect EPO in plasma samples. Confirmation that patients with AD have higher levels of EPO will require the analysis of a more significant number of samples. However, the constructed biosensor is characterized by its simplicity and rapidity of measurement, and the method does not require any special sample preparation.

1. Introduction

Human erythropoietin (EPO), a 165-amino acid protein with a molecular weight of ~30 kDa, is a highly glycosylated hormone whose primary function is the regulation of erythropoiesis. While the kidney is the primary source of EPO, it is also expressed in the liver, brain, and uterus [1]. By binding to the EPO receptor (EPOR) on the surface of erythroid progenitor cells, EPO activates JAK2 (Janus kinase 2) and subsequently phosphorylates EPOR [2]. As a result, several signalling pathways are activated downstream, including the Ras/MAPK (mitogen-activated protein kinase), PI3K (phosphatidylinositol 3-kinase)/AKT (protein kinase B) and STAT (signal transducer and activator of transcription) pathways. These pathways promote erythroid progenitor cells' growth, differentiation, and survival [2]. EPO is primarily used to treat anaemia. Patients with chronic kidney disease, cancer chemotherapy, and HIV infection benefit significantly from the use of EPO [3]. However, overuse has negative consequences. One of the most crucial side effects is an increased risk of thrombosis (blood clots) due to EPO's procoagulant activity [4]. EPO use has also been associated with a higher risk of hypertension, seizures, and cardiovascular events [5]. This protein use also reduces overall survival in cancer patients receiving chemotherapy [6]. However, prolonged use of EPO in cancer patients leads to the formation of neutralizing antibodies that reduce the hormone's effectiveness and result in pure red cell aplasia (PRCA) [7]. A further limitation to the use of EPO is its low blood-brain barrier (BBB) permeability [8], requiring higher concentrations and thus more susceptibility to adverse effects.

Assaraf et al. [9] have shown that EPO receptors (EPOR) are highly expressed in the temporal cortex and hippocampus of people with AD. Upregulation of EPOR in AD-affected neural tissues facilitates the action of EPO. It may protect host cells from the potentially deleterious effects of excess b-amyloid, proinflammatory cytokines, and redox-active

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metals implicated in the pathophysiology of this degenerative disease [10]. EPO concentrations in cerebrospinal fluid (CSF) of patients with AD have been found not significantly different and comparable to those found in healthy patients of the same age [11]. A possible explanation is that CSF EPO concentrations in neurodegenerative conditions may be influenced by post-translational modifications such as glycosylation, which could affect CSF EPO concentrations by influencing biological half-life or interfering with antibody binding [12].

There are no significant studies about EPO levels in the blood plasma of Alzheimer's patients. From the aforementioned information on the importance of EPO in protecting neuronal cells from the detrimental effects of the onset of AD and the fact that the development of the disease triggers through various pathways the overexpression of EPOR and thus enhances the biological activity of EPO, it is conceivable that this protein may provide insight into the clinical status of the Alzheimer's patient and the diagnosis of AD.

AD is currently diagnosed using neuroimaging techniques such as positron emission tomography (PET) or magnetic resonance imaging (MRI). CSF analyses are also carried out. These techniques are effective but time-consuming and relatively expensive [13]. The cheaper alternative, i.e., biosensors, can significantly reduce the research budget and help find more biomarkers in biological fluids, which, as a diagnostic panel, will allow accurate diagnosis and broader analysis of AD.

Biosensors convert biochemical signals generated by specific combinations of molecular recognition elements and target molecules into optical, electrical, or other physical signals for analysis [14]. This process is based on particular binding and signal transduction. The specific binding process is primarily related to the recognition element [15]. Conventional recognition elements include antibodies, enzymes, whole cells, polymeric [16], and aptamers [17]. Based on different signal transduction processes, biosensors can be divided into optical, electrochemical, piezoelectric, temperature sensing, magnetic, and micromechanical [18].

The most common form of assay used in research into the construction of biosensors and their usefulness for determinations in biological fluids is detection in plasma or blood serum [19–21]. These are standard matrices in biological and clinical research derived from whole blood, which undergoes various processes after collection. Because CSF is in close contact with the cerebral cortex and other parts of the brain, its analysis for biomarkers of AD provides the most reliable results regarding the levels of such markers in people with AD [22].

Electrochemical biosensors for detecting and quantifying EPO in human blood plasma samples with fair sensitivity have recently been developed [23,24]. However, electrochemical biosensors face problems such as temperature dependence, interference from other gases, and the necessity to modify the analyte electrochemically.

An SPR biosensor using BiaCore X instrumentation for the detection of EPO in urine samples has also been reported recently, showing a limit of detection (LOD) of 23 ppb and a Coefficient of Variation (CV) of around 20 % [25].

In this work, an SPRi biosensor has been constructed, i.e. a device that allows the concentration of a given protein to be obtained using a transducer with optical detection, i.e. the Surface Plasmon Resonance in imaging mode (SPRi). The SPR phenomenon is used as a detection method in biosensors designed to detect potential biomarkers. The method uses plasmons, i.e., surface electron oscillations between two media (metal-dielectric) [26]. In this area, high sensitivity to introduced changes is observed – adsorption of new particles or immobilization of molecular layers. The basis of operation is the transfer of energy to surface electrons by photons from a light beam. Excitation of plasmons by a light beam can only occur when the component of the light wave vector, which is parallel to the metal surface on which the light falls, corresponds to the plasmon component. Gold is the most commonly used metal as the base layer of the SPRi biosensor. The measurement in this article is based on the Kretschmann configuration [27]. P-polarized light is incident on the glass prism of the SPRi device and is reflected by a 50 nm gold layer placed on the prism (Fig. 1.) Appropriate conditions – the angle of incidence of polarized light and the wavelength – allow the transfer of photon energy to surface electrons between the two media, which causes a change in the angular dependence and spectral intensity of light reflection [28].

Current commercial SPR sensors are based on monitoring the decrease in reflectance with angular or wavelength conditions that occur upon adsorption of successive layers of biomolecules (Fig. 2).

Biological recognition elements on the metal surface recognize and capture the analyte present in the liquid sample, which causes an increase in the refractive index on the metal surface. The increase in the refractive index causes an increase in the propagation constant of the plasmon spreading along the metal surface, which can be accurately measured by optical devices - including a CCD camera, which is used in the SPRi method [29]. Using a recorder instead of a camera allows us to bypass the analysis of sensograms, which are essential in conventional SPR. The change in reflected light is measured in SPRi using images from the CCD camera [30].

Surface Plasmon Resonance enables us to obtain information on the processes occurring at the metal-dielectric interface, such as binding constants, chemical reaction rate constants, and concentration changes [31]. The gold surface can be modified by immobilizing an appropriate thiol (linker) layer, such as cysteamine, octadecanethiol [32], or 11-mercaptoundecanoic acid, used in this study. Forming a linker layer then allows for the attachment of an appropriate receptor layer, where antibodies, inhibitors, enzymes, aptamers, or nucleic acids can be used. This is an excellent basis for constructing biosensors in diagnostics, where it is necessary to search for new solutions to determine potential biomarkers [33].

2. Experimental

2.1. Materials and reagents

The following reagents were used for the research: EDC (*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride) (SIGMA, Steinheim, Germany), NHS *N*-hydroxysuccinimide (Aldrich, Munich, Germany), buffered saline solution (PBS buffer) (Biomed, Lublin, Poland), absolute ethyl alcohol (POCh, Gliwice, Poland), ethanolamine solution

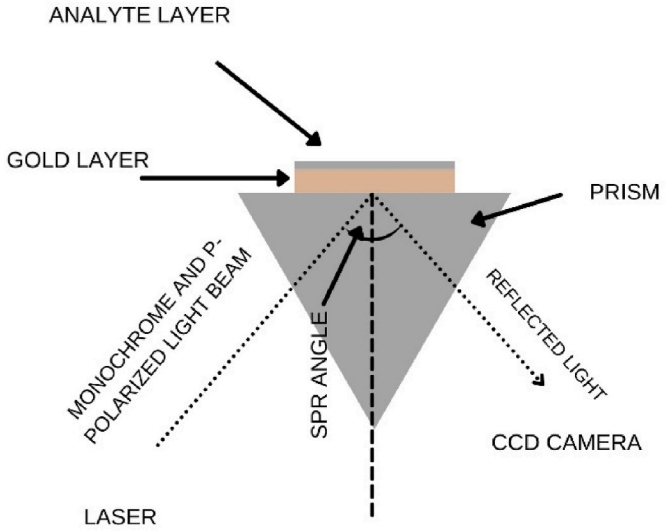

Fig. 1. Schematic of Kretschmann Configuration used in SPRi biosensor designs. A light source incident from a laser hits a gold layer arranged on the surface of a glass prism and is then reflected from its surface at an equivalent angle, called the SPR angle. Own elaboration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

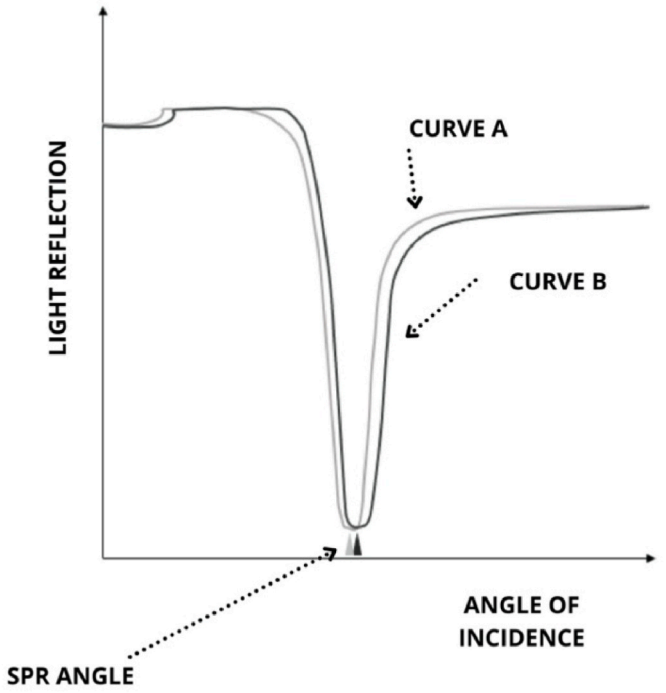

Fig. 2. Shift observed during the recording of signals from SPRi. When another layer of molecules (curve B) is adsorbed on the gold layer (curve A), the reflection curve shifts towards higher angles about the previous layer. Own elaboration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(SIGMA, Steinheim, Germany), thiol - 11-Mercaptoundecanoic acid (SIGMA, Steinheim, Germany), recombinant human erythropoietin protein (R&D Systems, Minneapolis, MN, USA), mouse monoclonal antibody against erythropoietin (R&D Systems, Minneapolis, MN, USA), recombinant human total tau protein (R&D Systems, Minneapolis, MN, USA), ptau-181 protein (Abcam, Cambridge, UK).The base of the biosensor is a plate with a gold layer (Ssens, Enschede, The Netherlands).

2.2. SPRi apparatus and formations of layers of the biosensor

The SPRi measurement was carried out using a Surface Plasmon Resonance apparatus in the imaging version (SPRi) in the Bioanalytical Laboratory of the Faculty of Chemistry, University of Bialystok. The apparatus consists of an LED light source of wavelength $\lambda = 635$ nm and a collimator, a lens system focusing the light beam, and a polarizer responsible for the polarization p. A beam of monochromatic and p-polarized light then passes through a prism on which a biosensor is placed, and the reflected light is collected by a CCD camera with a resolution of 1.4 MP and converted into an image. The device also consists of movable elements, allowing it to operate in an angular range from 30° to 75°. The user can select the appropriate angle, which is determined individually for each biosensor, that is, for each glass plate with a gold layer immobilizing the corresponding antibody layer. The angle is selected during the analytical method validation process for each biosensor—each time a new gold plate and photopolymer layer is used—using WinSpall SPR curve modeling software. The selected SPR angle value is where the most significant change in reflectance from the baseline curve is monitored. The chip is made of BK7 glass on which titanium (1 nm) and gold (50 nm) layers have been sputtered. For further immobilization of the antibody on the gold plate, the chip is immersed in an alcoholic solution of 11-MUA (11-mercaptoundecanoic acid) thiol at a concentration of 1 mM for 24 h. The chip is then washed with ethyl alcohol and dried in an argon stream. A foil that separates the nine measurement sites is placed on the prepared chip. The components and structure of the sensor are shown in Fig. 3.

2.3. Biological material

The biological material consisted of eight plasma control plasma samples from patients who were not affected by AD but were smokers, as smoking causes hypoxia in the brain and consequently can lead to neurodegenerative conditions, and seven plasma samples from patients diagnosed with Alzheimer's Disease. The relevant Bioethics Committee of MUoB agreed to conduct research using this material (approval APK.002.596.2024–March 20, 2025).

3. Procedures

3.1. Immobilization of the receptor layer – antibody

A mixture of EDC (0.4 M) and NHS (0.1 M) is applied to nine sample places in a gold chip. EDC transforms the carboxyl group of the thiol into an ester group, while NHS converts the resulting ester into an active, short-lived NHS ester. After 10 min, the solution is removed from the biosensor's surface, and the antibody-ligand solution is applied for 10 min. The NHS ester covalently binds to the amino groups of the antibody. The next step is the application of ethanolamine (1.0 M) to each of the nine active sites of the biosensor. Ethanolamine deactivates active NHS esters that are not bound to the antibody and removes the OH group from the carboxyl group of the thiol so that other protein molecules do not bind to the same sites - preventing non-specific adsorption. The chip is then washed with PBS buffer and prepared to capture the target analyte from the sample. The diagram is shown in Fig. 4.

3.2. SPRi measurement

After preparing an antibody layer specific for EPO and washing the biosensor with PBS buffer, it was placed on the prism of the SPRi device using oil immersion. The SPRi signal of the receptor layer was measured. 3 μL of EPO samples were applied to the chip and left for 10 min. The association time was experimentally investigated by measuring the SPRi signal about the association time of EPO (10 pg/mL) on the sensor surface. A graph presenting this relationship is presented in Fig. 5. After rinsing with PBS buffer, subsequent measurements were taken and saved

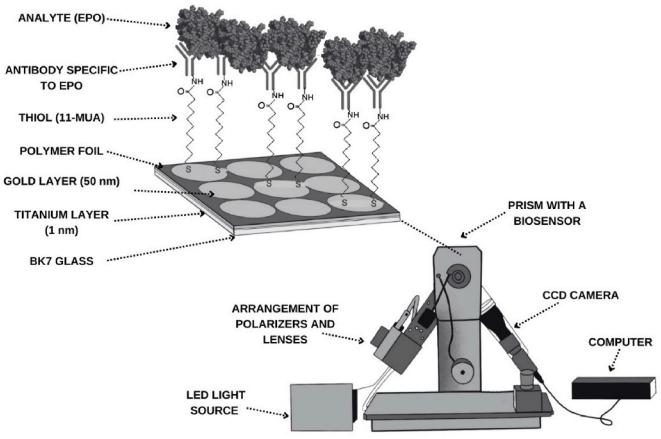

Fig. 3. Schematic diagram of the SPRi apparatus with device components and structure of EPO biosensor. The glass plate (BK7) has a sputtered titanium layer (1 nm), which facilitates the adsorption of a gold layer (50 nm) on its surface. After creating a thiol layer - 11-MUA, an appropriate polymer film is applied to the chip to isolate 9 active sites. Own elaboration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

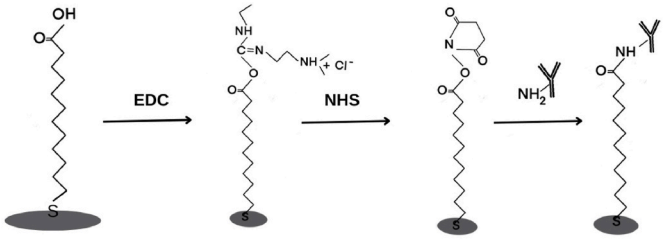

Fig. 4. Schematic diagram of the antibody layer formation on the biosensor's gold surface. EDC modifies the carboxyl group of the antibody into an ester group, while NHS forms an active, short-lived NHS-ester. The applied antibody solution causes the NHS-ester to react with the amine groups of the antibody, forming a covalent amide bond. This is a classic example of EDC/NHS chemistry. Own elaboration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

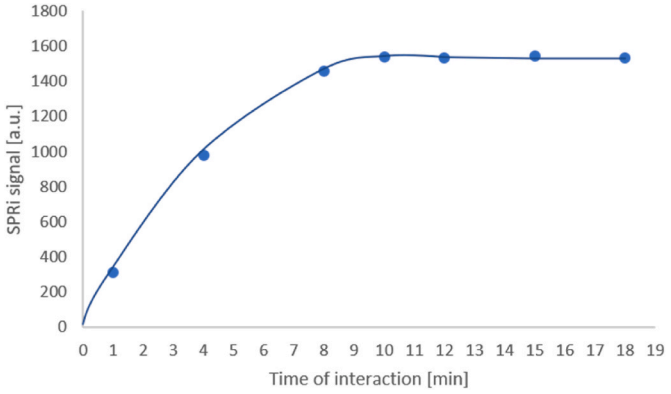

Fig. 5. Changes in the SPRi signal depend on the reaction time. After 10 min, minimal changes in the signal occur – a plateau appears on the curve. The signal increases to a particular value and remains at specific SPRi signal values. Data acquisition was performed for EPO concentration = 10 pg/mL. Ten minutes was selected as the receptor layer interaction time.

as images. The analytical signal is the difference in reflected light before and after interaction with the analyte. ImageJ software (version 1.53, National Institutes of Health, NIH) converted the images into a quantitative signal. Biosensor validation was performed on three other measurement chips to establish appropriate receptor layer concentrations, calibration curves, and precision and accuracy of the method. Determinations in natural samples were eventually performed. Biosensors with SPRi detection can be multiplexed due to the possibility of depositing several antibodies specific for different proteins on the gold surface after applying thiol. In this study, the sensor was used only to determine EPO in samples. An antibody sensitive only to erythropoietin was immobilized in each of the nine measurement sites.

4. Results and discussion

4.1. SPR model curves. Proof of layer formation on the biosensor surface

The formation of successive layers of the biosensor was confirmed by plotting SPR curves. Images were activated in the angular range from 34 to 37°. Measurements were taken of the exposed gold layer and, after forming the 11-MUA thiol layer, the immobilized antibody layer and the attached analyte. The concentrations of the EPO-specific antibody were five μg/mL. The EPO protein concentration was 50 ng/mL. The SPR curves were fitted to the experimental data using the SPR curve modeling software WinSpall (version 3.02, Res Tec, Rosenheim, Germany). Each of the SPR curves is shifted to higher SPR angle values relative to the SPR curve of the previous layer, confirming the presence of newly formed layers. The SPR curves are shown in Fig. 6.

4.2. Optimization of receptor layer concentration

The appropriate concentration of ligand-antibody was determined by applying different concentrations of antibodies specific to EPO to the active sites of the biosensor. The following concentrations were tested for each compound: 10 ng/mL, 50 ng/mL, 10 ng/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL, and 25 μg/mL. The chip with the formed 11-MUA layer was placed on the prism of the SPRi instrument, and the appropriate angle was selected. The first measurements were performed. Then, EDC and NHS were applied, and after 10 min, washing with PBS buffer, the appropriate antibody was used. After another 10 min, excess solutions were removed from the measurement sites, and ethanolamine was applied to avoid non-specific adsorption. In the final step, the ethanolamine was removed, the chip was washed with PBS buffer, and further readings were taken. The analytical signal is the difference in intensity of the reflected light before and after applying the appropriate antibody. The SPRi signals and antibody concentration increase to a specific value, and then a plateau is observed in the saturation curve. The saturation curve is shown in Fig. 7. Above a concentration of 5.00 μg/mL, no ligand binding occurs on the biosensor surface. These highest concentrations will accurately capture the analyte of interest from the sample.

4.3. Analytical response to EPO concentrations

A calibration curve was drawn to check the biosensor response. The SPRi signal was measured in the following concentration range for EPO: from 5.00 to 50.00 pg/mL. Standard solutions of erythropoietin protein were used for this purpose, and the concentrations were obtained by dilution with PBS buffer. Measurements were performed, and the analytically sound ranges, i.e., the rectilinear part of the calibration curve, were selected from the obtained curves. An antibody at a 5.00 μg/mL concentration was immobilized on each active site as a receptor layer. The results are shown in Fig. 8. The detection and quantification limits for EPO were also determined. The calculated results are shown in Table 1. The detection and quantification limits were determined as described below.

The only sample preparation performed before testing was dilution. Appropriate dilution allows the concentration values to be read from the range of the calibration curve. The range of the curve due to the dilutions used in the study of biological samples is sufficient for the tests. The following formulas were used to calculate the limit of detection (LOD) and the limit of quantification (LOQ): $LOD = (3.3 \times SD)/a$ and $LOQ = (10 \times SD)/a$, where SD is the standard deviation, and a is the slope of the calibration curve.

4.4. Precision, accuracy, and repeatability of the biosensor

The precision and accuracy of the determinations made using the SPRi EPO sensitive biosensor were determined by measuring the concentrations of standard solutions in triplicate using the following actual concentrations: 5.00, 10.00, 15.00, 25.00 pg/mL. The standard deviation (SD), the arithmetic mean of the concentrations from three replicate measurements (Cmean), and the coefficient of variation (CV) were then calculated. Low values for the standard deviation indicate high precision. A CV value of less than 20 % indicates high accuracy of the determinations. The results are presented in Table 2.

A three-fold quantitative analysis of an actual sample tested the repeatability of the biosensor. The repeatability was determined by the mean value of the coefficient of variation (CV). The CV value for EPO was 1.82 %. This CV value indicates that the determinations made in a sample of biological material are repeatable.

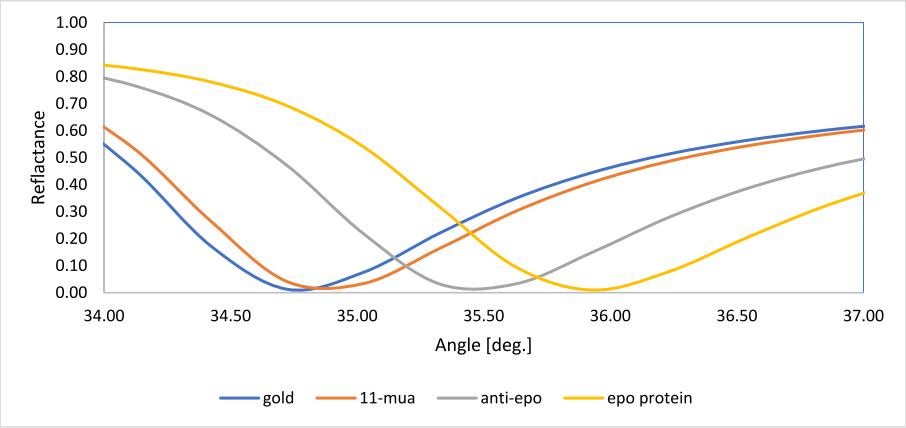

Fig. 6. The course of the SPR model curves. For each of the distinguished layers present on the biosensor surface, data were collected at angles from 34 to 37° in 0.1-degree increments. Each of the curves for a given layer is shifted towards larger angle values in relation to the curve of the previous layer.

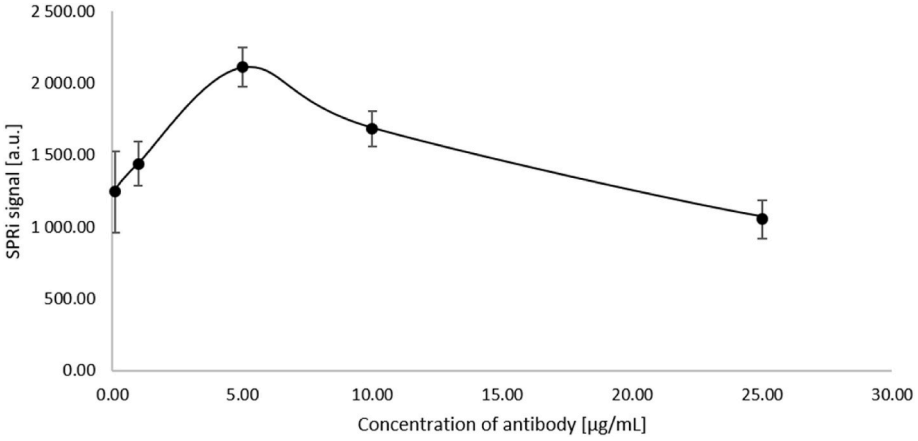

Fig. 7. Saturation curve for anti-EPO. Above a 5 µg/mL concentration, no more ligand binding occurs on the biosensor surface. This is the highest concentration that will enable capture of the analytes of interest from the sample with the greatest accuracy.

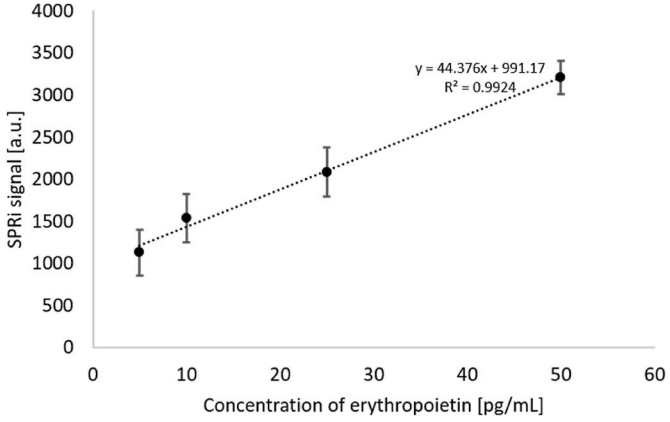

Fig. 8. EPO calibration curve. The straight-line part of the calibration curve. Ligand/antibody concentration for EPO = 5.00 µg/mL.

Table 1
Values of detection and quantification limits.

LOD [pg/mL]	0.03
LOQ [pg/mL]	0.10

Table 2
Results of precision and accuracy parameters of protein determinations using the SPRi biosensor.

Concentration of the Standard Solution of EPO [pg/mL]	SD [pg/mL]	C_{mean} [pg/mL]	CV [%]
5.00	0.68	5.34	12.80
10.00	0.65	10.01	6.48
15.00	1.95	15.08	12.93
25.00	0.63	25.07	2.52

4.5. Potential influence of interferents

In determining by a given method, it is necessary to check whether other components present in the sample subjected to the tests do not affect the final result. As indicated in previous studies on SPRi biosensors, albumin does not cause changes in the analytical signal in any of the earlier cases. Tau protein and its phosphorylated form (p-tau) and changes in their levels compared to healthy patients are indicated as biomarkers of AD [23]. These compounds play a role in the development of the disease, and changes in their levels in the blood can be used for diagnosis. They can be called interferents in blood plasma when determining another AD marker. Each EPO solution was mixed with solutions of selected interfering substances at concentrations of 1:10 and 1:100. The selected protein interfering substances were total tau and p-tau. EPO-specific antibodies were immobilized on the gold surface according

to the procedure. Then, the measurement was performed. The biosensor is specific for the compound to be detected and thus selectively captures the analyte of interest from the sample in the presence of other components contained in biological fluids. The recovery value determines the specificity test parameter. The average recovery value obtained for EPO (103.84 %) indicates the high specificity of the designed biosensor. The results of the analysis are presented in Table 3.

4.6. Determinations in biological samples

To verify the performance of the biosensor using the developed method, EPO was determined in eight blood plasma samples from patients who were not affected by AD but were smokers, as smoking causes hypoxia in the brain and can lead to neurodegenerative conditions, and seven samples from patients diagnosed with AD. This was done to demonstrate the method's usefulness for assaying biological material. An antibody layer was generated on the gold plate according to the EDC/NHS covalent immobilization protocol, and then ethanolamine was applied to prevent non-specific adsorption. Each measurement site with an exposed gold layer was intended to analyze EPO levels. After SPR analysis of the receptor layers, samples were applied to these sites. After 10 min of washing, further SPR measurements were performed. Plasma samples were not diluted; the results obtained for concentrations are presented in Table 4. Study group samples numbered 1AD to 7AD, and control samples numbered 1c to 8c. The preliminary results obtained indicate that the biosensor is suitable for the selective determination of EPO. The average concentration of the tested compound is higher in the study group than in the control group. Still, the concentrations within the same group are highly variable, as shown by the SD values (concentration of EPO: 30.73 ± 17.18 pg/mL vs.

6.25 ± 3.04 ng/mL). Information on the utility of this biosensor as a biomarker will require analysis of a more significant number of samples and ELISA testing of the same samples. A literature review was conducted to confirm whether the EPO levels determined in the plasma samples were correct. Hessanain et al. constructed an electrochemical sensor to detect EPO in blood plasma. According to the results obtained using DPV and ELISA, they received an agreement of 104.85 ± 3.35 %. The concentration in the plasma samples was about 211 p.m., corresponding to 6.33 pg/mL of EPO. This level is consistent with the average obtained in our study from the control group, 6.25 ± 3.04 ng/mL. The samples used in the study from the literature did not come from patients with diseases, so the obtained results are realistically consistent [34].

A preliminary statistical analysis of the obtained results was performed to compare them. Analysis of the normality of the distribution (Shapiro-Wilk test) indicates a distribution different from usual. Therefore, the statistical analysis used the nonparametric Mann-Whitney *U* test. The graphical presentation of the Mann-Whitney *U* test is presented in Fig. 9. A high statistical significance of the differences in concentrations between the group of AD patients and the control group was obtained ($p = 0.000626$, $\alpha = 0.05$). The median concentration in the group with Alzheimer's disease is higher.

Table 3
Influence of interference factors on the analytical signal of the biosensor. Erythropoietin was mixed in a concentration ratio of 1:10 and 1:100 with the interferent being tested.

Interferent	Concentration ratio	Searched concentration [pg/mL]	The average concentration marked [pg/mL]	Recovery [%]
total tau protein	1:10	50.00	52.07	104.4
	1:100		52.13	104.25
phospho-tau protein	1:10		51.13	102.26
	1:100		52.36	104.71
			MEAN	103.84

Table 4
Measurements of 7 plasma samples of patients diagnosed with AD and 8 plasma samples of smoker patients without neurodegenerative diseases.

Sample number and type (AD – patient with diagnosed Alzheimer Disease, c – healthy control)	Concentration of EPO [pg/mL]
1AD	32.96
2AD	43.53
3AD	20.90
4AD	61.84
5AD	21.94
6AD	23.34
7AD	10.61
1c	4.35
2c	5.74
3c	8.01
4c	5.19
5c	3.66
6c	6.74
7c	12.76
8c	3.58

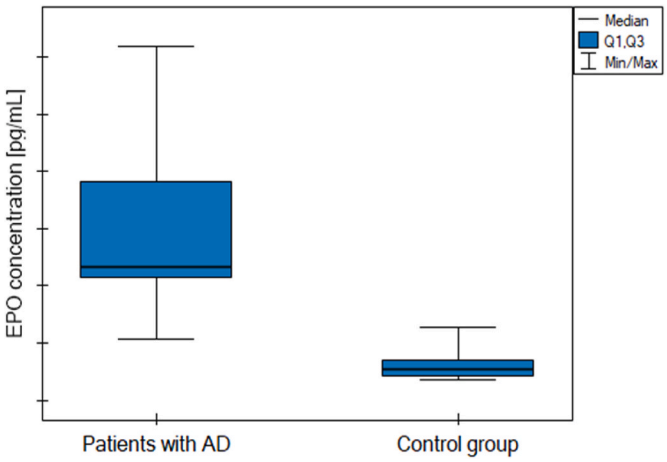

Fig. 9. Graphical representation of the Mann Whitney *U* test.

Each new method requires confirmation of diagnostic usefulness. For this purpose, ROC curves were used (Fig. 10). The results were obtained with high statistical significance ($p = 0.00178$). For erythropoietin, an increase in concentration means an increase in the chance of having Alzheimer's disease. The AUC value accurately calculates patients into the sick or healthy group. The closer to one, the more accurate the matching to the group. At AUC = 0.98, we can say that the classification into the group is not accidental. The test also shows a sensitivity of 100 %. The probability of detecting a potentially healthy person and specificity remains at 87.5 %. The positive predictive value (PPV) indicates that 87.5 % of a person with a positive test has AD. The obtained negative predictive value NPV at 100 % means that a person with a negative test is entirely sure about the absence of the disease. The cut-off point of the tested method is 10.61 pg/mL. Above this EPO concentration, there is a high probability of AD.

5. Conclusions

A biosensor sensitive to EPO has been constructed for its detection in blood plasma samples of patients with AD. The experiments were performed using the SPRi technique. The method did not require labels, and the measurement procedure for one measurement cycle (9 samples), including the preparation of the antibody-ligand layer, took about 2 h, an advantage of this method. A minimal sample is used for the assay (3 μL). The first step in the validation process was determining the optimal concentration of the ligand-receptor layer, which was found to be five

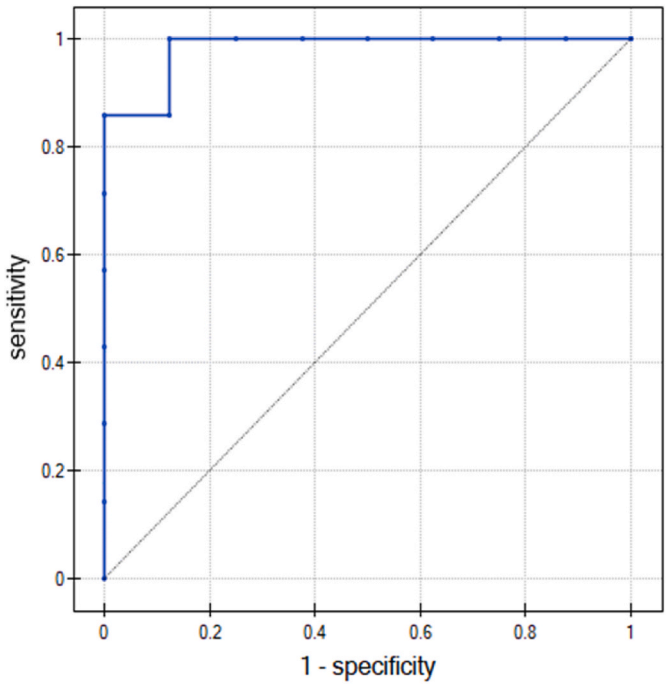

Fig. 10. Graphical ROC curves.

ug/mL. A calibration curve covering concentrations from 5 to 50 pg/mL of EPO was plotted, showing the concentration dependence of the SPRi signal. Determining the curve equation then allowed the determination of concentrations in biological samples. As a result of the validation, the LOD and LOQ values of the method were obtained. The results indicate the possibility of determining very low concentrations in the order of pg/mL (LOD = 0.03 pg/mL, LOQ = 0.10 pg/mL). The determination of other validation parameters (precision, accuracy, repeatability) and the achievement of low SD and CV values indicated that the results of the developed method were highly accurate and repeatable. The biosensor is also specific for EPO, with a recovery value of approximately 100 %. To verify whether the constructed biosensor can determine EPO in natural samples, testing was performed on blood plasma from patients diagnosed with AD and a control group of patients not affected by AD but who were smokers. Comparing the results for the study and control groups, the average concentration of the tested compound is higher in the study group than in the control group. Still, the concentrations are highly variable within the same group, as shown by the SD values (concentration of EPO: 30.73 ± 17.18 pg/mL vs. 6.25 ± 3.04 ng/mL). Due to the small number of samples tested, we cannot determine whether EPO is a marker characteristic of AD. Initial statistical analysis showed significant concentration differences between the patient and control groups. Initial ROC curve studies indicate a high probability of diagnostic usefulness, which should also be tested on larger samples. However, the results showed that the constructed SPRi biosensor is sensitive and selective to erythropoietin, which was the primary assumption of the studies. The construction of a sensor with good parameters was expected, which could then be used for determinations in which we plan to check the effectiveness of EPO as a biomarker of AD, but in combination with other biomarkers of this disease. Samples will be collected progressively to obtain a more significant number of samples from patients diagnosed with AD.

Nevertheless, the assay is suitable for detecting EPO in blood plasma samples. It is also necessary to perform measurements using a certified method, such as an ELISA test, to determine if they are convergent. A comparison with the results from the literature indicates the correctness of the determinations performed in the control group. However, the results in the group of patients are not unequivocal - no EPO levels were found in the literature in the blood plasma of patients with Alzheimer's disease. Although there is no direct link, research suggests that EPO may have some effect on the brain. Some studies suggest that EPO may help regenerate brain cells and protect against neurodegenerative damage. However, this research is in its early stages, and the effect of EPO on Alzheimer's requires further study. There is no evidence that EPO can treat or cure Alzheimer's, nor that it is an effective treatment or prevention. In addition, a broad statistical analysis of the results is needed to verify the diagnostic utility of this biomarker, taking into account a wide range of biological samples. However, studies indicate that EPO secretion in the brain may allow for the introduction of appropriate treatment strategies due to its neuroprotective effect. It can, therefore, be stated that in healthy patients, EPO levels will be lower than in the first stages of Alzheimer's disease - through neuroinflammation and oxidative stress, which play an essential role in the pathogenesis of AD [35]. EPO, as a neuroprotective factor, appears in a reduced amount of oxygen in cells, i.e., in conditions of oxidative stress. Its action can be combined with the hypoxia-inducible factor (HIF-1) to which the EPO gene binds explicitly, which may be another research subject on potential AD biomarkers [36].

CRediT authorship contribution statement

Antony Chirco: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Zuzanna Zielinska:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Ewa Gorodkiewicz:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Elisabetta Meacci:** Writing – review & editing, Supervision, Conceptualization. **Giancarlo Margheri:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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