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Amperometric determination of endoglin in human serum using disposable immunosensors constructed with poly(pyrrolepropionic) acid-modified electrodes

E. Martínez-Periñán [a], E. Sánchez-Tirado [a], A. González-Cortés [a], R. Barderas [b], J.M. Sánchez-Puelles [c], L. Martínez-Santamaría [d], S. Campuzano [a], P. Yáñez-Sedeño [a, *], J.M. Pingarrón [a]

[a] Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040, Madrid, Spain
[b] UFIEC, CROSADIS, National Institute of Health Carlos III, Majadahonda, 28222, Madrid, Spain
[c] Departamento de Medicina Celular y Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28040, Madrid, Spain
[d] Department of Bioengineering, UC3M, CIEMAT, IIS-Fundación Jiménez Díaz, CIBER de Enfermedades Raras (CIBERER), Madrid, Spain

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ABSTRACT

An amperometric immunosensor for the determination of the biomarker endoglin (CD105) to comply with the requirements of sensitivity and accuracy demanded in clinical practice is reported in this work. The immunosensing platform is implemented onto disposable electrodes modified with poly(-pyrrolepropionic) acid (pPPA). The methodology involves a sandwich configuration and labeling of the biotinylated detector antibody with poly-HRP-streptavidin for signal amplification. Amperometric detection of hydrogen peroxide reduction in the presence of HQ was employed as analytical readout. The different steps involved in the immunosensor preparation were monitored by electrochemical impedance spectroscopy. The resulting immunosensor provided a linear range between 0.18 and 20 ng mL^{-1}, adequate for the determination of CD105 in serum, with a detection limit (LOD) of 140 pg mL^{-1}. These analytical characteristics improve those reported previously for other electrochemical immunosensors. A good reproducibility of the measurements, an excellent storage stability of the anti-CD105-pPPA/SPCE bioplatforms and an excellent selectivity of the resulting immunosensors were found. The usefulness of the immunosensors was tested by analyzing human serum samples collected from healthy individuals and patients of colorectal, breast and lung cancer and epidermolysis bullosa. The results were successfully validated against those provided by an ELISA kit.

1. Introduction

Human endoglin (CD105) is a 180 kDa homodimeric hypoxia-inducible cell transmembrane type III glycoprotein, densely expressed on the surface of angiogenic proliferating endothelial cells that acts as an auxiliary receptor for the transforming growth factor (TGF) family of cytokines. This protein plays a significant role in angiogenesis, a crucial process involved in several physiological and pathological conditions such as cancer [1,2]. The stronger CD105 expression found in a wide range of endothelial tumors, including colon, breast, brain, lung, prostate and cervical cancer, compared to normal tissues, suggests the possible involvement of CD105 in tumor angiogenesis [3–5]. In fact, increasing levels of CD105 in biological fluids from affected patients may be used as an indicator for disease progression and risk of metastasis [6,7]. CD105 circulating levels have been found to be altered in response to chemotherapy and, therefore, their monitoring can be useful for both the evaluation of the response of patients to treatment, especially to antiangiogenic therapies, and in tracing disease recurrence.

Increased CD105 levels are also detected in inflamed tissues. The protein modulates TGF-β induced production [8] and, therefore, is not involved only in cancer but also in the development and progression of other important diseases related to angiogenesis such as

* Corresponding author.
E-mail address: yseo@quim.ucm.es (P. Yáñez-Sedeño).

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preeclampsia [9], rheumatoid arthritis [10] and skin diseases such as epidermolysis bullosa [11].

Various immunoassay methods for the detection of CD105 have been reported so far [12] related with cancer treatment [2] or diagnostics of preeclampsia [13]. ELISA kits available from Thermo Scientific [14] or SigmaAldrich [15] are examples of commercial kits involving sandwich-type configurations with anti-CD105 capture antibodies, biotinylated anti-CD105, and peroxidase (HRP)-streptavidin conjugates. These kits provide dynamic ranges extending from units to thousands of pg mL^{-1} with minimum detectable doses (MDD), calculated statistically from the zero signal value, of units of pg mL^{-1}. An important disadvantage of these methods is the long analysis time required (five hours). More simple immunoassays involving capture and HRP-labeled detection antibody as immunoreagents are also available. For instance, the ELISA kit from Sino Biologicals [16] provides a calibration plot ranging from 62.5 to 4000 pg mL^{-1} CD105 with a MDD value of 36 pg mL^{-1} in an assay time of 3 h 40 min.

Regarding electrochemical immunosensors, to our knowledge only two configurations have been reported so far. A sandwich type immunosensor involved immobilization of anti-CD105 capture antibody on a mercaptoethylamine self-assembled monolayer (SAM) onto a gold nanoparticles (AuNPs)—modified gold electrode and the use of a detection antibody chemically linked to the electron mediator thionine and platinum nanoparticles (PtNPs) for signal amplification [17]. This approach provided a linear range from 1.3 to 200.0 ng mL^{-1} and a detection limit (LOD) of 0.9 ng mL^{-1}. However, it implies laborious and time consuming protocols for SAM modification of the electrode surface (26 h) and preparation of the labeled immunoconjugates (12.5 h). More recently, a sandwich-type immunosensor was implemented on the surface of magnetic microbeads (MBs) and used capture and HRP-detection antibodies as well as amperometric detection of hydrogen peroxide in the presence of hydroquinone (HQ). A linear relationship between 0.8 and 10.0 ng mL^{-1} and a LOD of 0.2 ng mL^{-1} were achieved [18]. It is worth to mention that although both immunosensors provide higher LODs than ELISA kits, the sensitivity is sufficient for the determination of the target protein in serum of patients diagnosed with cancer, where the mean values found using a radioimmunoassay were of 34.0 ± 26.8 and 63.8 ± 72.5 ng mL^{-1} in metastasis-negative (42) and positive (59) patients, respectively [19]. However, the great variability observed in these results highlights the need for developing methodologies that provide not only good sensitivity but also good reproducibility for the determination of this circulating biomarker.

Nowadays the use of electrodes modified with electropolymerized conducting polymers containing suitable functional groups offers an interesting strategy to develop electrochemical immunosensing platforms with enhanced performance. For example, poly(pyrrolepropionic) acid (pPPA), a conducting polymer with abundance of carboxyl groups, allows the immobilization of large bioreagents loadings and permeation of electroactive species to the electrode surface due to its porous structure [20]. Despite these interesting properties, only few examples of electrochemical immunosensors involving the use of pPPA as modifier of glassy carbon electrodes [21–24] and SPCEs [25] have been found in the literature.

In this work, a strategy able to comply with the requirements of sensitivity and accuracy demanded by the determination of CD105 is described. The immunosensing platform was implemented onto a disposable electrode modified with pPPA and involved a sandwich configuration as well as labeling of the biotinylated detector antibody with poly-HRP-streptavidin for signal amplification. Amperometric detection of hydrogen peroxide reduction in the presence of HQ was employed as analytical readout. The immunosensor provided a calibration plot with a linear range between 0.18 and 20 ng mL^{-1} CD105, a LOD value of 140 pg mL^{-1} CD105 and was successfully applied to the determination of the target analyte in serum samples from cancer or epidermolysis bullosa patients with minimal sample treatment.

2. Experimental

2.1. Apparatus and electrodes

Screen-printed carbon electrodes (SPCEs, 110 DRP) were used as electrochemical transducers DropSens-Metrohm (Oviedo, Spain). They are constituted of a 4 mm ∅-working electrode, a silver pseudo-reference electrode and a carbon counter electrode. A μ-Autolab type III potentiostat, operated by the GPES 4.7 software (EcoChemie), was employed for electropolymerization of PPA onto SPCEs. Electrochemical impedance spectroscopy (EIS) was carried out using an Autolab type III potentiostat controlled by the FRA2 software (EcoChemie). Amperometric measurements were performed with an INBEA Biosensor S.L. potentiostat using the Ib-Graph software. An Elmasonic Se60 (Elma) ultrasonic bath, a Vortex homogenizator (Velp Scientifica) and a Crison Basic 20+ pH-meter were also used. All electrochemical experiments were performed at room temperature.

2.2. Reagents and solutions

Mouse anti-human endoglin capture antibody (anti-CD105), from Human Endoglin/CD105 DuoSet Elisa Catalog Number: DY1097 from R&D Systems, was reconstituted with 1.0 mL 0.01 M phosphate buffer saline solution (PBS) of pH 7.4 containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. Anti-CD105 solutions were prepared by appropriate dilution with 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, from Gerbu) buffer solution of pH 5.0. CD105 standard solutions and biotinylated goat anti-human endoglin (Biotin-anti-CD105), also from Human Endoglin/CD105 DuoSet Elisa, Catalog Number: DY1097 from R&D Systems, were prepared in 0.01 M PBS solution of pH 7.4 supplemented with 1% (w/v) BSA.

Pyrrole propionic acid (PPA) (Sigma-Aldrich, 97%) and KCl (Scharlau, 99.5%) were used. The electro-synthesis of pPPA polymer was made from 5 mM PPA +0.5 M KCl aqueous solutions. N-(3-dimethyl-aminopropyl)-N′-ethylcarbodiimide (EDC, Acros Organics) and N-hydroxysulfo-succinimide (Sulfo-NHS, Sigma-Aldrich) were used as activation agents for carboxyl groups confined at the electrode surface. 50 mg mL^{-1} solutions of EDC and Sulfo-NHS each in 25 mM MES buffer of pH 5 were used for this purpose. The blocking agent was a commercial 1% (w/v) casein solution prepared in 0.01 M PBS of pH 7 (Blocker™ Casein, Thermo Fisher). Solutions of streptavidin labeled with horseradish peroxidase (HRP-Strept, Roche), prepared in 0.1 M phosphate buffer (PB) of pH 7.4, and poly-HRP-Strept (85-R200), diluted in stabilizer solution (Fitzgerald), were also used. Hydrogen peroxide (Aldrich, 30% (w/w)) and hydroquinone (HQ, Sigma) were also employed. 0.05 M $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (Sigma) solutions were prepared in 0.01 M PB of pH 7.4. Bovine serum albumin (BSA), immunoglobulin G (IgG), hemoglobin (HB), uric acid (UA) and ascorbic acid (AA), all from Sigma, transforming growth factor beta-1 (TGF-β1), interleukin 1 beta (IL-1β), E-cadherin (E-Cad), cadherin 17R (Cad17R), and fibroblast growth factor receptor 4 (FGFR4), all from R&D System Inc., interleukin 6 (IL-6, Abcam), recombinant human ErbB2 protein (ERBB2, Sino Biological Inc.), and recombinant full length human p53 protein (p53, EMD Millipore Corporation), were tested as potential interfering compounds. Solutions of these reagents were prepared in 0.01 M PBS of pH 7.4. All other chemicals