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Developing electrochemical sensor for point-of-care diagnostics of oxidative stress marker using imprinted bimetallic Fe/Pd nanoparticle

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ABSTRACT

A novel electrochemical-sensing platform based on imprinted bimetallic Fe/Pd (BI-Fe/Pd) nanoparticle has been fabricated for point-of-care diagnostics of oxidative stress marker (3-nitrotyrosine) in biological fluids. Herein, BI-Fe/Pd nanoparticles are used as a platform on which 3-nitrotyrosine imprinted cavities are created using acrylamide as monomer and *N*-*N*′-methylene bisacrylamide as cross-linker. The performance of the obtained imprinted sensor is investigated by cyclic, differential pulse, and square wave voltammetry in stripping mode. The imprinted sensor exhibits high recognition ability and affinity for 3-nitrotyrosine in comparison with the non-imprinted one. In addition, the proposed sensor is capable of measuring 3-nitrotyrosine in aqueous as well as in human blood serum, plasma, and urine samples within the range of 4.90–867.57 $\mu g\ L^{-1}$ and 9.90–867.57 $\mu g\ L^{-1}$ with detection limit of 1.20 $\mu g\ L^{-1}$ and 3.25 $\mu g\ L^{-1}$ by square wave and differential pulse stripping voltammetry, respectively. Imprinted BI-Fe/Pd nanoparticle modified sensor shows high affinity and no interference from blood or urine components. Modified sensor was stored for 45 days at room temperature without any detrimental effects to their binding properties. The high affinity of proposed sensor and the lack of requirement for cold chain logistics make them an attractive alternative to the enzyme-linked immunosorbent assay (ELISA) technique.

1. Introduction

Nitration of tyrosine residue is a common post-translational modification to proteins that occurs *in vivo* especially under conditions of oxidative and nitrosative stress [1]. Tyrosine nitration of proteins (in *ortho* position to the phenolic hydroxyl group) is generated by reactive nitrogen species (peroxynitrite, nitrogen dioxide, nitrous acid, nitryl chloride *etc.*) either from the inflammatory cell defense mechanism or from a combination of nitric oxide synthase activity with a high rate of oxidative metabolism [2]. Tyrosine nitration can dramatically change protein structure and conformation. Subsequently it alter the biological half-life, inactivate enzymes and receptors that depend on tyrosine residues for their activity. This prevents phosphorylation of tyrosine residues, which is important for signal transduction. This modification is also associated with a number of pathologies [1], including neurodegenerative disorders such as Alzheimer's [2], atherosclerosis [3], osteoarthritis [4], rheumatoid arthritis [5], Parkinson's [6] and cardiovascular disease [7].

Now-a-days, 3-nitrotyrosine (3-NT) has been suggested and used as a biomarker for diagnosis of oxidative stress-induced pathological conditions. The predictive value of 3-NT was independent of other measures such as C-reactive protein, an inflammatory marker. Because of its utility in monitoring disease pathology and therapeutic intervention, a sensitive, specific and reliable method for measuring nitrotyrosine is crucial. Several analytical methods have been developed and used to measure free nitrotyrosine and/or nitrated proteins. Among all these methodologies reported so far, three major methodological approaches have been regularly used to measure 3-NT levels in biological matrices. The first methodology, mass spectroscopy (MS) technique, is usually coupled to either gas chromatography (GC–MS) [8] or liquid chromatography (LC–MS) [9]. Second, HPLC coupled with electrochemical detection has been widely used for the measurement of free and protein bound 3-NT [10]. The third and commercialized method reported for 3-NT detection is ELISA. According to the literature, more than five ELISA kits are available for 3-NT determination (Table 1). Based on the reaction with reactive nitrogen species, two forms of 3-NT are found *in vivo*, *i.e.* free

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Table 1
List of commercially available ELISA techniques reported for determination of 3-nitrotyrosine.

S. nos.	Company name	Range ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	Detection technique
1.	Abcam-ab116691	8.0–1000.0	0.001	Spectrophotometer
2.	HycultBiotech-HK501	0.45–339.28	0.45	Spectrophotometer
3.	USCN	3.7–3000	1.38	Spectrophotometer
5.	MyBiosourse.Com	0–20	0.001	Spectrophotometer
6.	Milipore	$0–12 \times 10^5$	–	Chemiluminescence
7.	This method	(a) 9.90–867.57 (b) 4.90–867.57	(a) 3.25 (b) 1.20	Electrochemical

and protein-bound. Typically, identification and quantification of 3-NT has involved measuring the free 3-NT either in plasma or urine sample [11] and only ELISA techniques are able to detect protein bound 3-NT. All the ELISA kits are based on monoclonal and/or polyclonal natural antibodies against 3-NT. Although natural antibodies are highly selective and sensitive to their target molecule, they exhibit poor stability; require long production time, difficult handling, low temperature storage, and high costs.

For these reasons, today, the research is focusing on the production of artificial antibodies which are capable of selectively binding target analyte with the same sensitivity of the natural antibodies but with simple steps and low cost of production. In this respect, molecularly imprinted polymers (MIPs) are in demand, in place of antibody, to design robust molecular receptors that are capable in recognizing target biological molecules at molecular level. Such MIPs can also mimic natural recognition entities, such as antibodies and biological receptors.

Mergola et al., have prepared 3-NT imprinted polymer as solid-phase extraction (SPE) sorbent material by bulk polymerization with methacrylic acid as functional monomer and acetonitrile as porogen [12]. Although their result showed high selectivity, but their application in real sample analysis (human blood) is not explored, which is the major agenda in 3-NT detection. Similarly, Schöneich et al., have done the 3-NT labeling with fluorogenic compound to visualize the cellular protein 3-NT by confocal microscopy [13]. But how much protein got converted or nitrated at tyrosine residue, *i.e.* the quantitative study, is not explored.

Bimetallic nanoparticles are of wide interest since they lead to many interesting size-dependent electrical, chemical, and optical properties. Recently, zero-valent iron (ZVI, Fe^0) is commonly utilized in biotechnological and biomedical industry, due to its nontoxic, paramagnetic behavior. According to the literature, the reductive activity of ZVI nanoparticles get enhanced in combination with some other metals [14]. A number of combinations has been reported so far to produce bimetallic ZVI systems like Pd/Fe [14], Cu/Fe [15], Ni/Fe [16], and Ag/Fe [17]. Among these alloys, Fe/Pd nanoparticle modification was preferred due to their biocompatibility, high electrical conductivity and inexpensiveness [14].

In this work, first time, we have synthesized 3-NT imprinted polymer as artificial receptor onto the surface of bimetallic nanoparticle for the fabrication of electrochemical sensor. The main aim of this study was to apply artificial receptor modified electrochemical sensor for detection of 3-NT as well as nitrated tyrosine residues in biologically available protein molecules. We have synthesized (*in situ*) a vinyl group modified bimetallic Fe/Pd nanoparticle (BI-Fe/Pd), using acrylic acid as stabilizing agent. The vinyl group modified BI-Fe/Pd was used as a platform for synthesis of 3-NT imprinted polymer in the presence of acrylamide (co-monomer), *N-N′*-methylene bisacrylamide (cross-linker) and ammonium peroxodisulphate (initiator), resulting in a biocompatible polymer matrix. Furthermore, the imprinted BI-Fe/Pd nanoparticles were used for fabrication of electrochemical sensor by drop coating onto the pencil graphite electrode (PGE) surface. The proposed sensor is to be used for the *in vitro* quantitative determination of free amino acid and/or total 3-NT levels in biological samples, without any acid hydrolysis or pronase digestion to release nitrotyrosine from nitrated proteins and peptides. Since the sensor can detect the modified amino acid present in protein, the assay is useful for proteins of all species. Moreover, the comparative study of proposed sensor with popular ELISA technique was also performed and it was found that the proposed sensor is much better than ELISA in terms of cost, time of analysis, handling, storage and reusability.

1.1. Materials and instruments

Acryl amide, acrylic acid, ammonium peroxodisulphate (APS), 3-NT, ferrous sulfate heptahydrate ($FeSO_4 \times 7H_2O$) ($>99.0\%$), sodium borohydride ($NaBH_4$) ($>98.0\%$), palladium acetate ($PdCl_2$), *N-N′*-methylene bisacrylamide (MBAA), and other interferents were purchased from Aldrich (Steinheim, Germany) and Fluka (Steinheim, Germany). Solvents, dimethyl sulphoxide (DMSO) and ethanol, were procured from Spectrochem Pvt. Ltd., (Mumbai, India). Standard stock solution of 3-NT ($5.0\ mg\ L^{-1}$) and interferents were prepared in double distilled water. For the binding characterization between imprinted nanoparticle and template, Fourier transform infrared spectroscopy (FT-IR) analysis was carried out on Varian FT/IR (USA) spectrometers. Morphological images and EDAX spectra of bare and modified SPE surfaces and BI-Fe/Pd nanoparticles were recorded using scanning electron microscope (SEM), Hitachi, model S-3400N. For BI-Fe/Pd nanoparticle characterization, UV–vis, X-ray diffraction (XRD), and high resolution transmission electron microscopy (HRTEM) analyses were performed on Perkin Elmer Lambda-35 spectrophotometer, Bruker D8 Focus X-ray diffractometer, and Tecnai T-30 (300 kV FEG TEM), respectively. Three kinds of biological fluids; human plasma, human serum, and human urine; were used to confirm the applicability of the proposed MIP sensor. All the pathological samples were obtained from the local pathology and were used as obtained. All the experiments were performed at room temperature ($25 \pm 1\ ^\circ C$).

1.2. Nitration of protein molecules

To explore the applicability of proposed sensor for protein bounded 3-NT analysis, bovine serum albumin (BSA) and ferritin molecules were nitrated in the laboratory by an earlier reported method [18]. First, protein was dissolved in 50 mM NH_4HCO_3 ($1\ mg\ mL^{-1}$) and nitrated with peroxynitrite ($ONOO^-$) by injecting the nitrating reagent into the protein solution while vortex mixing for 30 s at room temperature. Peroxynitrite was prepared, method described previously [19], by ozonolysis of sodium azide in 0.1 M NaOH and was stored at $-80\ ^\circ C$ until it was used. After nitration, the protein samples were dialyzed overnight against 50 mM NH_4HCO_3 buffer and stored at $-4\ ^\circ C$, before use.

1.3. Preparation of BI-Fe/Pd nanoparticle

Bimetallic Fe/Pd NPs were synthesized by earlier reported method with some modification [14]. Initially, dispersed solutions of acrylic acid with two different concentrations (0.5 v/v % and 1 v/v %) were prepared by deionized double distilled water. For preparation of BI-Fe/Pd nanoparticle, 10.0 mL of dispersant solution was added into 100.0 mL $FeSO_4$ solution ($0.05\ mol\ L^{-1}$). The resultant solution was homogenously mixed by vigorous magnetic stirring for 30 min. Then 125.0 mL of $NaBH_4$ solution (0.1 M) was drop wise added into the mixture for the formation of vinyl group modified zero-valent iron. The reaction mechanism is shown below for clarity: