Reagentless Electrochemical Immunoassay Using Electrocatalytic Nanoparticle-Modified Antibodies

Ronen Polsky, Jason C. Harper, David R. Wheeler, Shawn M. Dirk, Julia A. Rawlings, and Susan M. Brozik*

Supplementary Information

Materials and Reagents: All solutions were prepared with 18 MΩ water using a Barnstead Nanopure water purifier (Boston, MA). Monoclonal anti-mouse/rat TNF-α antibody (clone TN3-19, for capture) polyclonal anti-mouse TNF-α antibody (for detection) and recombinant mouse TNF-α were purchased from eBioscience (San Diego, CA). Mono-Sulfo-NHS Nanogold was purchased from Nanoprobes Inc (Yaphank, NY). Sodium phosphate monobasic, sodium phosphate dibasic, ascorbic acid, sodium chloride, potassium chloride, ethanolamine, Tween 20, and 2-aminobenzoic acid (ABA), were purchased from Sigma. Fluoroboric acid was purchased from Aldrich. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Acros Organics. Palladium chloride was purchased from Fisher. All electrochemical measurements were performed on a PGZ 100 Voltalab potentiostat (Radiometer Analytical, Lyon, France) and were measured versus an Ag/AgCl reference and Pt counter electrode (Bioanalytical Systems, West Lafayette, IN). Glassy carbon electrodes, (GCE) 3 mm electrodes (Bioanalytical Systems) were polished successively by 1, 0.3, and 0.05 μm alumina slurry on a cloth polishing pad (Buehler, Lake Bluff, IL) with sonication in ethanol and water in between steps. TEM pictures were taken on a Tecnai F-30ST TEM/STEM operated at 300kV

Preparation of Nanoparticle Modified Antibody: 200 ppm anti-TNF-α antibody was mixed with 0.06 nmol mono-NHS 1.4 nm Au particles in 200 μl 20 mM phosphate buffer saline (PBS) for 1.5 hours. The solution was then washed three times with 200 μl deionized water through a 100k MW cutoff Centricon (Millipore). For TEM pictures of the Au-NP antibody conjugate the antibody solution was brought up to a volume of 200 μl with deionized water and a 10 μl drop of solution was placed onto a formvar-coated copper TEM grid. For Pd deposition the Au-NP-modified antibody was brought up to a volume of 66 μl in deionized water. 60 μl of 1 mM $PdCl_2$ and 10 μl 100 mM ascorbic acid was added to the Au-NP-modified antibody solution and allowed to react for 15, 30, and 60 min before being washed three times with 200 μl deionized water through the Centricon. Afterward, the solution was brought up to a final volume of 200 μl with deionized water. A 10 μl drop of these solutions was placed onto the copper TEM grid for imaging and allowed to dry under ambient lab conditions.

Preparation of NP-Modified-Antibody Electrodes: A 10 μl drop of the appropriate NP-antibody solution was placed onto the working area of a GCE and evaporated to create a NP-modified-antibody glassy carbon surface. The response of the modified electrodes was analyzed in 10 ml 20 mM PBS (pH 7.4) buffer using linear sweep

voltammetry at a scan rate of 100 mV/sec versus an Ag/AgCl reference and a Pt wire counter electrode.

Preparation of Carboxyl Diazonium Molecule: 4-aminobenzoic acid (2.74 g, 20.0 mmol) was dissolved in fluoroboric acid (48%, 14.6 g, 80 mmol) and water (20 mL). The solution was heated until the aniline completely dissolved then cooled in an ice water bath. Sodium nitrite (1.46 g, 21.2 mmol) dissolved in water (4 mL) was added dropwise while the reaction mixture stirred. The solution was allowed to warm to room temperature and concentrated via rotary evaporation to half the original volume. The solution was cooled in an ice bath, the white solid was collected and washed with cold ether to give 1.24 g (26%) of the desired diazonium salt.

Diazonium protein modification: 10 mg of 4-carboxybenzenediazonium tetrafluoroborate, 20.6 mg EDC, and 11.5 mg NHS were mixed in 1 ml of water for 10 min. From this solution 10 µl was added to 200 µl of 500 µg/ml antibody solution in carbonate buffer, pH 11, and was allowed to react for 2 hours. The solution was then run through a 100,000 MW cutoff Centricon centrifugal filter (Millipore, Billerica, MA) for 5 min at 4000 rpm and washed twice with 200 µl of water for 5 min at 4000 rpm before being brought up to a final volume of 200 µl with water. Final protein concentrations were determined with a Pierce GCA^{TM} protein assay kit (Pierce, Rockford, IL).

Protein Deposition Procedure: Protein Deposition Procedure: A 20 µl drop of 40 µg/ml diazonium-modified antibody in 5 mM HCl solution was placed onto the working area of a GCE in connection to a Ag/AgCl reference and Pt wire counter electrode respectively. A cyclic voltammogram was run from 0 to -1000 mV for 5 cycles at 100 mV/sec.

Protocol for Sandwich Immunoassay: Following deposition of diazonium-modified antibody, the GCE was treated with a 200 µl drop of a given concentration of cytokine in 2x PBS and incubated for 2 hours. This was followed by washing (twice) with 2x PBS and reaction with 20 µl of NP-modified capture antibody solution for one hour, washed three times with 2x PBS with 0.1 % Tween and a linear sweep voltammogram was run in 0.1 M phosphate buffer at pH 7.4 with a scan rate of 100 mV/sec.

Preparation of Aminobenzoic acid modified electrode and electrochemical immunoassay: A freshly polished and washed glassy carbon electrode was modified by cycling its potential between 0.0 and 1.0 V at 40 mV/sec for 8 cycles in a 1.0 M sulfuric acid solution containing 0.05 M ABA. Following electropolymerization the electrode was activated with 100 mM NHS and 400 mM EDC in water. After 20 min the electrode was washed with water and placed into a 30 ppm anti-TNF-α antibody solution for 12 hours at 4° C. Then, residual reacting groups were blocked with 1 M ethanolamine-HCl (pH 8.5) for 20 min. The electrode was then reacted with either 1 ppm BSA or 500 ppb TNF-α in 20 mM PBS for 2 hours. The electrodes were then washed with with 20 mM PBS, 0.1% Tween 20 and incubated with 20 µl of the Au/Pd-antibody solution for 1 hour. The electrodes were washed with 20 mM PBS, 0.1 % Tween and a linear sweep

voltammogram was run in 0.1 M phosphate buffer at pH-7.4 with a scan rate of 100 mV/sec against an Ag/AgCl reference and Pt counter electrodes respectively.

Results Below: