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Amperometric magnetoimmunoassay for the direct detection of tumor necrosis factor alpha biomarker in human serum

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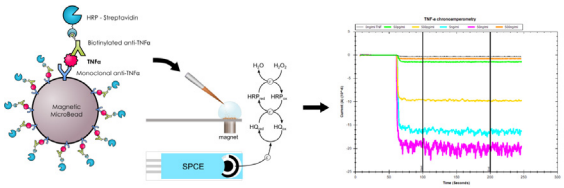
HIGHLIGHTS

- Electrochemical magnetoimmunosensor for tumor necrosis factor alpha (TNFα) biomarker.
- Sensitive and selective detection of TNFα in undiluted serum.
- LOD achieved lower than the cut-off value established for relevant illnesses.
- Useful and affordable alternative to ELISAs for TNFα determination in serum.

GRAPHICAL ABSTRACT



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ABSTRACT

An amperometric immunoassay for the determination of tumor necrosis factor alpha (TNFα) protein biomarker in human serum based on the use of magnetic microbeads (MBs) and disposable screen-printed carbon electrodes (SPCEs) has been developed. The specifically modified microbeads were magnetically captured on the working electrode surface and the amperometric responses were measured at −0.20 V (*vs.* Ag pseudo-reference electrode), upon addition of hydroquinone (HQ) as electron transfer mediator and H_2O_2 as the enzyme substrate. After a thorough optimization of the assay, extremely low limits of detection were achieved: 2.0 pg mL^{-1} (36 fM) and 5.8 pg mL^{-1} (105 fM) for standard solutions and spiked human serum, respectively. The simplicity, robustness and this clinically interesting LOD proved the developed TNFα immunoassay as a good contender for real clinical application.

1. Introduction

Tumor necrosis factor-α protein (TNFα) is a pro-inflammatory cytokine secreted by activated macrophages that plays a relevant role in a wide variety of immune and inflammatory processes [1]. It is present in serum as a biologically active molecule in the form of a 55 kDa homotrimer whose concentration increases when the inflammatory cascade is activated, which makes TNFα a very attractive protein biomarker for several pathologies [2]. Therefore, the determination of TNFα concentration in real samples is highly interesting for many immunological studies. A worth-mentioning case is that of rheumatoid arthritis or Crohn's disease, in which the binding inhibition of circulating TNFα by biological drugs significantly alleviates the symptoms [3]. It has been suggested that the knowledge of circulating TNFα levels could improve the drug dosage to reduce the high treatment costs and to avoid

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unnecessary side effects [4]. Other authors have studied also the prognostic effect and the effect of chemotherapy on serum levels of TNFα in patients with advanced stage non-small cell lung cancer (NSCLC) [5].

Normal concentrations of circulating TNFα lie on the range of a few pg mL^{-1} [2] and, consequently, the assay of TNFα in blood or serum requires extremely sensitive detection methods. The current gold-standard method for assaying TNFα levels remains being the conventional enzyme-linked immunosorbent assay (ELISA). However, ELISA methods are difficult to be automated, miniaturized and performed with cost-effective instrumentation and hence hardly used *in situ* and not recommended to perform routine and decentralized analyses. As a result, the use of this marker is not widespread in clinic and is only used to research level. With the aim of overcoming these limitations, a wide variety of assays and biosensor techniques including optical, acoustic, capacitive and electrochemical methods have been reported for the detection of TNFα. However, probably due to the unavoidable difficulty added by the matrix effects, only a few works deal with clinically interesting samples such as blood or serum. Martinez-Perdiguero et al. used a surface plasmon resonance (SPR) immunoassay for the detection of TNFα in 25% serum and, via gold nanoparticle signal amplification, achieved a LOD of 54.4 pg mL^{-1} [6]. Luo et al. employed chemiluminescence imaging and carried out recovery tests in serum [7]. Using different electrochemical approaches, several authors have reported TNFα assays in complex media: Liu et al. developed an aptasensor using methylene blue as a label and square wave voltammetry (SWV) measurements to obtain a LOD of 10,000 pg mL^{-1} in pure blood [8]; Sun et al. used self-assembled peptide nanowires, glucose oxidase functionalized gold nanorods and SWV [9]; Weng et al. proposed a label-free strategy based on $K_3[Fe(CN)_6]$ and cyclic voltammetry (CV) [10]; and finally, Li et al. employed Prussian Blue-functionalized ceria nanoparticles and CV [11]. Despite the very low LODs reported in some of the mentioned assays, their inherent complexity can prevent them from reaching clinical use.

MBs are a powerful and versatile tool employed in a variety of analytical applications, including electrochemical immunosensing [12]. They facilitate efficient target analyte retrieval and concentration, largely reduce the assay time and allow easy application of analytical procedures to higher sample throughput and automation [13]. Its use minimizes matrix effect due to washing and separation procedures and allows faster assay kinetics to be achieved because the beads are in suspension and the analytical target does not have to migrate very fast [14]. The use of MBs has also been considered a methodological approach that enhances sensitivity and reduces detection time in electrochemical immunosensors and hence different electrochemical magnetogenosensing approaches have been successfully applied to the detection of relevant clinical biomarkers [15,16]. Interestingly, Betazzi et al. reported an electrochemical bioassay including MBs and alkaline phosphatase (AP) as enzymatic label for the detection of TNFα [17]. Using ELISA reagents and a sample diluting solution as serum-mimic, they reached a LOD of 44 pg mL^{-1}. As they suggested, this value was still an order of magnitude larger than it is necessary for real clinical interest [2] and further investigations in real samples are necessary.

In order to achieve the direct determination of ultralow concentrations of TNFα in undiluted human serum, we report here an amperometric magnetoimmunoassay using a sandwich configuration, SPCEs and HOOC-modified MBs (HOOC-MBs). The developed methodology involved the immobilization of the capture antibody on the HOOC-MBs, and successive incubation steps of the modified MBs with the analyte, a specific biotin-labeled detection antibody and a streptavidin–horseradish peroxidase (SAv–HRP) conjugate. Amperometric measurements at −0.20 V (*vs.* the Ag pseudo-reference electrode) of the enzyme reaction product were carried out at SPCEs using HQ as electron transfer mediator and H_2O_2 as the enzyme substrate. The implemented magnetoimmunoassay, tested against standard solutions and spiked human serum samples, demonstrated a much better performance in serum samples than the only one electrochemical magnetobioassay reported previously [17].

2. Experimental

2.1. Reagents

For all the experiments, carboxylic acid-modified MBs (HOOC-MBs, 2.8 μm, 30 mg mL^{-1} Dynabeads M-270 carboxylic acid) from Invitrogen were used as received. Recombinant human TNFα protein, monoclonal mouse anti-human TNFα (anti-TNFα) as capture and biotinylated polyclonal rabbit anti-TNFα (biotin-anti-TNFα) as detector antibodies were purchased from PeproTech (Refs. AF-300-01A, 500-M26 and 500-P31ABT, respectively). A TNFα ELISA Kit, was purchased from Immunotech (ref. IM1121). Mouse immunoglobulin G (MIgG), human immunoglobulin G (HIgG), bovine serum albumin (BSA) and streptavidin conjugated HRP enzymatic label (SAv–HRP) were from Jackson ImmunoResearch (Refs. 015-000-003, 009-000-003, 001-000-161 and 016-030-084 respectively). Human serum from human male AB plasma was purchased from Sigma (Ref. H4522). Moreover, a serum sample from a patient with a non-detectable TNFα concentration (analyzed by the commercial ELISA method) was used to perform the recovery studies.

N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride, hydroquinone (HQ) and Tween 20 were from Sigma–Aldrich. Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), 2-(*N*-morpholino)ethanesulfonic acid (MES), sodium chloride and potassium chloride were purchased from Scharlau. All other chemicals were of analytical grade and ultrapure water (Millipore Milli-Q) was used for all solutions and buffers.

The buffers and solutions employed in this work were: 0.01 M phosphate buffered saline at pH 7.5 containing 137 mM NaCl and 2.7 mM KCl (PBS); 0.05 M MES buffer at pH 5.0; 0.05 M phosphate buffer at pH 6.0 (PB); 0.1 M Tris–HCl buffer at pH 7.2; and PBS containing 0.05% (w/v) Tween 20 (PBST).

2.2. Apparatus

All the electrochemical measurements were performed using a PGSTAT302N potentiostat from Autolab. The SPCEs, consisting of a circular 4-mm carbon working electrode, a carbon counter electrode and a Ag pseudo-reference electrode were purchased from DropSens and were used in conjunction with a specific connector (DRP-CAC, Dropsens). A MixMate mixer (Eppendorf) was used for the homogenization and incubation of the solutions and a DynaMag-2 magnet (Invitrogen) for the magnetic separation of the MBs. A homemade poly(methyl methacrylate) (PMMA) dock with an encapsulated neodymium permanent magnet (AimanGZ) was employed to magnetically capture the MBs onto the working electrode surface during the electrochemical measurements.

2.3. Procedures

2.3.1. Biofunctionalization of MBs and sandwich immunoassay

An aliquot of HOOC-MBs suspension (12 μL) was transferred to a 1.5 mL microtube and washed twice with MES buffer (pH 5.0) for