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Covalent linking of peptides onto oxygen-terminated boron-doped diamond surfaces

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Abstract

This paper reports on the formation of semicarbazide boron-doped diamond surfaces and their use for the preparation of peptide microarrays through site-specific α-oxo semicarbazone ligation. Hydrogen-terminated diamond substrates were first photochemically oxidized and the resulting surface hydroxyl groups were coupled with aminopropyltriethoxysilane to yield amine-terminated surfaces. Chemical reaction of the terminal amino groups with triphosgene and Fmoc-protected hydrazine, followed by a deprotection step led to the formation of a semicarbazide termination. Peptides bearing a glyoxylyl group were linked to the semicarbazide-terminated BDD surfaces through site-specific ligation. X-ray photoelectron spectroscopy and fluorescence measurements were used to characterize the resulting surfaces.
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1. Introduction

Since the first report by Geysen et al. on the immobilization of different peptides on the same solid substrate to map for B-cell epitopes [1], advances in modern proteomic research are highlighted by the exploration of many new strategies for the site-specific grafting of proteins which preserve the proteins' native form and spatial orientation [2–5]. Consequently, protein-based microarrays as well as peptide arrays have been exploited for the high-throughput evaluation of complex protein functions and have been the subject of much discussion in recent years [6,7]. In comparison to proteins, which unfold readily and subsequently loose their biological activities, peptides are rather stable and retain their activities under most reaction conditions. This makes peptides the preferred molecules for robust screening assays for protein studies especially in microarray-based formats. Besides some recent reports on the use of supramolecular hydrogels as array platform for peptide/protein immobilization [8], the preferred interface for the site-specific peptide immobilization is glass substrate [9–16]. While Mrksich et al. developed a peptide array using modified peptides immobilized on a glass surface coated with a self-assembled monolayer of alkane dithiols and used antibodies for the detection of peptide phosphorylation [10], Yao et al. took advantage of the chemistry involved in the native chemical ligation and biotin–avidin interactions [15]. Peptide microarrays for the determination of protease substrate specificity were reported by Ellman et al. and are based on linking peptides to glass slides via a chemoselective oxime formation using an aldehyde-derivatized surface and alkoxylamine-functionalized substrates [16]. Melnyk et al. described the preparation of novel peptide–protein microarrays based on the utilization of glass slides functionalized by a semicarbazide layer [11–14]. This surface was used for the site-specific immobilization of glyoxylyl peptidic

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antigens through α-oxo semicarbazone ligation or for the immobilization of recombinant proteins through physisorption. The microarrays displayed high levels of sensitivity and specificity for the detection of HIV, HCV, HBV, EBV and syphilis antibodies.

Extension of the surface chemistry of glass to electrically conducting interfaces appears as an attractive alternative. Next to silicon, which is a widely used substrate for the preparation of miniaturized devices due to its electronic and well-defined chemical properties, boron-doped diamond (BDD) has gained remarkable interest in the biomedical field [17–24] due to its excellent mechanical properties, extreme chemical stability, good electrical conductivity, low background current densities, a large potential window in aqueous electrolytes (about −1.35 V to 2.3 V/NHE) as well as its biocompatibility [25,26]. While most of the reported work in the literature regarding biomolecule linking to the BDD surface deals with different ways of DNA immobilization, a limited number of papers highlight the use of diamond for protein immobilization. Hamers et al. covalently link ethylene glycol oligomers and amines bearing a terminal vinyl group to hydrogen-terminated p-type diamond films via a photochemical process [18,24,27] to which fluorescence tagged proteins such as avidin, bovine serum albumin, fibrinogen, and casein were linked through adsorption. The focus of this work was on the establishing of a method for minimizing non-specific binding of proteins. Garrido et al. [23] used the same photochemical approach reported by Hamers et al. [27] to covalently attach proteins on n-type hydrogen-terminated nanocrystalline diamond films, previously patterned using photolithography. Green fluorescence proteins could be linked to the diamond surface through the formation of a peptide bond, by first acylating the primary amine and converting the terminal carboxyl groups on the diamond into a reactive *N*-hydroxysuccinimide (NHS) ester intermediate by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and NHS.

In this paper, we report on the preparation and characterization of semicarbazide BDD substrates using a multistep procedure. First, oxidized BDD surfaces are obtained by UV irradiation of hydrogen-terminated surface in air at room temperature [28]. The reaction generates surface hydroxyl groups that can easily be coupled to organosilanes. Reaction of the oxidized BDD surface with aminopropyltriethoxysilane yields an organic layer covalently attached to the surface. The surface amine termination was then transformed to Fmoc-protected semicarbazide using triphosgene and Fmoc-hydrazine. After removal of the protecting group, the resulting semicarbazide surface was used for site-specific immobilization of peptide molecules. In a model experiment using fluorescence detection, glyoxylyl peptide: 3×FLAG-COCHO was printed on the semicarbazide-terminated BDD surface to capture antibody anti-peptide FLAG labeled with tetramethylrhodamine.

2. Experimental section

2.1. Peptide synthesis

The synthesis of glyoxylyl peptides **1** and **2** was performed on a 0.2 mmol scale using the Fmoc/*tert*-butyl strategy on a Novasyn TG resin (Novabiochem) modified by an isopropylidene tartrate-based linker. The preparation of peptide amide **3** was performed on a 0.2 mmol scale using the 9-fluoromethoxy-carbonyl (Fmoc)/*tert*-butyl strategy on a Rink amide resin ([5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid]–polyethyleneglycol–polystyrene) (Fmoc-PAL–PEG–PS) resin) [29].

2.2. Preparation of H-terminated and oxidized diamond samples

Polycrystalline diamond layers were synthesized on a silicon high purity p-type wafer by microwave plasma-enhanced chemical vapor deposition (PECVD) in a conventional reactor [30]. The dopant source was boron oxide set in a Pt crucible placed on the substrate holder near the silicon substrate. The dopant concentration in the diamond layers, as estimated from Raman spectroscopy measurements is in the range 10^{19} to 10^{20} B cm^{-3}. The film resistivity was ≤0.1 Ω cm as measured with a four-point probe.

Oxidized diamond samples were obtained through UV irradiation in air for 2 h using a low pressure mercury arc lamp as reported previously [28]. The UV lamp displays lines at 185 and 254 nm.

2.3. Preparation of semicarbazide-terminated diamond surfaces

The oxidized diamond surfaces were first amine-terminated by reaction with 3% aminopropyltriethoxysilane (APTES) in methanol/water: 95/5 (v:v) for 30 min under sonication. The resulting surfaces were then washed with methanol, water (two times), and methanol and finally annealed at 110 °C for 15 min. The NH_2-terminated surfaces were treated with 0.1 M triphosgene/0.8 M diisopropylethylamine solution in 1,2-dichloroethane for 2 h under sonication followed with a 22 mM Fmoc–NH_2NH_2 solution in DMF containing 1% ethanol for 2 h under sonication. Finally, removal of the Fmoc groups was performed with 0.2% piperidine and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF (30 min). The substrates were washed with DMF, water (two times), and methanol and dried under reduced pressure. The resulting surfaces are stable and can be stored for several weeks at room temperature in a dust-proof container without any apparent degradation.

2.4. Peptide microarray

Peptides 3×-FLAG-NH_2, 3×-FLAG-COCHO and HA-COCHO (10^{-4} M), dissolved in 0.1 M, pH 5.5, sodium acetate buffer were printed five times each as nanoliter drops on the diamond substrate using a 4 piezo tips Perkin-Elmer BioChip Arrayer I. The printed wafers were incubated at 37 °C in a humid chamber (60% relative humidity) overnight then soaked in a saturated solution (0.1% Tween 20 (by volume), 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 0.01 M, pH 7.2)) for 60 min under sonication. A washing solution containing 0.05% Tween 20 (by volume) in PBS (0.01 M, pH 7.2) was used three times to remove excesses of peptides and BSA. Incubation was performed using 100 μl of

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