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Surface Modification of Highly Ordered Pyrolytic Graphite (HOPG) by a Mussel-Inspired Poly(norepinephrine) Coating: Characterizations and Cell Adhesion Test

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Carbon substrates, including highly ordered pyrolytic graphite (HOPG), carbon nanotubes, and graphene, have gained a great deal of attention because of their useful features that stem from their favorable mechanical, electrical, and thermal properties.[1,2] Potential applications include electronics, sensors, catalysts, and medical devices. To achieve these applications, suitable surface functionalizations that modulate the interfacial properties of carbon substrates are necessary. The covalent approach to surface modification of carbon substrates has been extensively investigated and utilized because it provides covalent linkages between the carbon and chemical modifiers that produce strong and stable bonds. Photoreactions of phenylazide,[3] alkylthiol,[4] and olefin derivatives,[5] electro-oxidation of alkylamine,[6] and electro-reduction of diazonium are examples of covalent approaches.[7,8] Briefly, photo- or electro-chemically triggered radical generation followed by coupling to aromatic carbons led to the stable bond formation. However, these methods have some drawbacks for practical use, such as the requirement of an external light source and/or electrochemical instruments to generate the radicals.

Recently, a bio-inspired approach to functionalize carbon substrates has been developed. Since mussel adhesive protein contains a significant amount of 3,4-dihydroxy-L-phenylalanine (DOPA) and lysine (Lys),[9] catecholamines that contain the key chemical functionalities of DOPA and Lys have been identified as minimalist adhesive protein mimics; it was determined that catecholamines can functionalize a wide range of materials in a simple manner, resulting in biocompatible surfaces.[10-15] For example, incubation of solid substrates in an alkaline dopamine solution resulted in polydopamine-coated substrates, and the coating has been widely utilized to prepare the functional surfaces such as, biomolecule-conjugated or mineral-deposited surfaces.[13,16] Moreover, we showed that norepinephrine also forms adherent films on virtually all material surfaces.[17] Unlike polydopamine coating, the coating showed the ability to initiate ring-opening polymerization on the surface due to the additional functionality in the side chain of norepinephrine. The method which is based on the oxidative polymerization of norepinephrine has been successfully applied to nanostructured carbon substrates (*i.e.*, graphene oxide),[18] and resulted in simultaneous reduction and surface modification of graphene oxide. The success of the poly(norepinephrine) (PN) coating suggested the potential application of graphene oxides as multifunctional nanohybrids *via* ring-opening polymerization and deposition of nanoparticles on the surface.[18]

Among various types of carbon substrates, graphene and graphene oxide have been of interest in biomedical devices, due to the bioactivity.[19] In the biomedical application of artificial devices, it is important to study initial adhesion of cells onto the artificial materials, because it determines the fate of cells such as, proliferation and differentiation.[20,21] In this respect, the PN coating which is expected to enhance the initial adhesion of cells is advantageous. Considering its wide applicability, cell-adhesive property, and ease of use, we reasoned that the PN coating is superior to the previously investigated surface modification method of carbon substrates. Herein, we report the facile modification of graphene layers using a mussel-inspired PN coating. The top graphene layer of HOPG was used as a model material to study interfacial properties of graphene. Although there are some differences between the top graphene layer of HOPG and graphene from a physical point of view, the chemical reactivity of the top layer of HOPG is comparable to that of graphene.[22] In addition, there are benefits of using HOPG over graphene or graphite; the preparation of large-scale graphene requires an expensive and complicated instrument,[23] and the physical property of HOPG is superior to that of graphite.

A PN coating on the surface of HOPG was applied by

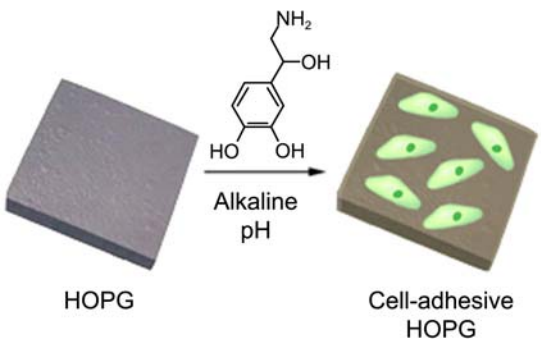

Figure 1. A schematic description of PN coating on the HOPG surface.

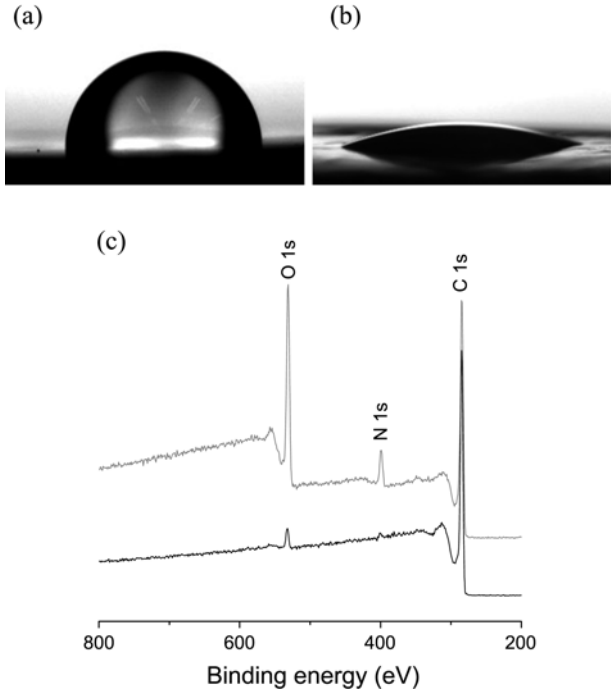

Figure 2. Water contact angle images of (a) unmodified and (b) PN-coated HOPG. (c) XPS spectra of unmodified (black, bottom) and PN-coated (grey, top) HOPG.

simply immersing the substrate in an alkaline norepinephrine solution, as described in Figure 1. After coating overnight, the HOPG surface became hydrophilic, as evidenced by a dramatic decrease in the water contact angle from 85.7° to 24.2°, due to the hydrophilic nature of the PN layers (Figures 2(a) and (b)). Further surface characterization by X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) confirmed successful modification of HOPG surface with PN. The XPS spectra revealed the change in the surface chemical composition caused by the PN coating (Figure 2(c)). In the XPS spectrum of unmodified HOPG, carbon and oxygen peaks, which correspond to the aromatic carbons of HOPG and oxygen adsorbed in HOPG, were observed (Figure 2(c), black). After coating with PN, we observed a new N 1s peak (399.4 eV, Figure 2(c), grey) originating from PN. Quantitative analysis of the surface chemical composition also supported the presence of the PN on the HOPG surface: The amounts of nitrogen (N 1s) and oxygen (O 1s) increased from 0 to 6.65% and 5.5 to 23.18%, respectively, with a concurrent decrease in the amount of carbon (C 1s) from 94.5 to 70.2%.

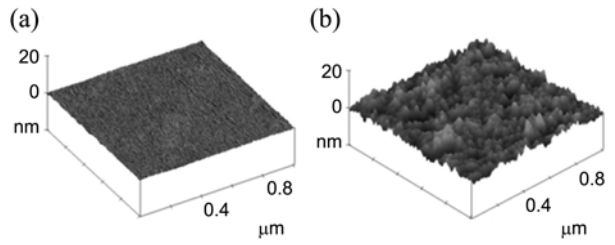

Figure 3. 3-Dimensional AFM images of (a) unmodified and (b) PN-coated HOPG.

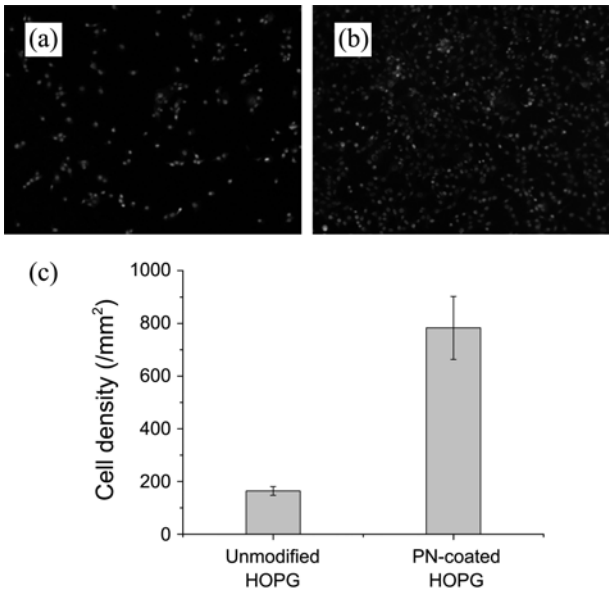

Figure 4. Fluorescence images of cells adhered onto the (a) unmodified and (b) PN-coated HOPG. (c) Quantitative analysis of cells adhered on the unmodified and PN-coated HOPG.

AFM analysis was also performed to characterize the PN layers on the HOPG surface, and changes in the morphology and root-mean-square (RMS) roughness were analyzed.

Figure 3 shows that PN-coated HOPG has a rougher surface than unmodified HOPG. Moreover, the RMS roughness of the HOPG surface increased after overnight PN coating: The roughness of the PN-coated HOPG surface was 1.33 nm, whereas the roughness of the unmodified HOPG surface was 0.23 nm (Figure 3). These data imply that PN layers completely covered the surface of HOPG, thereby forming a rough structure on the surface.

The enhancement of cell adhesion on the PN-coated HOPG was validated *via* an adhesion test of fibroblast cells (NIH-3T3) under the following conditions: DMEM containing 10% FBS and a cell-seeding density of 1.2×10^5 cells/mL. The surface of PN-coated HOPG showed higher cell adhesion than the unmodified HOPG surface (Figures 4(a) and (b)). A quantitative analysis of the density of cells adhered to the HOPG surface was achieved by counting the nuclei of the cells after staining with DAPI: A four-fold increase in cell density from 164 to 783 cells/mm^2 was observed after coating with PN (Figure 4(c)). This result is indicative of the highly enhanced cell adhesion property of the surface.

The enhanced cell adhesion is expected to originate from the increased surface energy after coating with PN. It has been reported that protein denaturation occurs on substrates with low surface energies, resulting in reduced cell adhesion.[24] Accordingly, various approaches to provide sufficient surface energy have been investigated, of which the mussel-inspired polymer coating was found to be a versatile method for increasing the surface energy of a wide range of materials.[14] Similarly, in our system, the PN layers are expected to

provide sufficient surface energy to the HOPG substrate, resulting in greatly enhanced cell adhesion on the PN-coated HOPG surface.

In summary, cell-adhesive HOPG was prepared using a mussel-inspired poly(norepinephrine) coating. Simple immersion of the HOPG substrate into an alkaline solution of norepinephrine resulted in poly(norepinephrine)-coated HOPG; the modified surface was characterized by contact-angle analysis, X-ray photoelectron spectroscopy, and atomic force microscopy. The resulting PN-coated HOPG surface showed highly improved cell adhesion. We believe that this method could be applied to the preparation of bioactive carbon materials which is applicable in tissue engineering or regenerative medical devices.

Experimental

Materials. HOPG (Veeco, United States), DL-norepinephrine hydrochloride (97%, Sigma), trizma base (99%, Sigma), and trizma HCl (99%, Sigma) were used as received. Ultrapure water (18.3 MΩ·cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

Poly(norepinephrine) Coating. A fresh surface of HOPG was prepared by peeling off top layers using an adhesive tape, and the resulting surface was used for further experiments. Poly(norepinephrine) coating was performed by immersing HOPG substrates in a buffer solution (2 mg of norepinephrine per milliliter of 10 mM Tris, pH 8.5) at room temperature. The coated substrates were rinsed with deionized water, and dried under a stream of argon.

Cell Adhesion Test on the HOPG Substrates. For cell adhesion experiments, all substrates were pretreated with cell culture media (DMEM) containing 10% fetal bovine serum (FBS) for 1 h at 37 °C and 5% CO_2. NIH-3T3 cells were seeded onto the substrates at a density of 1.2×10^5 cells/mL and incubated at 37 °C with 5% CO_2. After 4 h, nonadherent cells were removed by aspirating the medium and carefully washed twice with PBS. Adherent cells were fixed in 4% paraformaldehyde for 10 min and nuclei of cells were stained with DAPI. Cell attachment onto the substrates was characterized by fluorescence microscopy (IX 71 fluorescence microscope, Olympus, Japan).

Characterizations. The XPS study was performed with a VG-ScientificESCALAB250 spectrometer (U.K.) with a monochromatized Al Kα line as an X-ray source. Emitted photoelectrons were detected by a multi-channel detector at a takeoff angle of 90° relative to the surface. During the measurements, the base pressure was 10^{-9}-10^{-10} Torr. Survey spectra were obtained at a resolution of 1 eV from three scans. AFM imaging was performed in a tapping mode on a Nanoscope IIIa multimode scanning probe microscope (Veeco, United States) with a tapping mode etched silicon probe (TESP). Static water contact angle measurements were performed using a Phoenix 300 goniometer (Surface Electro Optics Co., Ltd., Korea).

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