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Electrochemical detection of tyrosine derivatives and protein tyrosine kinase activity using boron-doped diamond electrodes

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ABSTRACT

In this report, we determined protein tyrosine kinase (PTKs) activity in human epidermoid carcinoma cells (A431) by employing a novel electrochemical method using boron-doped diamond (BDD) electrodes that enables the electrochemical oxidation of tyrosine (Tyr), phosphorylated Tyr (Tyr-P) and sulfated Tyr (Tyr-S) in water-based solutions. Cyclic voltammetry for Tyr, Tyr-P and Tyr-S showed well-defined oxidation peaks at 0.8 V for Tyr, 1.4 V for Tyr-P and 1.7 V for Tyr-S, respectively. Very little work has been reported previously on the detection of Tyr-P and Tyr-S, probably due to their high oxidation potentials. We utilized electrochemical methods for the detection of kinase activity in connection with poly(Glu-Tyr) modified magnetic beads. Linear-sweep voltammograms for the electrochemical detection of PTKs activity were carried out using BDD electrodes consisting of peptide-modified magnetic beads. Without phosphorylation of the peptide-modified magnetic beads using PTKs, we observed clear oxidation peaks for Tyr oxidation and no significant electrochemical responses for Tyr-P oxidation at 1.4 V for the background. On the other hand, with phosphorylation of the beads using PTKs, the peak oxidation current at 1.4 V clearly increased, while the peak oxidation current for Tyr oxidation decreased. This indicates that PTKs activity could be successfully detected by using electrochemical methods employing BDD electrodes. This method was utilized for the *in vitro* kinase activity detection of human cell lysate, and the electrochemical measurements were compatible with the Enzyme-Linked ImmunoSorbent Assay based method. Our results indicate that the electrochemical method can be applied to real samples such as cell lysate.

1. Introduction

About one-third of the potential 30,000 proteins in the human proteome are estimated to be substrates for phosphorylation at some stage in their life cycle (Cozzone, 1988; Olsen et al., 2006; Ullrich and Schlessinger, 1990; Cohen, 2000). The transfer of a phosphoryl group from ATP to a nucleophilic acceptor group on the amino acid side chain of a protein is catalyzed by protein kinases (Cohen, 2002; Jensen and Hunter, 2001). The side chains that can be phosphorylated in proteins are most commonly serine, threonine, and tyrosine (Tyr). Phosphorylation of Tyr occurs at the hydroxyl group on its aromatic ring, via the action of protein Tyr kinases (PTKs). Dephosphorylation of phosphotyrosine (Tyr-P) occurs through the action of protein Tyr phosphatases. Tyrosine kinases play an important role in the regulation of normal cell signaling patyways (Jaiswal and Simon, 2007; Jorissena et al., 2003; Schlessinger, 2000). Furthermore, tumor cells express high levels of tyrosine kinases (Noble et al., 2004; Mendelsohn and Baselga, 2000).

Protein kinase activity has been extensively investigated by a number of techniques including fluorescence, radioactive, and surface-plasmon resonance-based systems that employ enzymatic reactions or phospho-specific antibodies to detect kinase activities (Sun et al., 2007; Houseman et al., 2002). However, the above-mentioned techniques are either labor- or time-intensive, or require highly specialized laboratory instruments and well-trained personnel. Simple and cost-effective methods that can be readily adapted for multiplexed kinase detection are desirable to enable kinase activity profiling for diagnostic applications and to accelerate the *in vitro* elucidation of cellular signal transduction pathways.

Recently, kinase-catalyzed biotinylation of substrate peptides and proteins has become a powerful alternative to radioactive or fluorescence methods for phosphoprotein detection and identification (Green and Pflum, 2007). Streptavidin-coated gold nanoparticles have been used as optical and electrochemical labels in connection with the kinase-catalyzed biotinylation of the substrate peptides (Wang et al., 2005, 2006; Kerman et al., 2007a).

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However, both of these methods require tedious post-labeling procedures that make handling a difficult task.

Kerman et al. (2007b) demonstrated a new label-free electrochemical method for the small-molecule inhibition of tyrosine phosphorylation. These authors found that the phosphorylation of Tyr residues causes suppression of the oxidation current response, and this effect can be used to monitor peptide phosphorylation. However, one disadvantage of their method is that loss of signal is monitored for the detection of phosphorylation. Given that there are many phenomena that can lead to the loss of electrochemical signals in complex samples (e.g., electrode fouling), methods in which the signal increases are much more compelling as diagnostic techniques.

On the other hand, boron-doped diamond (BDD) electrodes have attracted much interest due to their superior properties, including low background currents, a wide working potential window, favorable electron transfer kinetics, and surface inertness, which result in high resistance to deactivation (Mitani and Einaga, 2009; Watanabe and Einaga, 2009). Our group has recently reported the quantitative electrochemical detection of bovine serum albumin (BSA) and also the detection of conformational changes in BSA by the direct electrochemical oxidation of tyrosine, tryptophan, and cysteine residues using BDD electrodes (Chiku et al., 2008a,b). BDD electrodes are well-faceted, hydrophobic, and have low surface energy, so they are a viable material for the direct oxidation of proteins.

Herein, we report the results of an investigation into the electrochemical detection of PTKs activity using BDD electrodes. BDD electrodes are demonstrated to be the best electrode material for the detection of Tyr-P, since they possess high sensitivity even at high oxidation potentials. Moreover, we have also demonstrated the electrochemical detection of sulfated tyrosine (Tyr-S) (Moore, 2003) by using BDD electrodes. We applied BDD electrodes to Kerman's methods (Kerman et al., 2007b) for the electrochemical detection of PTKs activity. Finally, we applied our method for the detection of PTK activity in real samples. The levels of PTKs activity in human cells (A431) were determined by our method, and were then compared with the results from the commercial Enzyme-Linked ImmunoSorbent Assay (ELISA) based assay.

2. Materials and methods

2.1. Materials

Poly(Glu, Tyr) 4:1, a Tyrosine kinase assay kit, ATP, human Epidermal growth factor receptor (EGFR) and EDAC were obtained from Sigma. Carboxylated super-paramagnetic polystyrene beads (3 μm i.d.) were obtained from Polysciences, Inc. (Warrington, PA). The other regents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The water used in the experiments was purified using a Direct-Q system (Millipore) to a specific resistivity of 18.2 M.

2.2. Instruments

The BDD electrodes were deposited on Si (100) wafers in a microwave plasma-assisted chemical vapor deposition system (ASTeX Corp.). The procedure has been described in detail previously (Ivandini et al., 2002). A mixed acetone/methanol (9:1, v/v) solution of B_2O_3 with a B/C atomic concentration ratio of 10,000 ppm was used as the carbon source. The typical grain size of the resulting BDD thin films was up to ~5 μm, with a thickness of ~20 μm for a deposition time of 7 h using 5 kW of plasma power.

The electrochemical measurements were conducted using a potentiostat (HZ-100 and HZ-5000, Hokuto Denko) with a standard three-electrode configuration and a single-compartment glass cell. An Ag/AgCl electrode was used as the reference electrode, and a Pt wire was used as the counter electrode. A magnetic plate was attached to the back-side of the BDD electrodes to adsorb the magnet beads onto the BDD surface.

2.3. Immobilization of peptides on beads

The manipulation of the magnetic beads was carried out on a BioMag processing platform. Briefly, 0.5 mL (10 mg) of carboxylated super-paramagnetic polystyrene beads were transferred into a 15 mL centrifuge tube. Then, 5 mL of a 50 mM 2-(N-morpholino)ethanesulfonic acid sodium salt (MES) buffer solution (pH 5.2) was added and the solution was mixed. The application of a magnetic field to the side of the centrifuge tube attracted the beads to the sidewalls. The MES buffer was carefully removed using a micro-pipette. The washing step was repeated three times. The beads were then suspended in 5 mL of 50 mM MES. For the covalent activation of the carboxylated beads, stock solutions of EDAC were prepared and added to yield a final concentration of 16.7 mM in MES. The mixture was incubated for 30 min at room temperature with gentle mixing on a 'microtiter' plate shaker. After magnetic separation, the beads were washed once with a 5 mL MES buffer solution. After the final wash, the beads were re-suspended in 5 mL of MES. Then, substrate peptides were added to yield the desired final concentration in MES. The mixture was incubated for 24 h at room temperature with gentle mixing. The peptide-modified beads were washed twice with 5 mL of MES, and 5 mL of a Glycine solution was added at a final concentration of 100 mM. The resultant mixture was incubated for 30 min at room temperature to deactivate and block the excess reactive ester groups remaining on the surfaces of the beads. Finally, the peptide-modified beads were washed three times with 5 mL of MES and suspended in 2 mL of MES. The peptide-conjugated beads were then stored at 4 °C until use.

2.4. Tyrosine kinase assay on beads

Tyrosine kinase assay was performed by adapting the protocol recommended by Sigma. A reaction mixture containing ATP (10 μL of kinase assay buffer containing 500 mM HEPES buffer, 150 μM ATP, 30 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM Na_3VO_4) and kinase (10 μL of the desired concentration in kinase assay buffer) was incubated in the presence of the magnetic bead-bound peptides for 30 min at 30 °C in a 0.5 mL centrifuge tube with gentle mixing. The beads were then separated by the application of a magnetic field, washed three times with 1 M NaCl in 50 mM Tris–HCl buffer (pH 6.5), and suspended in 10 μL of 50 mM Tris–HCl buffer.

2.5. Electrochemical measurement

The magnetic beads suspended Tris–HCl buffer solution (10 μL) were dropped onto the BDD electrode surface and left for 5 min so that the beads could become magnetically adsorbed onto the BDD electrode. After the beads were adsorbed, 300 μL of a 50 mM Tris–HCl buffer solution was added into the electrochemical cells and we started the electrochemical measurements.

2.6. Cell culture

Cells derived from human epidermoid carcinoma cell line A431 were donated by the Cell Resource Center for Biomedical Research (Tohoku University). The A431 cells were cultured in a DMEM medium (Gibco Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Gibco) and 50 μg/mL penicillin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2.