STRATEGIES FOR THE DEVELOPMENT OF an ELECTROCHEMICAL BIOASSAY FOR TNF-ALPHA DETECTION BY USING A NON-IMMUNOGLOBULIN BIORECEPTOR

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Abstract

TNF-α is an inflammatory cytokine produced by the immune system. Serum TNF-α level is elevated in some pathological state such as septic shock, graft rejection, HIV infection, neurodegenerative diseases, rheumatoid arthritis and cancer. Detecting trace amount of TNF-α is, also, very important for the understanding of tumor biological processes. Analysis of this key biomarker is commonly achieved by use of ELISA or cytofluorimetric based methods. In this study the traditional optical detection was replaced with differential pulse voltammetry (DPV) and an affinity molecule, produced by evolutionary approaches, has been tested as capture bioreceptor. This molecule, namely a combinatorial non-immunoglobulin protein (Affibody®) interacts with TNF-α selectively and was here tested in a sandwich assay format. Moreover magnetic beads were used as support for bioreceptor immobilization and screen printed carbon electrodes were used as transducers.

TNF-α calibration curves were performed, obtaining a detection limit of 38 pg/ml, a quantification range of 76–5000 pg/ml, and RSD%=7. Preliminary results of serum samples analysis were also reported.

Keywords: Affibody®, magnetic beads, electrochemical detection

1. Introduction

Tumor necrosis factor-α protein (TNF-α) is a pro-inflammatory cytokine produced by some cells of the immune system 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 47-55 kDa trimer [2], whose concentration increases when the inflammatory cascade is activated, which makes TNF-α a very attractive protein biomarker for inflammatory-based diseases. Nearly every type of disease has involvement of cytokines, including TNF-α, as potential biomarkers (from rheumatoid arthritis or Crohn's disease [3], to cancer [4] [5], neurodegenerative diseases (Alzheimer, Parkinson) [6], rejection to clinical transplantation [7], sepsis [8], and other [9]. Therefore, measuring TNF-α is important for the understanding of inflammation, predicting progression, monitoring the effects of treatment and discovering new drugs that alleviate it [10].

Typical standard methods for TNF-α detection are based on immunoassay-based techniques including enzyme-linked immusorbent assays (ELISA) and bead-based immunoassays read by flow cytometers [9].

Recently increasing attention has been devoted to develop new bioanalytical methods for cytokine detection [4], that should increase the analytical performance in term of reducing the analysis time or decrease the sample volumes (microliters), as well as decrease the detection limit of standard ELISA (10-1000 pg/ml range). These innovative methods rely on different transducing principles [11] [12] [13] [14] [15] or in different bioreceptors (capturing agents) [9] [16].

Electrochemical biosensors appear as promising tools for point-of-care testing due to low cost, ease of miniaturization, and possibility of integration with multi-array devices [16]. In the last years some examples of electrochemical bioassay for TNF-α detection have been reported, that well addressed the issues listed above [17] [18] [19] [20] [21] [22] [23] [24]. The majority of these electrochemical immunoassays rely on antibodies as biorecognition elements, whereas very few papers are based on the use of innovative non-immunoglobulin bioreceptors such as nucleic acid aptamers [25] [16]. For instance, to the best of our knowledge, only Rezvin's group reported the development of an electrochemical aptasensor for TNF-α [26] [27]. The principle of this device operation was based on conformational changes of the aptamer immobilized onto the surface of a gold electrode. Binding event of the target analyte changed the distance between redox reporters and the electrode surface, generating an electrochemical signal. Using methylene blue as label and square wave voltammetry measurements a LOD of 10 ng/ml in pure blood was achieved [26].

Recently a new class of combinatorial non-immunoglobulin affinity protein, known with the commercial name of Affibody®, has received particular attention and found application in several studies, especially for in vivo diagnostic imaging [28]. Affibody® molecules [28] are small single

domain proteins that can be isolated for high affinity and specificity to any given protein target. Affibody® molecules were originally derived from the B-domain in the immunoglobulin-binding region of staphylococcal protein A. This B domain is a relatively short cysteine-free peptide of 58 amino acids that is folded into a three-helical bundle structure and which has been engineered into a variant denoted the Z domain. The Z domain retained its affinity for the Fc part of the antibody, while the weaker affinity for the Fab region was almost completely lost [28]. Affibody® molecule libraries are generally constructed by combinatorial randomization of 13 amino acid positions in helices one and two that comprise the original Fc-binding surface of the Z-domain. The libraries have typically been displayed on phages, followed by biopanning against desired targets. Using this strategy, Affibody® molecules showing specific binding to a variety of different proteins, including TNF-α [29] [30], have been generated. The Affibody® anti TNF-α has been studied as capture ligand on fluorescent protein microarrays [31]. However, despite the interesting feature of this affinity molecule towards TNF-α, no reports regarding its use in an electrochemical bioassay has been reported so far.

Having in mind these considerations, we pursued the development of electrochemical non-immunoglobulin based biosensors for detection of TNF-α in complex media such as human serum. An assay format based on the coupling of magnetic beads (MBs) with electrochemical transduction was here reported. The use of MBs has been considered a methodological approach that enhances sensitivity and reduces detection time in electrochemical immunosensors [32]. In particular, their use improves the performances of the affinity interaction for the faster assay kinetics achieved because the beads are in suspension and for the minimized matrix effect due to improved washing and separation. Moreover, they allow the analysis of complex samples without any pre-enrichment or purification steps. Their application can also be automated in order to further decrease the assay time. Thus, the Affibody® was tested as capturing agent using a sandwich configuration, screen-printed carbon electrodes (SPCEs) and functionalized MBs. Detection assays using a sandwich format, where an unlabeled analyte is captured by a primary affinity reagent and detected by a secondary affinity reagent, are widely used methods for protein detection. The sandwich assay benefits from two recognition events, which can greatly increase the selectivity of detection, and this feature is of particular importance for the analysis of proteins in complex sample mixtures.

The method described in this paper, involve the immobilization of the capture agent on the MBs and successive incubation steps of the modified MBs with TNF-α, and then with a specific labeling system based on a specific antibody and an alkaline phosphatase conjugated secondary antibody (secondary affinity reagents). Differential Pulse Voltammetry (DPV) measurements of the enzyme reaction product were carried out at SPCEs using α-naphthyl phosphate as enzyme substrate. The

bioassay was tested against standard solution and spiked human serum samples.
This work is the first example in the realization of an assay for TNF-α based on the use of the specific Affibody® molecule exploiting the advantages of these biomimetic receptors. Results demonstrated that this Affibody® molecule has interesting analytical features that allows its use in bioassay even in complex matrices such as serum.

2. Materials and Methods

2.1 Chemicals

Mouse Antibody anti TNF-α, anti mouse Alkaline-Phosphatase conjugated antibody (IgG-AP) and anti TNF-α Affibody® were from Abcam (UK). Bovine serum albumine (BSA), NHS (*N*-hydroxysulfosuccinimide sodium salt), ethanolamine, diethanolamine, di-sodium hydrogen phosphate dodecahydrate, dithiothreitol (DTT), Tween 20, serum from human AB male plasma were from Sigma-Aldrich-Fluka (Italy). Carboxylic acid MyOne magnetic beads were from Dynal (Invitrogen, Italy). EDAC (N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride), was from Calbiochem (Italy).

2-(N-morpholino)ethanesulfonic acid (MES), sodium chloride, potassium chloride, potassium dihydrogenphosphate, Tris-HCl, and other chemicals were from Merk (Italy). Water from Milli-Q Water Purification System (Millipore, UK) was used for all solutions and buffers.

Illustra Nap-5 desalting columns were purchased from GE HealthCare (Italy).

The compositions of the buffers are:

PBS: 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.2

PBST:PBS + 0.05% Tween 20

DEA: DiEthanolAmine 0.1 M pH 9.6, 0.1 M KCl, 1 mM $MgCl_2$, 0.05% Tween 20

2.2 Disposable screen-printed electrodes and electrochemical procedure

SPCE consists of a carbon working electrode (3 mm diameter), a carbon counter electrode and a silver pseudo-reference electrode. Screen-printed gold electrode consists of a gold working electrode (3 mm diameter), a carbon counter electrode and a silver pseudo-reference electrode. Materials, dimensions and procedures to screen-print the transducers are described elsewhere [23],[33], [34]. Electrochemical measurements were performed using a μAutolab type II potentiostat/galvanostat with a GPES 4.9 software package (Ecochemie, The Netherlands). All potentials were referred to the Ag/AgCl screen-printed pseudo-reference electrode. All electrochemical experiments were carried out at room temperature (25°C). To perform electrochemical measurements using magnetic beads, SPECs were kept horizontally and a magnet holding block was placed on the bottom part of the electrode, to better localize the beads onto the working surface. Then, a known volume of a solution containing the enzymatic substrate was added on the SPEC surface to close the electrochemical cell.

Measurements were performed in DPV with the following parameters: potential range 0/+600 mV, step potential 5 mV, modulation amplitude 70 mV, standby potential +200 mV, interval time 0.1 s.

2.3 Non-immunoglobulin protein based assay

2.3.1 anti TNF-α Affibody®

The Affibody® molecules contain a unique C-terminal cysteine and tail-to-tail dimers are spontaneously generated via a disulfide bridge between the C-terminal cysteines. Prior to use the Affibody® molecules need to be reduced. Thus, as recommended by the supplier, the lyophilized Affibody® molecules were dissolved in 0.02 M DTT solution (prepared in 0.01 M Tris buffer, pH 8.0) and left to incubate for 2 h at room temperature. The excess of DTT was removed by passage through a desalting column (NAP-5 column) with PBS buffer.

The Affibody® stock solution (100 μg/ml) was then divided in aliquots and stored frozen (−20 °C) for further experiments.

2.3.2 Bioassay using gold screen-printed electrodes

8 μl of a solution containing 7.5 μg/ml Affibody®, in PBS buffer, was dropped on a gold electrode surface. The solution was then incubated over night at 4°C. Sensors were then washed twice with PBS buffer and a blocking solution of 0.5 % BSA in PBS buffer was dropped onto the electrode surface for 1 h. After, a further washing step in PBS buffer was performed. Then, solutions containing different concentrations of TNF-α in PBS buffer were dropped onto the electrode surface and then incubated for 1 h. Sensors were washed twice with PBS buffer and a solution of 1μg/ml anti TNF-α antibody in PBS buffer was added. After 1 h of incubation time, sensors were washed as described before and then incubated with a solution of anti mouse IgG-AP in DEA buffer containing 10 mg/ml of BSA (30 min). Sensors were then washed with DEA buffer and the electrochemical cell was then covered using 60 μl of 1 mg/ml α -naphthyl phosphate in DEA buffer. After 20 min of incubation, the electrochemical signal of the enzymatically produced α-naphthol was measured by DPV.

2.3.3 Biomodification of paramagnetic carboxylated MBs

For the activation of the carboxylic groups of the MBs as well as the corresponding washing steps, the procedure used was the one recommended by the MBs supplier. Briefly, 10 μl of MBs were washed twice with 300 μl of 25 mM MES buffer pH 6, containing 0.01% Tween 20, for 10 min. For magnetic separation of the beads, a magnetic particle concentrator (MagneSphere Magnetic Separation Stand, Promega) was used throughout the work. The tube was placed on the magnet for

2 min and the solution was removed. Immediately before use, two solutions containing 50 mg/ml EDAC and 50 mg/ml NHS in cold MES buffer were prepared. 200 µl of EDAC and NHS solutions were added to the beads. Particles were mixed well and then incubated for 30 min. The tube was, then, placed on the magnet for 2 min and the solution was removed. MBs were then washed twice with 300 µl of MES buffer containing 0.01% Tween 20. 200 µL of Affibody® solution dissolved in PBS buffer were added and incubated for 2 h at room temperature. The tube was, then, placed on the magnet for 2 min and the solution was removed. Beads were then washed twice with 300 µl of MES buffer. In order to quench the non reacted activated carboxylic acid groups, beads were incubated with 300 µl of 50 mM ethanolamine in PBS pH 8 for 60 min. After incubation the tube was placed on the magnet for 2 min and the solution was removed.
Beads were then washed three times with 300 µl of 10 mM Tris buffer pH 7.4, containing 0.01% Tween 20. Finally beads were re-suspended in 500 µl of 10 mM Tris buffer pH 7.4 containing 0.01% Tween 20 and stored at + 4°C.

2.3.4 Bioassay procedure using MBs

250 µl of TNF-α solution was added to 50 µl of modified beads and incubated for 1 h. PBS buffer was used as blank solution. After incubation the tubes were placed on the magnet for 2 min and the solutions were removed. MBs were then washed twice with 300 µl of PBS buffer containing 0.01% Tween.
MBs were then incubated with 0.5 µg/ml of anti-TNF-α antibody for 1 h at room temperature. After incubation the beads were washed three times with 250 µl of PBS-T, to remove non-specifically adsorbed target.
After washing steps with DEA buffer, beads modified with mouse anti-TNF-α antibody were then incubated with a solution of anti mouse IgG-AP in DEA buffer containing 10 mg/ml of BSA (30 min). After incubation time beads were washed three times with 250 µl of DEA buffer containing 0.01 % Tween. Beads were then re-suspended in 50 µl of DEA buffer.

2.3.5 Electrochemical measurements of TNF-α using SPEC and modified MBs

The electrochemical measurements were performed placing the magnetic particles concentrator under the SPCE. 10 µl of beads suspension was deposited onto the working electrode surface; the electrochemical cell was then covered using 60 µl of 1 mg/ml α-naphthyl phosphate in DEA buffer. After 5 min of incubation, the electrochemical signal of the enzymatically produced α-naphthol was measured by DPV.

2.4 LOD and LLOQ calculation

The calibration plots (dose-response curves) were fitted by non-linear regression to the four-parameter logistic (4-PL) equation commonly used in immunoassays, using proper software (Origin Pro 2015, Origin Lab Corporation, USA) according to the following formula:

$$Y = A + \frac{(B - A)}{1 + \left(x / EC_{50} \right)^{m}}$$

where A is the Y-value at the bottom plateau of the curve, B is the Y-value at the top plateau of the curve, EC_{50} is the analyte concentration necessary to have the 50% of the signal and m Hill slope is the slope of the linear part of the curve and x is the analyte concentration.

The limit of detection (LOD) of each assay was evaluated as minimum detectable concentration, which is the lowest concentration of analyte which can be distinguished at a stated level of probability from a sample not containing the analyte. The lower limit of quantification (LLOQ) is considered as the level above which quantitative results may be obtained with a specified degree of confidence. The LLOD value was calculated by the evaluation of the average response of the blank plus three times the standard deviation, whereas the LLOQ was estimated considering the average response of the blank plus 10 times the standard deviation. The obtained values were converted in ng/ml by fitting it in the calibration function.

The Upper limit of quantification (ULOQ) was considered as the highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy; it was assessed as the concentration corresponding to 90% of the maximum signal, with an acceptable RSD% ≤ 15% (n=3).

The Quantification range was considered to be the range of concentration (including ULOQ and LLOQ) that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

A precision (RSD%) of 15% was considered acceptable following the recommendations of the European Medical Association (EMA) (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf)

2.5 ELISA test

Serum samples (diluted 1:10) were analyzed by a commercial ELISA Kit (Human TNF- α total Module Set ELISA ,BMS2034MST, eBioscience Europe) following the manufacturer instruction.

2.6 SPR measurements

SPR measurements were performed using the Biacore X instrument using bare gold sensor chip (General Electric Healthcare Bio-Sciences AB, Sweden).

Before use, the gold chip was cleaned by immerging it in a boiling solution containing H_2O_2:NH_3:H_2O (1:1:5) for 10 min and then thoroughly washed with Milli-Q water.

The cysteine modification has been used for the immobilization of the Affibody® onto the gold sensor chip. The immobilization procedure was performed at a constant flow rate of 2 µl/min and at a temperature of 25 °C. A solution of 200 ppm in PBS of anti-TNF-α Affibody® (50 µl) was injected. The surface was further blocked with 500 ppm BSA in PBS for 20 minutes (2 µl/min).

After the immobilization of the Affibody® molecule, the interaction between the immobilized bioreceptor and TNF-α at different concentrations was monitored with an association time of 15 min following by washing with PBS (running buffer). Binding interactions were monitored at a constant flow rate of 5 µl/min at a temperature of 25°C.

The kinetic analysis was performed according to a bivalent analyte interaction model using BIAeval 3.1 software (Biacore AB).

3. Results and Discussion

The goal of this study was to develop an electrochemical bioassay for TNF-α determination in serum, using a novel molecule as capturing agent. This molecule, selected by evolutionary selection methods, is an engineered protein, known as Affibody® [29][30].

A sandwich assay format was chosen with a specific labeling system based on a specific antibody and an alkaline phosphatase conjugated secondary antibody. The coupling of the Affibody® and the antibody as ligands for the sandwich assay results from the fact that only one Affibody® molecule has been commercially available for TNF-α, therefore the only way to perform a sandwich assay is to combine it with a TNF-α specific antibody. Preliminary experiments performed with several commercial anti-TNF-α antibodies (data not shown) revealed that the labeling reagents chosen are the only one able to work with this Affibody® molecule (at least among the reagent tested). Anyway, this work is the first step in the realization of an electrochemical assay for TNF-α based on the use of the specific Affibody®, exploiting the known advantages of this biomimetic receptor.

Affibody® immobilization was first optimized using gold electrode as both support and transducer of the affinity reaction. Due to the low sensitivity obtained, the assay was optimized using MBs as immobilization support and carbon electrode as transducers. The detection of spiked TNF-α in serum samples was, finally, demonstrated.

3.1 Surface plasmon resonance measurements

Surface plasmon resonance (SPR) experiments were, firstly, carried out in order to characterize the affinity binding between the immobilized Affibody® molecule and TNF-α. The affinity reaction was monitored by injecting TNF-α buffered solutions (prepared in PBS, pH 7.4) at different concentrations (25, 50, 100 ng/nl). An equilibrium constant $K_D = 0.4 \pm 0.1$ nM was estimated by fitting the experimental data to a bivalent interaction model, taking into account the ability of TNF-α to self associate into multimeric forms. This estimated value is in line with the value reported in a precedent report [30]. Moreover, the value was considered comparable to concentrations of cytokine secreted by cells *in vitro* or observed in blood upon mitogenic stimulation as reported in [26][35].

3.2 Affibody® based electrochemical assay

3.2.1 Bioassay based on gold screen-printed electrodes

Preliminary experiments were performed using gold electrodes for both the immobilization as well as the transduction step. Anti-TNF-α Affibody® molecules were immobilized on the gold surface via their C-terminal cysteine. Since tail-to-tail dimers can be spontaneously generated via a

disulfide bridge between the C-terminal cysteines, prior to use, as suggested by the supplier, the Affibody® molecules were treated with DTT in order to cleave the – S–S– bonds eventually present.
The different parameters that can influence the bioassay have been optimized and results reported in Table 1. The different parameters were optimized by taking as the selection criterion the ratio between the current of the α -naphthol oxidation peak in the absence (N) and in the presence (S) of 1 ng/ml TNF-α following the procedure described in Section 2.
The calibration curve for the detection of TNF-α under the optimized conditions was reported in Figure 1. A non-linear relationship between the current and concentration of TNF-α was obtained in the range of 0 and 1000 ng/ml with a LOD of 37 ng/ml LLOQ of 75 ng/ml, a quantification range of 75-950 ng/ml and an average relative standard deviation of 13%.
This value is too high to be considered interesting for diagnostic application; thus, in order to achieve a better sensitivity, the use of MBs has been considered. This because it is generally accepted that their use can improve the performances of the affinity interaction for the faster assay kinetics achieved because the beads are in suspension and for the minimized matrix effect due to improved washing and separation.

3.2.2. Optimization of Affibody-MB bioassay conditions

To develop an electrochemical assay for the detection of TNF–α using Affibody® molecule as capturing agent, carboxylated magnetic beads were modified with the well known carbodiimides chemistry to activate the carboxylic groups and to crosslink the Affibody® molecule trough the NH_2 terminus.
The different parameters that can influence the MB bioassay have been optimized and results reported in Table 1. The different parameters were optimized by taking as the selection criterion the ratio between the current of the a-naphthol oxidation peak in the absence (N) and in the presence (S) of 1 ng/ml TNF-α (unless otherwise stated) following the procedure described in Section 2. Table 1 collects the ranges tested for all the variables studied, as well as the selected values for each.
As an example of this optimization process, Figure 2 shows the anti TNF-α Affibody® loading on the activated MBs. Three different aliquots of modified beads were prepared using 200, 100, and 50 µg of Affibody® per mg of beads, respectively. The non-reacted carboxylic acid groups were quenched using ethanolamine. From our experimental results, the best performance was obtained with 100 µg of Affibody/mg of beads. In the case of immobilization of antibodies, a binding capacity up to 50 µg of protein/mg of beads was suggested by the manufacturer. Our results can be

consistent with the size of the Affibody® molecules. Affibody® molecules have a molecular weight of 13.7 kDa. Compared to the molecular weight of antibodies, which is 150 kDa, the Affibody® molecules are, thus, striking smaller. Thus, a higher amount of Affibody® molecules should be immobilized per mg of beads in comparison to antibodies.

3.2.3 Calibration curve in buffer

The sandwich assay was performed using different concentrations of TNF-α standard solutions. The corresponding dose-response curve, shown in Figure 3, covered the whole dynamic range from the region of signal noise to a saturation value. A LOD of 0.038 ng/ml (654 fM), a LLOQ of 0.076 ng/ml, and ULOQ of 5 ng/ml was obtained. The assay was repeated in order to evaluate the reproducibility; for this purpose, three repetitions of each standard solution in the concentration range 0 – 100 ng/mL were carried out. The average relative standard deviation (RSD) was 7%, calculated as mean of all the concentrations considered.

3.2.4 Analysis of human serum samples

Once the suitability of the assay to detect TNF-α in standard solutions was verified, experiments on spiked commercial serum samples were carried out. A commercially available serum, diluted 1 : 10, was tested alone or spiked with TNF-α (in the concentration range 0 – 100 ng/ml), and the corresponding dose-response curve is shown in Figure 4a. The signal increased with the increased concentration of the analyte and a matrix effect was observed considering the lower currents measured in serum with respect to buffer. Probably this decrease is due to a reduced concentration of TNF-α available for the binding caused by the interaction with some matrix components. However, a possible interference on the binding properties of the affibody-modified beads can not be excluded. The calculated LOD and LLOQ were 0.17 ng/ml and 0.99 ng/ml, respectively. A quantification range (concentration range between LLOQ and ULOQ) of 0.99-40 ng/ml was obtained and the average relative standard deviation was 12%.

The same samples were also analyzed by a conventional colorimetric ELISA kit obtaining a correlation significant at the level of 0.05 with a Pearson's coefficent r = 0.979 (degree of freedom=6), see Figure 4b. Thus, a linear correlation between the two techniques was found.

To the best of our knowledge, there is only another one report [31], describing the quantification of TNF-α using the anti-TNF-α Affibody®. In that case, a fluorescent sandwich assay format was developed. The Affibody® molecules were covalently immobilized on carboxymethyl dextran microarray slides and incubated with dilution series of unlabeled TNF-α, followed by incubation with a target-specific, biotinylated antibody and detected with a fluorescent-labeled antibody (Alexa

Fluor 555- labeled antibody). The corresponding dose-response curve allowed the estimation of a LOD for TNF-α in 1:100 diluted human plasma of 3.3 ng/mL.
The improved analytical performance of our electrochemical assay is attributable to the use of MBs, appropriately blocked with ethanolamine after Affibody® immobilization.

4. Conclusions

An electrochemical assays based on Affibody-coated magnetic beads and disposable electrochemical sensors were developed and applied to the detection of TNF-α in serum samples. The device was simple and cost-effective, since it involved low amounts of reagents and low-cost mass- produced sensors. The developed assays were based on a sandwich scheme in which an Affibody molecule and a specific TNF-α antibody were employed as capture and detection biorecognition elements, respectively. The performance of the assay in terms of sensitivity and reproducibility was studied in buffer and in serum. The LOD and LLOQ calculated in buffer were 0.038 ng/ml and 0.076 ng/ml respectively and the relative standard deviation (RSD) resulted 7 %. The calibration curve carried out in serum covered an interesting area to distinguish between ill and healthy subjects.
The optimized assay, tested against standard solutions and spiked human serum samples, demonstrated a much better performance in serum samples than other TNF-α Affibody-based assay previously reported.

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Graphical abstract

Scheme of the electrochemical assay based on magnetic beads and the disposable screen-printed sensor.

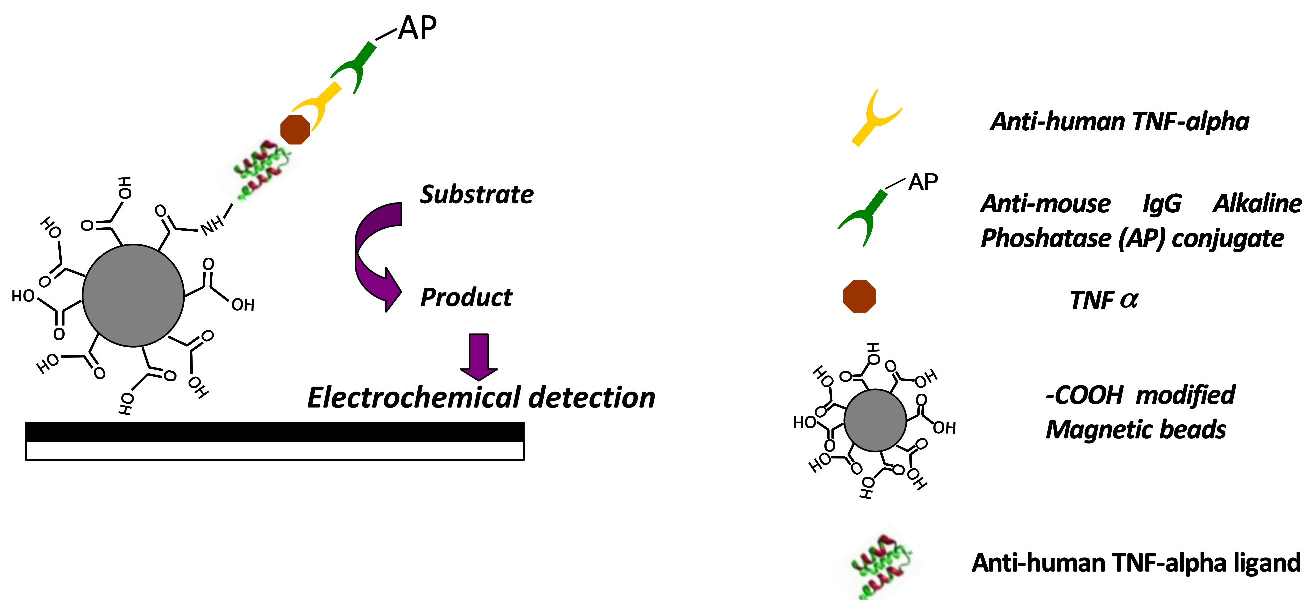


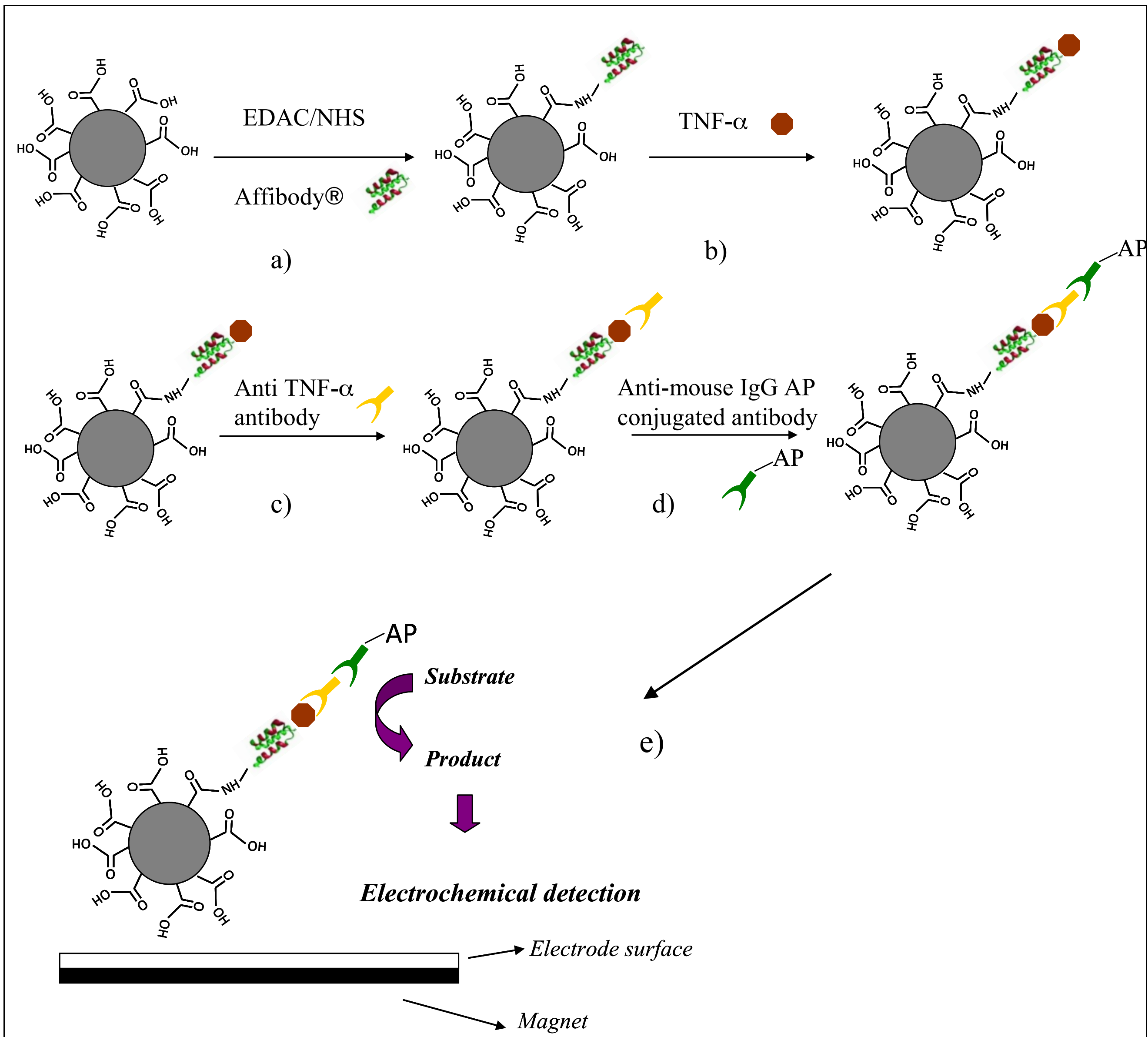
Table 1
Optimization of the different experimental parameters involved in the preparation of Affibody®-based assay using gold electrodes and Affibody®- based assay using MB.

Affibody®- based assay for TNF- α detection using gold electrodes		
Parameters	**Tested ranges**	**Selected values**
Affibody®	30-0.75 µg/ml	7.5 µg/ml *
Anti TNF alpha antibody concentration	0.1-2 µg/ml	1 µg/ml
TNF alpha incubation time		60 min
AP-anti mouse IgG dilution	1/10000-1/1000	1/2500
AP-anti mouse IgG incubation time	0-60 min	30 min
Affibody®- based assay for TNF- α detection using MB		
Parameters	**Tested ranges**	**Selected values**
Affibody®	100-25 µg/mL	50 µg/mL **
Anti TNF alpha antibody concentration	0.5-2 µg/ml	0.5 µg/ml
TNF alpha incubation time	0-60 min	60 min
AP-anti mouse IgG dilution	1/500-1/2000	1/500 ***
AP-anti mouse IgG incubation time	0-60 min	30 min

* TNF- α concentration 100 ng/mL
** TNF- α concentration 5 ng/mL
*** TNF- α concentration 10 ng/mL

Scheme 1

Scheme of the electrochemical assay based on magnetic beads and disposable screen-printed sensors. a) Carboxylic Acid-coated paramagnetic microparticles were activated with EDAC/NHS and functionalized with the anti-human TNF-α Affibody® and the remaining active sites were blocked with ethanolamine. b) Solutions containing TNF-α were incubated with functionalized MBs. C-d) The anti human TNF-α antibody and a anti-mouse IgG-AP conjugate were added. e) Then, the particles were magnetically blocked onto the working electrode by means of a magnet, and the electrochemical cell was covered with the substrate. The enzymatic product was measured by Differential Pulse Voltammetry.



AP: Alkaline phosphatase.

Figure 1

Calibration curve of TNF-α performed using gold screen printed electrodes. Experimental conditions described in table 1. The data points and the error bars represent average and standard deviation of measurements from three different affibody-modified electrodes (n=3).

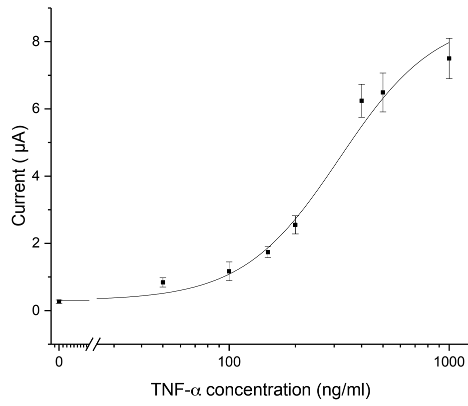


Figure 2

Effect of MBs loading on electrochemical responses measured with 0 and 5 ng/ml of TNF-α (S/N). The data and the error bars represent average and standard deviation of measurements from three different repetition on the same standard/sample (n=3).

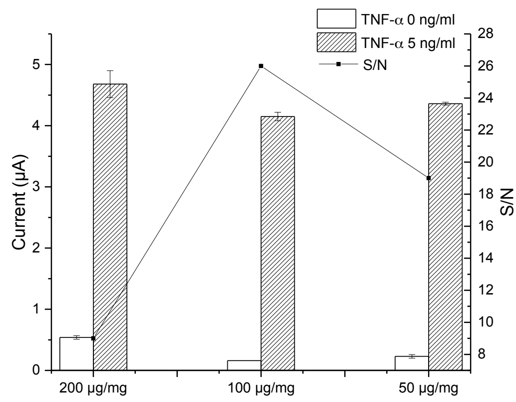


Figure 3

Calibration curve of TNF-α performed using Affibody® modified MB-based bioassay. Experimental conditions described in table 1. The data points and the error bars represent average and standard deviation of measurements from three different repetition on the same standard/sample (n=3).

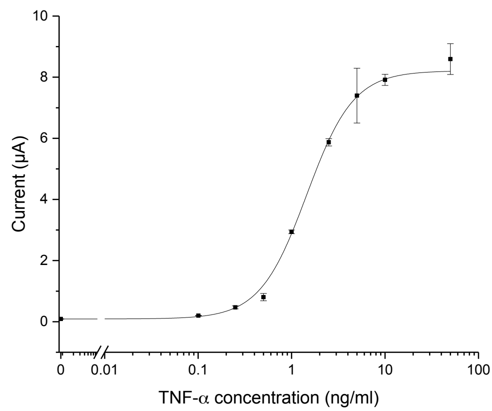


Figure 4:

Measurements carried out in serum. 4a) Calibration curve of TNF-α performed using Affibody® modified MB-based bioassay, in 1:10 diluted serum. Experimental conditions described in table 1. The data points and the error bars represent average and standard deviation of measurements from three different repetitions on the same sample (n=3). 4b) Correlation among the data obtained with the electrochemical bioassay and a commercial colorimetric ELISA on the same spiked serum samples. The data points and the error bars represent average and standard deviation of measurements from three different repetition on the same standard/sample (n=3).