# Nanoparticle Labels/ Electrochemical Immunosensor for Detection of Biomarkers

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## ABSTRACT

A sensitive electrochemical immunosensor based on poly(guanine) functionalized silica nanoparticle label has been developed for the detection of protein biomarker, recombinant human tumor necrosis factor-α(TNF-α).This method is simple, selective, and reproducible for trace biomarker analysis. A remarkable LOD has been achieved through dual signal amplification by poly(Guanine) functionalized silica NPs and catalytic guanine oxidation. The work demonstrates the feasibility of developing an inexpensive, sensitive, and portable device for multiplexed diagnoses of different protein biomarkers.

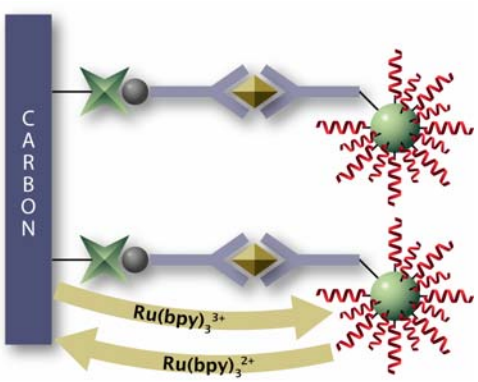
***Keywords***: Nanoparticle, Immunosensor, Biomarker, Electrochemical

## INTRODUCTION

A biological marker, or biomarker, is defined as a characteristic that is measured and evaluated objectively as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[1] Tumor necrosis factor α(TNF-α) is an extremely potent peptide cytokine which serves as an endogenous mediator or inflammatory, immuno and host defense functions. Elevated TNF concentrations in serum have been associated with a broad series of pathological states, such as neonatal listeriosis, severe meningococcemia, HIV infection, systemic erythema nodosum leprosum, endotoxic shock, graft rejection and rheumatoid arthritis. [2] Some procedures, such as enzyme-linked immunosorbent assays(ELISA), radioimmunoassay(RIA), fluorescence microplate-based assay, chemiluminescence, dissociation-enhanced lanthanide fluorometric assay (DELFLA), time-resolved immunofluorometric assay, Enzyme-amplified lanthanide luminescence, Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), immuno-PCR and chemiluminescence imaging, have been used for TNF detection.[4-12] Most methods, unfortunately, require highly qualified personnel, tedious assay time, or sophisticated instrumentation.

Immunosensor technology, alternative and simple immunoassay method, has been used for fast and sensitive protein analysis in connection with different transducers. [13] Here, the immunologic material is immobilized on a transducer; the analyte is measured through a label species conjugated with one of the immunoreagents. Immunosensors in connection with small and portable electrochemical devices have shown great promise for protein testing and are ideally suited for shrinking protein diagnostics.

The emergence of nanotechnology is opening new horizons for the applications of nanoparticles in bioassays and biosensors. Because of their excellent electrical and optical properties, nanoparticle(NP)-based materials offer excellent prospects for chemical and biological sensing. Recently, various nanomaterials, such as gold nanoparticle, quatumn dots, metal nanowires and carbon nanotubes have been used as labels for ultrasensitive protein and DNA detection in our group.[14-18] The power and scopes of such nanomaterial labels such nanoparticles have been greatly enhanced by coupling them with electrical processes and biological recognition reactions (i.e., nanobioelectronics).




**Scheme 1** Schematic illustration of an electrochemical immunosensing TNF-α based on the poly[G]-functionalized silica NPs on carbon electrode. Diamond stands for the TNF-α; "Y"shape for TNF-α antibody; red strings for poly [G].

In this work, we developed an electrochemical immunosensor based on polyguanine(polyG) functionalized silica nanoparticle label for sensitive detection of TNF-α. The new immunosensing TNF-α protocol involves a sandwich immunoassay on a streptavidin-modified disposable screen printed electrode and a electrocatalytic detection of the captured poly G-silica nanoparticle labels in the presence of $Ru(bpy)_3^{2+}$substrate. (Scheme 1) We use silica nanoparticles as carriers to load with numerous poly(guanine) and combine with the catalytic oxidation of $Ru(bpy)_3^{2+}$ of guanine to yield a dramatic enhancement of the sensitivity.

## EXPERIMENTAL

### Reagents and Instruments

Biotin- polyclonal anti-mouse/rat TNF-α and TNF-α, In vivo cytokine capture (IVCCA) in vivo capture anti-mouse antibody were bought from Ebioscience Inc.. Glutaric anhydride, glutaraldyde, poly(guanine) (poly[G]), 1-ethyl-3-

(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) , N-hydroxysuccinimide (NHS) and amino-modified silica nanoparticles were purchased from Sigma. Ethanol, DMF, potassium hexacyanoferrate ($K_3FeCN_6$), phosphate buffer (pH 7.4), Tris-HCl buffer, LiCl, NaCl, bovine serum albumin (BSA), and Tween 20 were purchased from Aldrich. Tris(2,2'-bypyridyl) ruthenium (II) chloride was purchased from Strem Chemicals. The bicinchoninic acid assay kit was obtained from Pierce. All stock solutions were prepared using deionized or autoclaved water.

**Synthesis of Antibody-Silica Nanoparticles-Poly[G] Conjugate**

1. Preparation of Carboxylic Acid-Functionalized Silica NPs A 1-ml amino functionalized silica NPs from stock solution was centrifuged, and the supernatants were discarded. The deposit was dispersed in a tube with dimethylformamide (DMF), and 11.4 mg of glutaric anhydride in DMF was slowly added. Then the mixture was left to stir overnight. The excess glutaric anhydride was removed by centrifuge, and the particles were redispersed in DMF, which was repeated twice. The silica NPs were finally redispersed in water by slowly increasing the ratio of $H_2O$/DMF.

2.. Preparation of NHS Ester-Functionalized Silica NPs The carboxylic acid groups of silica NPs were activated with EDC and NHS by adding 1 ml of a dispersion of the above silica NPs in a 2-[n-morpholino]-ethanesulfonic acid buffer to a tube containing 10 mM of EDC and NHS. After 1.0-H stirring, the particles were centrifuged and redispersed in carbonate buffer to produce the NHS ester-terminated silica NPs.

3. Preparation of TNF-α antibody and poly[G] Functionalized Silica Nanoparticles The above silica NPs were dispersed in 1 ml of the solution containing the TNF-α antibody (Ab) and poly[G], and the solution was stirred overnight. The excess of Ab and poly[G] was removed by successively cleaning the silica NP conjugates, and the solution was centrifuged until the supernatants no longer contained any poly[G] and Ab (the electrochemical test for poly[G] and the bicinchoninic acid [BCA] assay for Ab were used). The resulting silica NPs were redispersed into a buffer solution (pH 7.4) and stored at 4°C.

**Immunoassay procedure**

A 30 μl quantity of 0.1 mg/ml biotinylated TNF-α antibody was incubated on the avidin-modified electrode surface for 45 minutes. The electrode was rinsed in PBS buffer and then put in a tube containing the washing buffer (PBS buffer containing 0.1% tween), and the solution was shaken 10 more minutes. The 0.5% BSA solution in PBS buffer was incubated for 0.5 hours and then cleaned with washing buffer again. A series of 30-μl PBS buffers containing different concentrations of TNF-α were incubated on the electrodes for 45 minutes and then thoroughly rinsed with PBS buffer, These electrodes were shaken with washing buffer in a tube for 10 minutes. Finally, the solution of the TNF-α antibody conjugated with a poly[G]-covered silica NP was incubated for another 45 minutes. Then the electrode was thoroughly rinsed with PBS and shaken with a washing buffer in a tube to eliminate the NPs physically adsorption on the surface of the electrode. The electrode was rinsed with water, dried under an $N_2$ stream, and then used for experiments.

**Electrochemical detection**

Square wave voltametric (SWV), chronoamperometric, chronocoulometric, and cyclic voltametric (CV) measurements were performed with a screen-printed electrode consisting of a carbon or avidin-modified carbon as a working electrode as well as a carbon-counter electrode and a Ag/AgCl reference electrode. A sensor connector was used to connect the screen-printed electrode and CHI electrochemical analyzer. For SWV measurements, the potential was scanned from 0 to 1.3 V, and the conditions were 4 mV step height, 25 mV pulse height, and a frequency of 15 Hz. A 0.1 M PBS buffer solution (pH 7.4) containing desired concentrations of $Ru(bpy)_3Cl_2$ was used for electrochemical experiments. An electrochemical cell was formed by dropping 100 μL of the above solution on the electrode surface. For chronocoulometry, a single step was used from 0.5 to 1.15 mV with a pulse width of 50 second or 150 second.

## RESULTS AND DISCUSSION

**Characterization of functionalized silica nanoparticle label**

The synthesized silica NP conjugates were firstly characterized by atomic force microscopy (AFM) (Figure 1 ). It can be seen that the silica NP conjugates on the mica surface are uniform with a diameter of around 15±5 nm. In order to confirm the poly[G] and a protein were attached onto the surface of silica nanoparticle, The X-ray photoelectron spectroscopy (XPS) and cyclic voltammetry

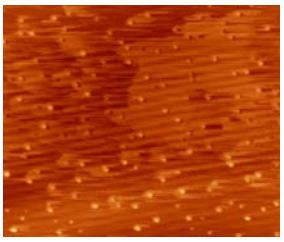


Figure 1. AFM image of the poly[G]-functionalized silica NPs on a silica surface

were employed. The XPS spectra of the silica NP conjugates and original silica NPs are shown in Figure 2(a) and Figure 2(b). As shown from XPS C 1s traces in Figure 2(a), both functionalized and unfunctionalized NPs show a peak at 284 eV, which is from C-H groups. However, for the silica NP conjugates, the antibodies are signified by the appearance of a prominent shoulder around 288 eV, which is attributed to carbonyl carbons. As shown in Figure 2(b) , the normalized XPS P 2p trace has a maximum at 133 eV for the silica NP conjugates [Figure 2(b), green line], which is from the phosphate backbone in poly[G]. However, the original

unfunctionalized silica NPs do not display this peak [Figure 2(b), red line]. Therefore, these spectra

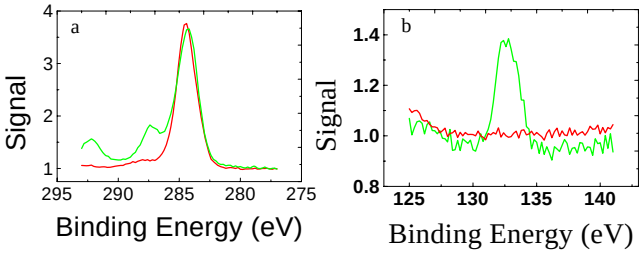



**Figure 2**. (a) XPS C 1s and XPS P 2p (b) spectra from the poly[G]-functionalized silica NPs (green) and original amino-modified silica NPs (red

indicate that both antibodies and poly[G]s are attached on the silica surface. Figure 3 shows the cyclic voltammograms at poly[G]-silica NPs modified screen printed carbon electrode (SPE) and silica modified SPE electrode in PBS solution containing 5.0 μM $Ru(bpy)_3^{2+}$. As shown in Figure 3, in the oxidative wave for $Ru(bpy)_3^{3+/2+}$ at 1.10 V, there is a large catalytic current at Poly[G]-silica NP modified electrode. In contrast, no catalytic current appeared in silica NPs modified electrode without ploy[G]. This indicates that the mediator, $Ru(bpy)_3^{2+}$ , can greatly enhance the current from guanine oxidation and the catalytic current is attributed to a large number of guanine on silica NPs.[19] and this further confirm that poly[G] was immobilized on the surface of silica NPs.

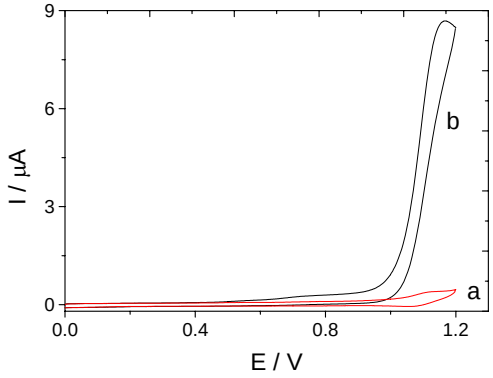



**Figure 3.** Cyclic voltammograms acquired at electrodes covered with (a) silica NPs and poly[G]-functionalized NPs (b) in 0.1 M PBS buffer containing 5.0 μM $Ru(bpy)_3Cl_2$.

Chronocoulometry was employed for quantifying the number of guanine on the silica surface. Figure 4 show the chronocoulometric responses obtained with a silica NP conjugate-modified SPE and a bare SPE in $Ru(bpy)_3Cl_2$ solution, respectively. It can be seen from this figure that the charge (green line) obtained at the silica NP conjugate-modified electrode is larger than that at the bared electrode, which is attributed to the attached guanine on the surface of silica NPs. The numbers of guanine residues on the electrode surface can be estimated by the difference of the charges of these two curves. According to the equation:[29]

$$Q = nFm\Gamma \quad (1)$$

(here, $Q$ is the charge, $F$ is the Faraday constant, m is the total number of silica NP conjugates on the electrode, and $\Gamma$ is the number of moles of guanine residues on each silica NP conjugate), the average number of guanine residues on each silica NP is estimated to be 1386 (two electrons are involved in the guanine oxidation, and the total number of silica nanoparticles can be estimated by the total volume of the solution of silica NP conjugates used).

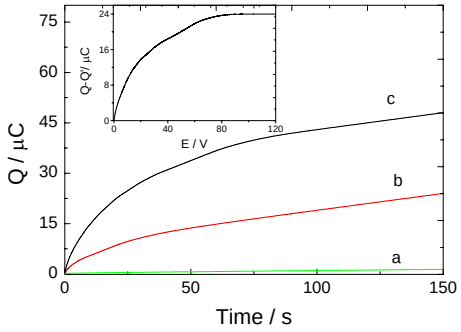



**Figure 4**. Q-t curves obtained at (a)silica NPs modified electrode without $Ru(bpy)_3^{2+}$ in 0.1 M PBS buffer (pH7.4) and (b) silica NPs modified electrode and poly[G]-functionalized silica NPs modified electrode in 0.1 M PBS buffer contaning 5.0 μM $Ru(bpy)_3Cl_2$. the scan rate is 0.1 V/ s.

## Analytical performance of immunosensor

The functionalized silica NPs can be used as a label for an amplified electrochemical immunosensing TNF-α. Scheme 1 illustrates the principle of an electrochemical immunoassay based on a poly[G] functionalized silica NP label. The procedure of TNF-α sandwich immunoassay can be described as followings: Biotinl-modified TNF-α antibody is immobilized on an avidin-modified screen-printed electrode (SPE) through avidin-biotin reaction. TNF-α is added to the assay mixture where it binds to the TNF-α antibody immobilized on the surface of the electrode. A second antibody against TNF-α labeled with poly[G] functionalized NPs is added to complete the immunoassay sandwich. After washing the sample, a solution of $Ru(bpy)_3^{2+}$ is added and current from catalytic guanine oxidation is measured. This current is proportional to the numbers of guanines in the vicinity of the electrode, which in turn depends on the concentration of TNF-α in the original sample. We investigated the specificity and sensitivity of this approach for a protein assay. The time for immunoreaction was 45 minutes. Notice that it is critical to use a bovine serum albumin (BSA) block step to prevent physical adsorption of the antibodies and TNF-α on the carbon electrode surface. A washing buffer containing 0.1% tween is also required to thoroughly clean the electrode surface. Figure 5 shows the typical square wave voltammetric (SWV) signals of this sandwich immunoassay with different concentration of the TNF-α It can be found that the voltammetric peaks are well defined ($E_{pa}$, 1.1 V), and the peak currents is creasing with increasing of the concentration of TNF-α. For a control

experiment (in the absence of TNF-α), a negligible current at the electrode was

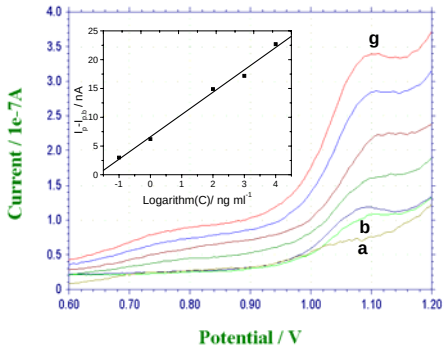



**Figure 5.** Typical square wave voltammograms (SWV) of immunosensors with different concentrations of TNF-α in 0.1 M PBS buffer solution (pH7.4) containing 1.0 μM $Ru(bpy)_3Cl_2$. (a) at avidin modified SPE electrode;(b) at TNF-α antibody modified electrode without TNF-α(control); (c to g) 0.1, 1, 100, 1000, 10000 ng $ml^{-1}$ TNF-α. Inset is the plot of background-subtracted peak current vs. logarithm of the different concentration of TNF-α.

found,(curve b) which might be caused by a small physical adsorption of the functionalized silica NPs on the electrode surface or the oxidation of W and Y residues in antibody by $Ru(bpy)_3^{3+}$ on the electrode surface. The response for the assay with non-specific protein IgG instead of TNF-α is also negligible, which indicates that the method is very selective (not shown). Inset of Figure 5 also shows the plot of background-subtracted peak current vs. the logarithm of TNF-α concentration. The current was linearly increased with the logarithm of concentration of the TNF-α ($R^2$=0.996). A series of six repetitive measurements of the 10 ng $mL^{-1}$ TNF-α yielded reproducible SWV peaks with a relative standard deviation of 10% (data not shown). The limit of detection (LOD) for this immunosensor based on S/N=3 is estimated to be about 0.05 ng/ml (about 2.0 pM), which corresponds to about 60 attomole mouse TNF-α in a 30-μl sample solution. Therefore, the sensitivity of immunosensor based on nanoparticle labels in this work is comparable with that of chemicluminescence imaging.[20]

In summary, we have successfully developed a sensitive electrochemical immunosensor based on poly G functionalized silica nanoparticle labels for the detection of TNF-α. This method is simple, selective, and reproducible for trace biomarker analysis. A remarkable LOD has been achieved through dual signal amplification by poly[G] functionalized silica NPs and catalytic guanine oxidation. The work demonstrates the feasibility of developing an inexpensive, sensitive, and portable device for multiplexed diagnoses of different protein biomarkers, and the technique developed in this work can be extended to study protein/protein, peptide/protein, and DNA/protein interactions.

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