Cell Adhesion Properties on Photochemically Functionalized Diamond

Kwok Feng Chong,[†] Kian Ping Loh,*[,†] S. R. K. Vedula,[‡] Chwee Teck Lim,[‡] Hadwig Sternschulte,[§] Doris Steinmüller,[§] Fwu-shan Sheu,[||] and Yu Lin Zhong[†]

Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Division of Bioengineering, National University of Singapore, 3 Science Drive 3, Singapore 117543, rho-BeSt coating, Hartstoffbeschichtungs GmbH, F Exlgasse 20a, A-6020 Innsbruck, Austria, and Department of Biological Sciences, National University of Singapore, 3 Science Drive 3, Singapore 117543

Received January 7, 2007. In Final Form: February 24, 2007

The biocompatibility of diamond was investigated with a view toward correlating surface chemistry and topography with cellular adhesion and growth. The adhesion properties of normal human dermal fibroblast (NHDF) cells on microcrystalline and ultrananocrystalline diamond (UNCD) surfaces were measured using atomic force microscopy. Cell adhesion forces increased by several times on the hydrogenated diamond surfaces after UV irradiation of the surfaces in air or after functionalization with undecylenic acid. A direct correlation between initial cell adhesion forces and the subsequent cell growth was observed. Cell adhesion forces were observed to be strongest on UV-treated UNCD, and cell growth experiments showed that UNCD was intrinsically more biocompatible than microcrystalline diamond surfaces. The surface carboxylic acid groups on the functionalized diamond surface provide tethering sites for laminin to support the growth of neuron cells. Finally, using capillary injection, a surface gradient of polyethylene glycol could be assembled on top of the diamond surface for the construction of a cell gradient.

Introduction

Diamond has been proclaimed as an excellent biocompatible material, although the basis of this statement has not been thoroughly qualified in the context of its surface chemistry and topography. The surface properties of diamond can be made hydrophobic or hydrophilic with hydrogen or oxygen termination,[1] respectively, which have implications for cellular adhesion. Photochemical coupling of an organic molecule onto a hydrogen-terminated diamond surface was pioneered by Hamers,[2] and biorecognition events based on impediemtric sensing had been achieved on these platforms.[3] In terms of cell growth, the ordered growth of neurons has been demonstrated by Specht and co-workers[4] on protein-coated diamond using microcontact printing. Neuronal cell excitability on functionalized diamond surfaces has been shown by Ariano and co-workers.[5] However, the cellular adhesion properties of as-grown diamond surfaces or functionalized diamond surfaces have not been studied in detail.

Cellular adhesion is of fundamental importance in many biological processes as the adhered cells will sense, interpret, integrate, and respond to the extracellular signals. Chemical and physical signals from the substrate such as surface energy, topography, electrostatic charge, and wettability play a vital role in stimulating cell adhesion and influencing cell growth behavior. The physiochemical effects of different functional groups on cellular attachment have been extensively studied.[6,7] In addition, surface topography is also an important biomimic stimulator for cell growth because the in vivo growth of cells occurs on biological interfaces[8,9] that usually have nanotopographical features.[9]

In order to develop diamond as a signal transduction platform for the optical or electrical monitoring of cellular activities, strong cellular adhesion on the surfaces of diamond must be achieved, because the cell and its adhesive components will be subjected to external perturbation in continuous flow systems. AFM operated in the force mode has been used in probing the interaction forces between cell−surface,[10] cell−cell,[11] biotin−streptavidin,[12] antibody−antigen,[13] and complementary strands of DNA oligomers.[14] Nebel and co-workers recently applied AFM to study the forces needed to remove bonded DNA from single-crystal diamond.[14] Here, we investigate the adhesion forces of NHDF cells on a diamond surface, with different surface chemistry and topography. Photochemical functionalization of the diamond using simple UV irradiation in air, or via alkene acids groups,[15] was evaluated comparatively in terms of biocompatibility. To assess the role of surface energy and topography on cell growth, the surface conditions and topography of diamond were correlated

* Author to whom correspondence should be addressed. E-mail: chmlohkp@nus.edu.sg.

[†] Department of Chemistry, National University of Singapore.

[‡] Division of Bioengineering, National University of Singapore.

[§] rho-BeSt coating.

[||] Department of Biological Sciences, National University of Singapore.

(1) Kawarada, H. *Surf. Sci. Rep.* **1996**, *26*, 205.

(2) Yang, W.; Auciello, O.; Butler, J. E.; Cai, W.; Carlisle, J. A.; Gerbi, J. E.; Gruen, D. M.; Knickerbocker, T.; Lasseter, T. L.; Russell, J. N., Jr.; Smith, L. M; Hamers, R. J. *Nat. Mater.* **2002**, *1*, 253.

(3) Wenmackers, S.; Christiaens, P.; Daenen, M.; Haenen, K.; Nesladek, M.; van deVen, M.; Vermeeren, V.; Michiels, L.; Ameloot, M.; Wagner, P. *Phys. Stat. Sol. A* **2005**, *202*, 2212.

(4) Specht, C. G.; WIllians, O. A.; Jackman, R. B.; Schoepfer *Biomaterials* **2004**, 25, 4073.

(5) Ariano, P.; Baldelli, P.; Carbone, E.; Gilardino, A.; Lo Giudice, A.; Lovisolo, D.; Manfredotti, C.; Novara, M.; Sternschulte, H.; Vittone, E. *Diamond Relat. Mater.* **2005**, *14*, 669.

(6) Lee, H. B.; Lee, J. H.; Jung, H. W.; Kang, I. *Biomaterials* **1994**, *5*, 9.

(7) Whitesides, G. M.; López, G. P.; Albers, M. W.; Schreiber, S. L.; Carroll, R.; Peralta, E. *J. Am. Chem. Soc.* **1993**, *115*, 5877.

(8) Teixeira, A. I.; Abrams, G. A.; Bertics, P. J.; Murphy, C. J.; Nealey, P. F. *J. Cell Sci.* **2003**, *116*, 1881.

(9) Shirato, I.; Tomino. Y.; Koide, H.; Sakai, T. *Cell Tissue Res.* **1991**, *266*, 1.

(10) Hyonchol, K.; Arakawa, H.; Osada, T.; Ikai, A. *Colloids Surf. B* **2002**, *25*, 33.

(11) Benoit, M.; Gabriel, D.; Gerisch, G.; Gaub, H. E. *Nat. Cell. Biol.* **2000**, *2*, 313.

(12) Florin, E.-L.; Moy, V. T.; Gaub, H. E. *Science* **1994**, *264*, 415.

(13) Dammer, U.; Hegner, M.; Anselmetti, D.; Wagner, P.; Dreier, M.; Huber, W.; Güntherodt, H.-J. *Biophys. J.* **1996**, *70*, 2437.

(14) Rezek, B.; Shin, D.; Nakamura, T.; Nebel, C. E. *J. Am. Chem. Soc.* **2006**, *128*, 3884.

(15) Zhong, Y. L.; Cong, K. F.; May, Paul; Chen, Z. K.; Loh, K. P. *Langmuir* **2007** (submitted).

10.1021/la070037y CCC: $37.00
Published on Web 04/04/2007

with the cell adhesion forces, cell attachment, and cell viability. In addition, the carboxylic acid groups present on the functionalized diamond surface were coupled to the active protein layer in order to support neuronal cell growth. Finally, by controlling the gradient of polyethylene glycol on the surface, a cell gradient had also been successfully generated on the diamond surface, and such a cellular gradient could potentially provide an experimental platform for combinatorial discovery and analysis.

Experimental Section

Chemicals. All of the chemicals used in this experiment were purchased from Sigma Aldrich unless otherwise stated and were used as received. A Polyethylene Glycol (PEG) derivative (molecular weight = 5197) possessing an amine end group, referred to as m-PEG-NH_2, was purchased from NOF Corporation.

Sample Preparation. The submicron grain size, 5 μm thick microcrystalline diamond was grown on silicon (100) substrate at a substrate temperature of 750 °C using hot filament chemical vapor deposition (conditions: 2 sccm CH_4, 200 H_2. 20 Torr for 7 h). A total of 2.5 μm of ultrananocrystalline (UNCD) diamond was deposited on p-doped Si(001) substrates which have been pretreated in a diamond powder/isopropanol slurry with ultrasonic technique. The deposition was performed by a modified hot filament process (patented ρ-BeSt process) with a CH_4 concentration of 2.91% in hydrogen. No additional gases have been used. The substrate temperature as measured by a thermocouple (type K, Chromel/Alumel) was about 780 °C. Diamond samples were cleaned and chemically oxidized with hot "piranha" solution (30% H_2O_2 : 97% H_2SO_4 = 1 : 3) for 1 h, followed by rinsing with ultrapure water. The samples were then rinsed with tetrahydrofuran followed by hexane. Cleaned diamond samples were hydrogen-terminated by hydrogen plasma treatment at 800 W in the microwave plasma CVD system under 300 sccm hydrogen gas flow for 15 min. All freshly prepared hydrogen-terminated diamond samples (denoted as H-terminated hereafter) were used immediately for surface treatment and cell culture.

UV Oxygenation. Hydrogen-plasma-treated diamond samples were exposed to UV irradiation (18 W, 254 nm) in air for 18 h (hereafter denoted as UV-treated diamond) in air.

UV Photochemical Grafting. UV photochemical grafting of the carboxylic acid functional group was achieved by covering the H-terminated diamond samples with a thin layer of undecylenic acid (UA) and introducing the sample into a chamber maintained in positive nitrogen pressure. Samples were exposed to the UV irradiation (18 W, 254 nm) for 18 h through an UV transparent quartz window. After UV functionalization, the samples (hereafter known as UA-functionalized diamond) were rinsed with ultrapure water, tetrahydrofuran, and finally with hexane. All prepared samples were used within 24 h.

X-ray Photoelectron Spectroscopy. X-ray photoelectron spectroscopy (XPS) was preformed with VG ESCALAB MkII spectrometer using an unmonochromatized Mg Kα X-ray source (1253.6 eV). The pass energy of the hemispherical analyzer was set at 50 eV for wide scan and 20 eV for narrow scan.

Morphology and Topography. Surface morphologies of microcrystalline and UNCD diamond were observed by JEOL 6701 FESEM (Field Emission Scanning Electron Microscope) and the surface topographies were investigated by Atomic Force Microscope XE-100 from PSIA.

Wettability Behavior. Contact angle measurements were performed with Rame-Hart Contact Angle Goniometer. A total of 3 μL of ultrapure water was placed on the prepared samples, and three measurements were taken for each sample.

Surface Carboxylic Acid Group Measurement. The UA-functionalized diamond samples (1 cm^2) were soaked in 5×10^{-4} M toluidine blue O (TBO) solution, adjusted to pH 10 with NaOH. Formation of ionic complexes between the surface carboxylic acid groups and the cationic dye was allowed to proceed for 5 h at room temperature, followed by rinsing the samples with NaOH solution to remove the uncomplexed TBO molecules. Desorption of the dye was performed in 50 wt % acetic acid solution, and its amount was calculated from its optical density at 633 nm, using a calibrated plot.

Cell Culture. Two cell lines were used in this work, namely normal human dermal fibroblast (NHDF) cells (PromoCell GmbH, Germany) and pheochromocytoma (PC12) cells. NHDF cells were routinely cultured at 37 °C and 5% CO_2 atmosphere with complete fibroblast growth medium (PromoCell GmbH, Germany) containing insulin (5 μg/mL), basic fibroblast factor (1 ng/mL), penicillin (1000 IU/mL), and streptomycin (1000 μg/mL). PC12 cells were routinely cultured on a collagen coated flask with RPMI1640 medium supplemented with fetal calf serum (10%), fetal horse serum (5%), penicillin (1000 IU/mL), and streptomycin (1000 IU/mL). NHDF cells or PC12 cells were seeded at 5×10^4 cells/mL onto 24-well tissue culture plates, containing 1 cm^2 of sample surfaces in each well. For cell seeding onto the PEG-modified gradient surface, the initial cell density at 1.5×10^5 cells/mL was used, and the sample with dimension of 2 cm × 2 cm was used. Cells were allowed to attach for 24 h, and the loosely attached cells were removed by rinsing with PBS 3 times.

The fibroblast cell is an anchorage-dependent cell line which is very useful in biocompatibility studies as it will contact with biomaterials upon implantation. Furthermore, it does not require stringent culture conditions and will proliferate readily into the confluence state on biocompatible material. PC12 cell is a tumorigenic cell line which will differentiate into sympathetic neurons upon exposure to the nerve growth factor (NGF). It is a very useful cell line for neurotransmitters study as sizable amount of neurotransmitter such as dopamine and norepinephrine can be stored in this cell line. However, it requires an extracellular matrix (ECM) such as collagen or laminin coating for the attachment and differentiation. This requirement is used in this work to assay the coupling efficiency of protein on functionalized diamond surface.

Attachment of Cell to AFM Cantilever. Concanavalin A-functionalized (Con A) cantilevers (spring constant of 0.01 N/m, Veeco) were prepared by cleaning tips in acetone for 5 min and then UV irradiated for 15 min followed by incubation in biotinamidocaproyl-labeled bovine serum albumin (0.5 mg/mL in 100 mM $NaHCO_3$) overnight at 37 °C. They were then incubated in streptavidin solution (0.5 mg/mL in PBS) for 10 min at room temperature after thorough rinsing with PBS. Following the removal of unbound streptavidin, the cantilevers were incubated in biotinylated Con A (1 mg/mL in PBS) for 1 h. The Con A-coated cantilever was finally rinsed with PBS three times before the AFM force measurements.

AFM Force Measurements. Force curves of cell−surface interactions were obtained on a MultiMode Picoforce AFM (Veeco) at room temperature, in a fluid cell with medium, using the functionalized cantilevers. The spring constant of the cantilever was obtained using the thermal tune module prior to attaching a cell. A small amount of cell suspension obtained by trypsinization was added on to the diamond surface being studied. The Con A-functionalized cantilever was positioned on top of a single NHDF cell and lowered gently. The contact was maintained for a few seconds to allow the NHDF cell to attach to the cantilever. The cantilever with the attached cell was then used to obtain force curves. Each force curve represents a single cycle of approach and retraction of the cell-cantilever system with respect to the surface. All force curves were obtained under similar interaction parameters (contact force of 3 nN, contact time of 200 ms and retraction velocity of 2 μms^{-1}), and a total of 150 force measurements were done for each samples.

Hoechst Stain Assay. The level of NHDF cell attachment was estimated by the total DNA content with Hoechst stain H33258.[15] Cell-attached samples were incubated in 0.02% sodium dodecyl sulfate/saline sodium citrate (SDS/SSC) for 1 h for cell membrane lysis, and they were subsequently stained with H33258 (1ug/mL in SSC) for 15 min to allow the binding of H33258 with DNA. Finally, the fluorescence intensity was assessed using an excitation wavelength of 355 nm and a detection wavelength of 460 nm. The total DNA content was calculated from the standard curve of known DNA concentrations.

MTT-ESTA Assay. The NHDF cells viability was determined by established MTT-ESTA assay.[16] First, the cell-attached samples were incubated in 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide (MTT) (0.3 mg/mL in PBS) for 2 h. Following cell lysis by SDS, the purple stain was eluted with acidified isopropanol, and the optical density was measured at 595 nm. The degree of optical density reflects the total cell viability.

Statistical Analysis. All results are tested with Student's *t*-test to identify the significant difference between results. All the results are presented as means ± standard deviation.

Live/Dead Cytotoxicity Kit. By using Live/Dead Cytotoxicity Kit from Molecular Probes, viable and nonviable cells can be recognized simultaneously without digesting the cells from the samples surfaces. Both viable cells (fluorescein filter) and nonviable cells (rhodamine filter) were visualized by fluorescent microscope (Olympus BX60) according to the standard protocol from Molecular Probe.

Protein Immobilization. The carboxylic acid group of the UA-functionalized diamond was activated to form an NHS-ester intermediate on the diamond surface by incubating with a solution containing EDC (0.4 M) and NHS (0.1 M) for 1 h. After rinsing with PBS, it was incubated with laminin solution (0.1 mg/mL in PBS) for 1 h. To eliminate the unspecific binding of protein, the sample was vortexed in Tween-20 solution (0.5% in PBS) for 30 min. After final rinsing with PBS, the sample was used immediately for neuron cell growth experiments.

Gradient Formation. A poly(ethylene glycol) (PEG) surface gradient was achieved by controlling the diffusion of PEG in the polymer gel.[17] Surface carboxylic acid group of diamond was activated similarly as protein immobilization step and after thorough rinsing with PBS solution, the NHS-ester modified surface was coated with a layer of 3% agarose gel. A small syringe containing *m*-PEG-NH_2 (4 mg/mL) solution was pierced into center of the gel. The syringe was connected to a syringe pump to deliver the PEG solution into the gel at a constant rate of 10 μL/hr for a period of 8 h. After peeling the gel from surface, the PEG-modified surface was rinsed with PBS followed by Tris (50 mM) to saturate the unreacted carboxylic acid group. The PEG-modified surface was used immediately after preparation for cell attachment experiment.

Results and Discussion

Surface Characterization. XPS wide scan spectra confirm that the H-terminated diamond consisted only of carbon and an insignificant amount of oxygen (Supporting Information, Figure S1). In contrast, both the UV-treated and UA-functionalized diamonds show appreciable O1s peak intensities at 533 eV, in addition to the C1s peak at 285 eV. The C1s narrow scans confirmed the presence of various functional groups by their respective chemical shifts (Supporting Information, Figure S2). H-terminated diamond shows only the bulk diamond peak at 284.5 eV, with a narrow full-width-at-half-maximum (FWHM). For UV-treated diamond, the FWHM is much wider, with a broad shoulder extending to 287.5 eV, which can be attributed to the presence of oxygen functionalities related to hydroxyl (OH) or carbonyl (C═O) groups. This proves that the UV irradiation of diamond in air resulted in the oxygenation of the hydrogenated diamond surface, possibly via attack by ozone or hydroxyl radicals generated from moisture. For UA-functionalized diamond, a chemically shifted peak at 289.6 eV due to the presence of carboxylic groups (COOH) can be observed.

The diamond surfaces became more hydrophilic after irradiation with UV or functionalization by UA, as evidenced by the wetting angle studies shown in Table 1. UV-treated diamond surfaces are more hydrophilic than the UA-functionalized diamonds, which can be attributed to the higher oxygen content on the UV-treated surfaces. The TBO method was employed to determine the amount of carboxylic acid group on the UA-functionalized diamond surfaces, as shown in Table 1. The UNCD diamond surface provides a higher surface area for the grafting of UA molecules compared to the microcrystalline diamond. The surface morphology of microcrystalline diamond is shown in Figure 1a, where a submicron grain size of between 100 and 300 nm can be seen. Figure 1b shows that UNCD has a finer grain size in the range of 5−10 nm. The three-dimensional surface topography map was also obtained using AFM (Supporting Information, Figure S3). It can be seen that UNCD has a finer grain size and smaller surface corrugation, which potentially can allow a larger surface contact area with the cells.

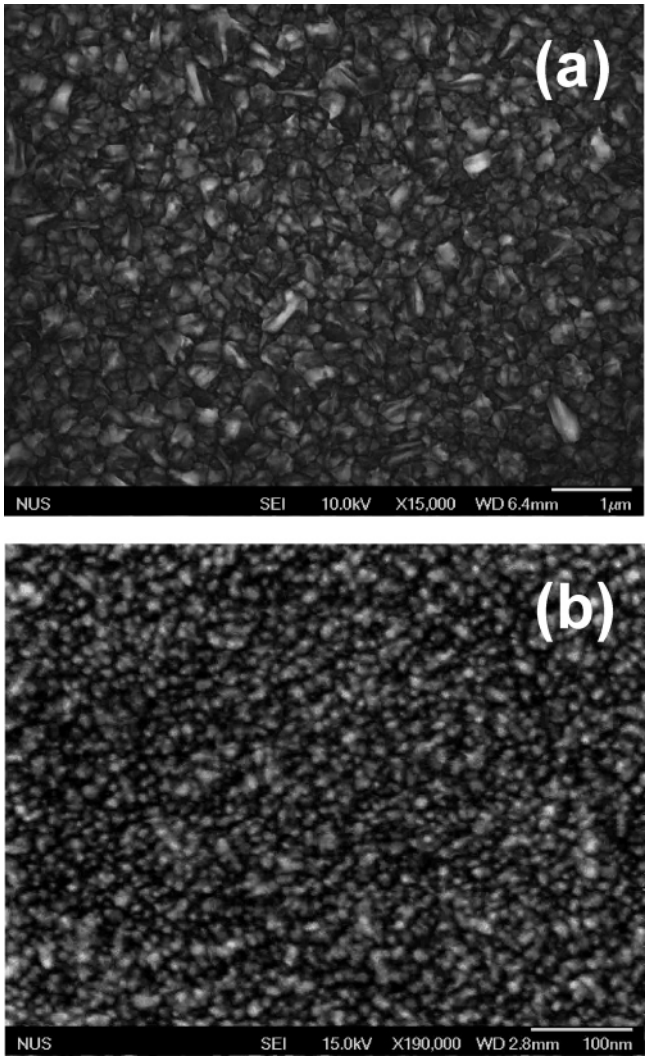

Figure 1. SEM micrographs showing the morphology of (a) microcrystalline diamond and (b) ultananocrystalline (UNCD) diamond.

Table 1. Wetting Angle of Water on Different Diamond Samples and Density of the Surface Carboxylic Acid Groups Determined by the TBO Method

diamond sample		wetting angle θ (°)	COOH density (molecules/cm^2)
H-terminated	micro	90.9 (± 1.0)	
	nano	88.7 (± 0.9)	
UV-treated	micro	69.3 (± 1.5)	
	nano	66.5 (± 1.5)	
undecylenic acid	micro	71.0 (± 0.9)	4.8 (± 0.5) $\times 10^{14}$
	nano	77.7 (± 1.1)	6.7 (± 0.5) $\times$ 10^{14}

(16) Ealey, P. A.; Yaterman, M. E.; Holt, S. J.; Marshall, N. J. *J. Mol. Endocrinol.* **1988**, *1*, 1.

(17) Liedberg, B.; Tengvall, P. *Langmuir* **1995**, *11*, 3821.

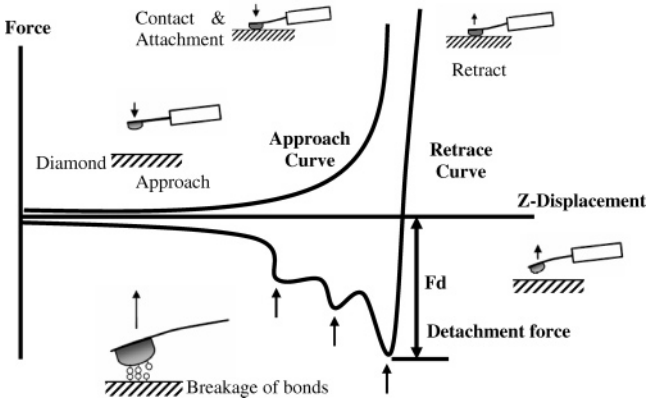

Figure 2. Schematic showing typical approach-and-retraction force curve.

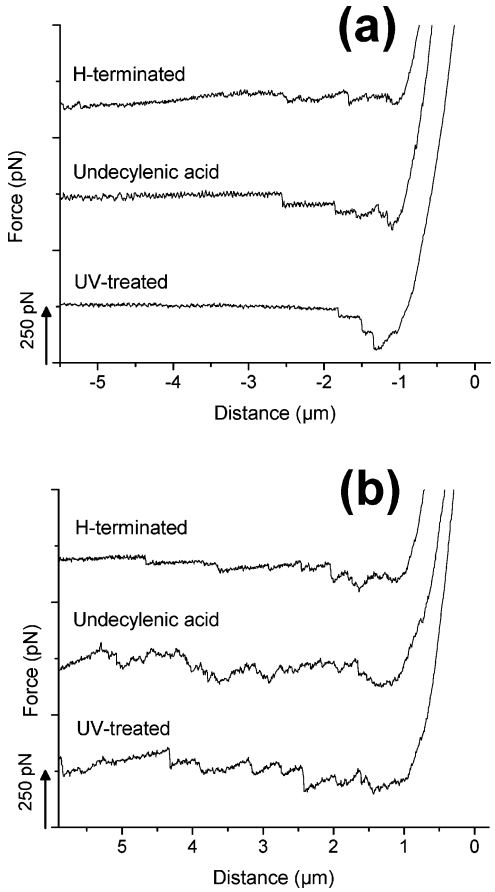

Figure 3. Force curves between a NHDF cell and (a) microcrystalline and (b) ultrananocrystalline diamond with different modifications.

Cell Adhesion Forces. Atomic force microscopy (AFM) in force mode is used to quantify the initial cell adhesion forces on different diamond surfaces. This is schematically illustrated in Figure 2. A single NHDF cell was first attached to a Con A-functionalized cantilever, and the cell−cantilever system was lowered on to the sample surface until a predefined contact force (3 nN) is established between the cell and the surface. After a contact time of 200 ms, the cantilever was retracted from the surface at a constant velocity of 2 μms^{-1} until they were completely separated. On the retraction process, the cantilever will be pulled downward if there are adhesive interactions established between cell and sample surface. Any adhesive interactions formed between the cell and the surface will rupture during the retraction process. The sequential rupture of one or more bonds is observed as sharp jumps in the retraction curve

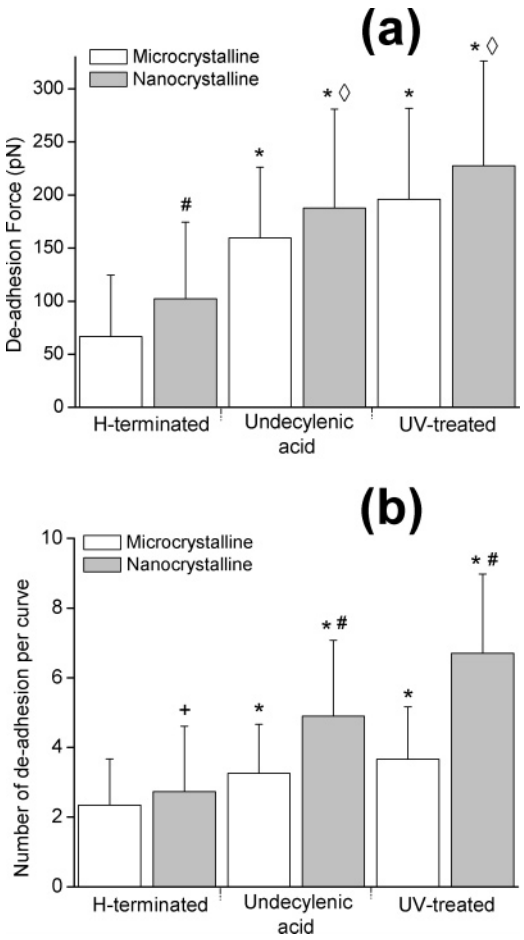

Figure 4. (a) De-adhesion forces and (b) number of de-adhesion events per curve between the NHDF cell and different diamond samples. (In the calculation of the de-adhesion event, peak transition higher than 40 pN with reference to the noise level is calculated as 1 de-adhesion event.) Data are presented as mean ± standard deviation of 150 experiments. Differences within samples were tested with Student's *t*-test: *, $P < 0.001$ compared with the respective H-terminated samples (microcrystalline or UNCD); #, $P < 0.001$; ◇, $P < 0.01$; +, $P < 0.05$ compared with the microcrystalline diamond samples under same surface treatment (H-termination, undecylenic acid functionalization or UV treatment).

and each of these sharp jumps (indicated by arrows under force curve) is considered as de-adhesion event. The maximal de-adhesion force is represented by the maximum (negative) peak in the retraction curve (Fd, Figure 2), which provides a quantitative measurement of cell adhesion force to the surface. The number of de-adhesion events per curve provides an indication of the total number of bonds breakages.

Figure 3 presents the representative force curves between a single NHDF cell and the diamond surfaces with different surface treatment. The "sawtooth" profile in the force curves indicate that multiple bond rupturing processes are involved in the retraction. It is noteworthy that additional de-adhesion forces are needed for complete cellular detachment on UV-treated diamond and UA-functionalized diamond compared with H-terminated diamond. For UNCD, the cell undergoes a multiple de-adhesion event which stretches for a few micrometers before the final separation from the surface (Figure 3b). In the case of microcrystalline diamond surfaces, the cell undergoes a smaller number of de-adhesion events and became detached from the surface at a shorter distance (Figure 3a).

A total of 150 force curves for each sample were analyzed in order to arrive at a statistical average. The average value of

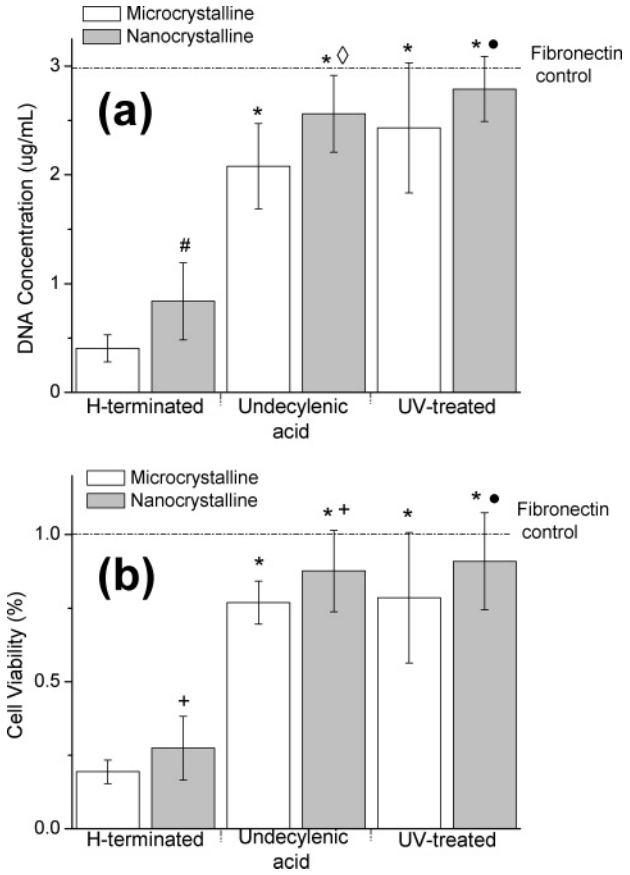

Figure 5. Level of NHDF cells attachment on different diamond samples was estimated from (a) total DNA concentration of cells; (b) cell viability. Data are presented as means ± standard deviation of 12 samples. Differences within samples were tested with Student's *t*-test: *, $P < 0.001$ compared with the respective H-terminated samples (microcrystalline or UNCD); #, $P < 0.001$; ◇, $P < 0.01$; +, $P < 0.05$ compared with the microcrystalline diamond samples under same surface treatment (H-termination, undecylenic acid functionalization); ●, $P > 0.05$ shows there is no significant difference between microcrystalline and UNCD samples with UV treatment.

maximum de-adhesion forces and the number of de-adhesion events per curve are summarized in Figure 4. For both microcrystalline and UNCD, the maximum de-adhesion forces were strongest on UV-treated surfaces, followed by UA-functionalized, and these forces were weakest on freshly prepared H-terminated surfaces (Figure 4a). On both types of diamond surfaces, UA functionalization increased the maximum cell de-adhesion forces by about 2-fold, and surface treatment with UV irradiation increased the maximum cell de-adhesion forces by about 2.5-fold, compared to that of the freshly prepared H-terminated surface. Under similar experimental conditions, the maximum de-adhesion forces for all UNCD samples were observed to be higher than those of microcrystalline diamond samples by about 30 pN. UNCD samples also had a higher number of de-adhesion events per curve compared to the microcrystalline diamond samples (Figure 3b). Cell adhesion was observed to be strongest on the UV-treated UNCD (227.60 ± 98.68 pN); on average about 7 bonds were formed during the adhesion event. In contrast, cell adhesion was weakest on H-terminated microcrystalline diamond (66.53 ± 58.01 pN), and on average, about two bonds were formed during the adhesion event. The maximum detachment force can be benchmarked against the forces measured on the fibronectin-coated diamond surface. In this case, the adhesion force is mediated by specific binding between the cell membrane protein and fibronectin, and it was determined to be

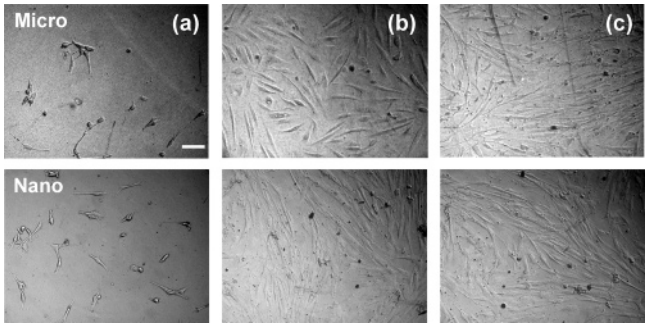

Figure 6. Representative optical micrographs (scale bar 150 μm) of NHDF cells after 24 h culture on (a) H-terminated, (b) undecylenic acid-functionalized, and (c) UV-treated diamond surfaces (top row, microcrystalline; bottom row, UNCD).

about 551 pN. Therefore, the cell adhesion force mediated by nonspecific interactions on UV-treated UNCD is about 50% of the specific adhesion forces between fibronectin and the same cell.

Cell adhesion on all diamond samples is mediated by the electrostatic interactions or the hydrogen bond formation between cell membrane proteins and diamond surfaces. The low de-adhesion forces on H-terminated diamond can be attributed to the hydrophobic characteristic of the surface. Surfaces which have been UV- or UA-treated have a high density of carbonyl or carboxylic acid groups which afford strong electrostatic interactions as well as hydrogen bonding with the cell membrane proteins. The topography of UNCD in this case provides a higher surface area for the functional groups to interact with the cell membrane, albeit nonspecifically. The fundamental basis of the biocompatibility of UNCD is due to its smaller grain size and higher surface area, which renders it intrinsically more reactive than microcrystalline diamond toward moisture and oxygen, especially when photochemically activated by light. The UNCD may also have a high density of defect states in the gap region which renders charge transfer with oxidizing species in the ambient to be more efficient, and as a result, the surface becomes consistently hydrophilic.

Cell Growth. To correlate the initial cell adhesion forces to the subsequent cell growth, cell culture experiments were carried out on microcrystalline and UNCD samples which have been subjected to the same series of surface modifications. NHDF cells were allowed to attach to the diamond samples and grow for 24 h, with a fibronectin-coated cover slip as control. After washing with PBS to remove the loosely attached cells, the NHDF cells were quantified by DNA assay and MTT-ESTA assay, and the results are summarized as Figure 5.

As shown in Figure 5a, the highest cell density could be found on the UV-treated surface, followed by the UA-functionalized surface. The lowest cell number was found on the freshly prepared H-terminated surface. For microcrystalline diamond samples, UA functionalization and UV treatment increased the cell density by about 5- and 6-fold, respectively, compared to the freshly prepared H-terminated surface. However, the surface treatment using either UV irradiation or UA on UNCD increased the cell density by only 3-fold because the original H-terminated UNCD surface already had a cell density which was 2-fold higher than the H-terminated microcrystalline diamond surface. Therefore, intrinsically, UNCD is more biocompatible than other diamond surfaces.

Figure 5b shows the cell viability studies where the percentages (with respect to the fibronectin-coated surface) of live cells were assayed on different diamond samples. A cell viability of 75%

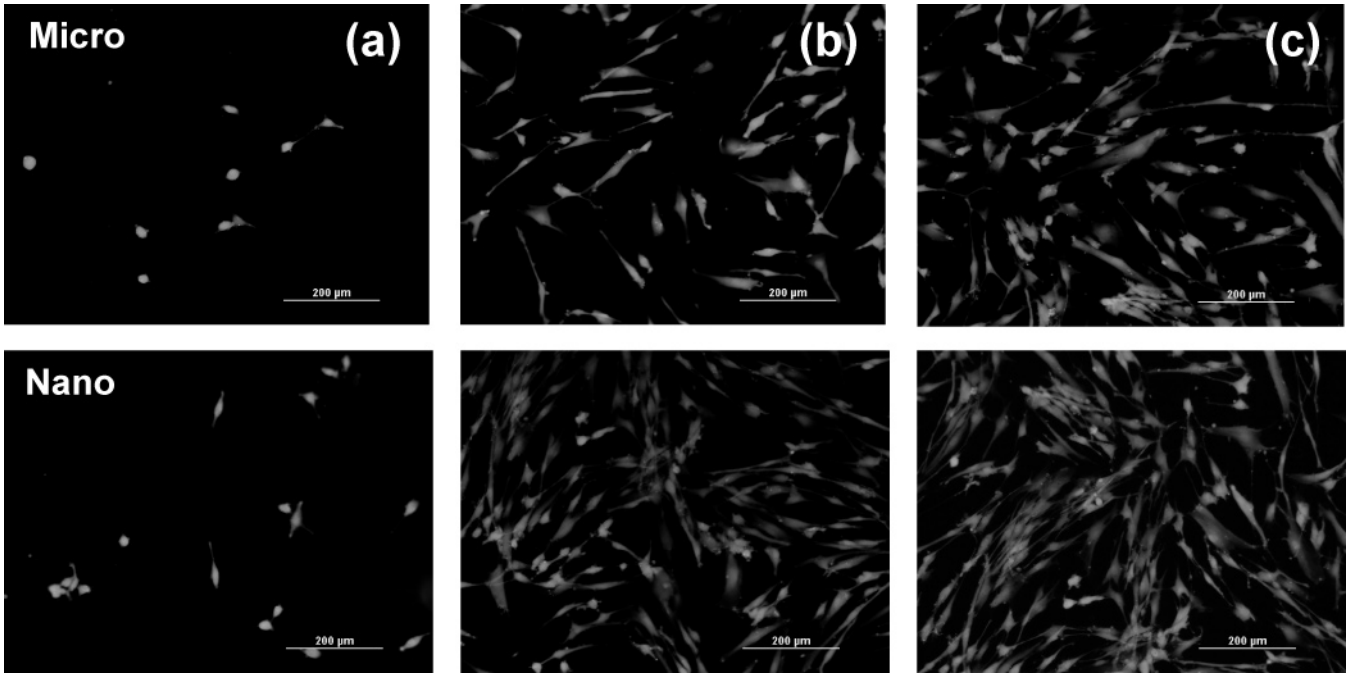

Figure 7. Fluorescence micrographs showing NHDF cells attachment on (a) H-terminated, (b) UA-functionalized, and (c) UV-treated diamond surfaces (top row, microcrystalline; bottom row, UNCD). The green fluorescence indicates that the cells have intact cell membranes and none of the surfaces are cytotoxic.

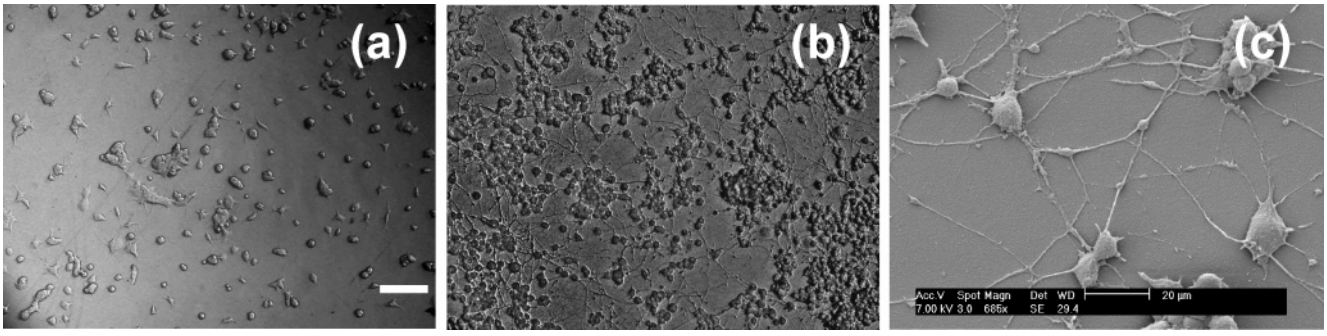

Figure 8. Morphologies of PC12 cells on laminin−UA-functionalized diamond surface after (a) 12 h culture in the absence of NGF; (b) 72 h culture in the presence of NGF (scale bare 150 μm), and (c) SEM showing neurites extensions from PC12 cells after 72 h culture in the presence of NGF.

or more could be observed on UV-treated or UA-functionalized diamond surfaces, whereas only 30% cell viability was observed on H-terminated diamond. Detailed cell morphology can be seen in representative optical micrographs, as shown in Figure 6. After surface treatment with UA or UV, NHDF cells spread and developed a spindle-like morphology on the diamond surfaces. This is very similar to the cell morphology on the fibronectin-coated cover slip, implying good cell attachment and healthy cell growth. However, NHDF cells exhibited round morphology on the H-terminated diamond surface, an indication of poor cell attachment due to the hydrophobic surface. A Live/Dead Cytotoxicity Kit from Molecular Probes was used to investigate the cytotoxicity effect of H-terminated and diamond surfaces after surface treatment. Representative fluorescence micrographs are shown in Figure 7, where green fluorescence could be observed from live cells with intact membranes and dead cells with damaged membranes were expected to show red fluorescence. It is clear that all of the cells attached to the diamond surfaces have intact membranes and that UV-treated diamond showed the highest viable cell count.

Protein Immobilization. In order to explore the possibility of the diamond surface as a platform for future neuronal cell studies, a protein layer was covalently bond to the diamond surface to support the neuronal cell growth. The surface carboxylic acid group of the UA-functionalized diamond was activated by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) to form a stable NHS-ester intermediate, and the latter reacts readily with the amine group from laminin to form amide bond. The laminin-coated platform favored the attachment of PC12 cells (Figure 8a) because these cells showed affinity only on surfaces coated with the extracellular matrix protein coating. To eliminate the possibility of nonspecific binding of protein on a diamond surface, the surface was rinsed copiously with Tween-20. A control experiment involving the incubation of H-terminated diamond with laminin showed no sign of PC12 cells attachment (Supporting Information, Figure S4). After attachment of PC12 cells onto the laminin−UA-diamond surface, their ability to differentiate into sympathetic neuron cells was tested by adding the nerve growth factor (NGF). It was shown that the PC12 cells were active to differentiate into neuron cells on the laminin−UA-diamond surface because numerous neurites extended from the cell body after nerve growth factor (NGF) was added (Figure 8, panels b and c). The ability of functionalized diamond to support the neuronal cells growth will enable it to be a useful platform for neurobiology and neurochemistry studies.

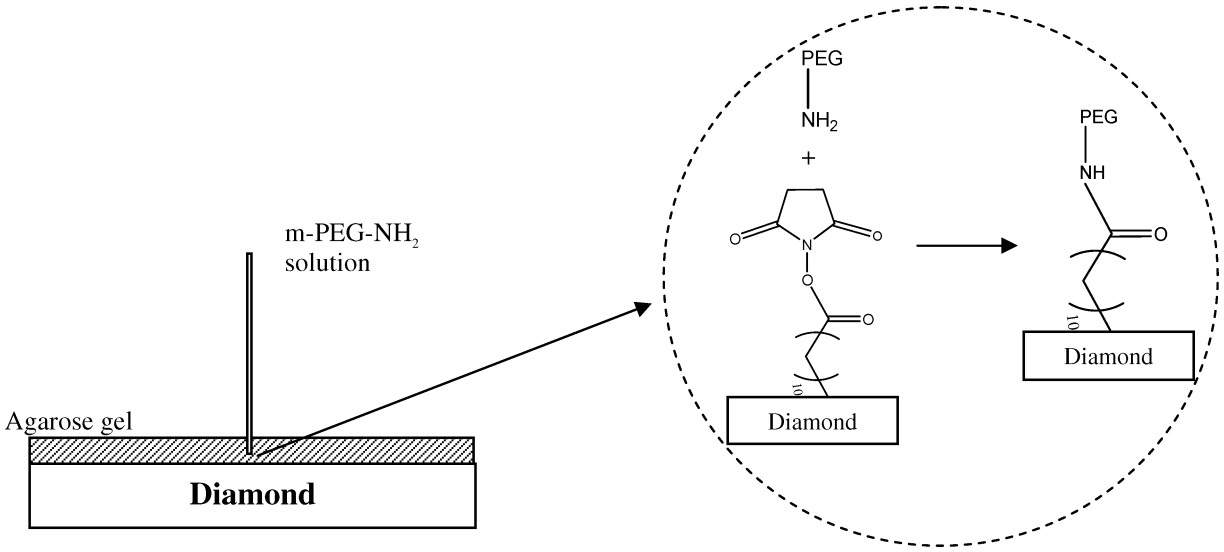

Figure 9. Schematic showing the construction of PEG surface gradient by gel diffusion method.

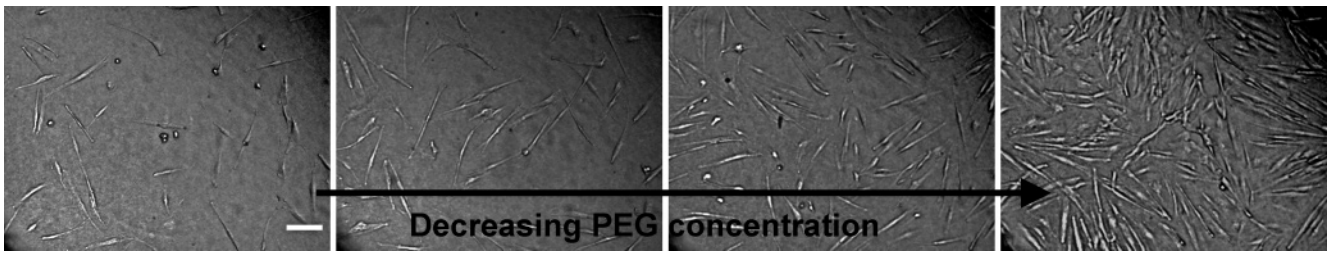

Figure 10. Micrographs showing attachment of NHDF cells on surface gradient of PEG, from left to right at 0, 4, 8, and 12 mm from PEG point source, respectively (white scale bar: 150 μm).

Cell Gradient Formation. The surface carboxylic acid group of UA-functionalized diamond was activated as described above to form a stable NHS-ester intermediate. This intermediate reacted readily with the amine group from m-PEG-NH_2. A syringe containing m-PEG-NH_2 served as a point source to deliver active PEG through the agarose gel, creating a diffusion gradient of PEG within the gel, as shown by the schematic in Figure 9. The region directly beneath the point source will have a higher concentration of PEG, and this concentration is decreasing with distance from the point source. PEG is well-known for its ability to reduce nonspecific adsorption of proteins and cells. The surface gradient of PEG coverage on diamond was subsequently used for cell gradient formation. NHDF cells at 1.5×10^5 cells/mL were seeded on to the PEG-gradient diamond surface and allowed to attach for 24 h. A series of optical micrographs in Figure 10 show the attachment of NHDF cells at different positions along the PEG gradient. Due to the greater cell inhibition at the regions of higher PEG concentration, a cell gradient was established following the diffusion profile of PEG, and the cell density was higher at the position farther away from the point source. Therefore, the controlled diffusion of PEG on UA-functionalized diamond allows us to establish a cell gradient on diamond, which can serve as an experimental platform for investigating combinatorial cellular chemistry.

Conclusions

The adhesion characteristics of NHDF cells on diamond surfaces with different surface topography and chemistry have been analyzed quantitatively using biochemical assays and atomic force microscope. UV irradiation of diamond surfaces in air was found to be effective for surface oxygenation, and such oxygenated surfaces are hydrophilic and provide a better platform for initial cell adhesion and subsequent cell growth compared to H-terminated surfaces. Photochemical coupling of undecylenic acid (UA) onto diamond surfaces also imparts equivalent biocompatible properties, although our study shows that UV irradiation provides a more convenient and effective route for promoting cell attachment and cell growth. The maximum detachment force (227 pN) of a NHDF cell from UV-treated UNCD was measured to be about 50% that of the specific binding force (551 pN) between fibronectin and the cell membrane and about 3.5 times larger than the detachment force on hydrogenated diamond (66 pN). Therefore, the term "biocompatibility" of the diamond surface refers to oxygenated diamond surfaces. It was also verified that UNCD exhibits better performance than microcrystalline diamond in terms of cell attachment and cell growth. UA-treated diamond surfaces however are amenable to further functionalization with biomolecules. By utilizing the surface carboxylic acid group of UA-functionalized diamond, an active protein layer supporting neuronal cells growth can be constructed on the diamond surface. Finally, we demonstrated the formation of a cell gradient by establishing a diffusion gradient of polyethylene glycol on UA-functionalized diamond.

Acknowledgment. K.P.L. and co-workers thank the funding support of NUS academic Grant R-143-000-275-112.

Supporting Information Available: XPS wide scan (Figure S1) and XPS narrow scan (Figure S2) spectra of H-terminated, undecylenic acid-functionalized and UV-treated diamond, AFM image of monocrystalline and UNCD (Figure S3), and optical micrograph of control experiment for PC12 cell growth on H-terminated diamond (Figure S4). This information is available free of charge via the Internet at http://pubs.acs.org.

LA070037Y