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Biocompatible Graphene Nanosheets Grafted with Poly (2-Hydroxyethyl Methacrylate) Brushes via Surface-Initiated ARGET ATRP

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Experimental

Materials. Milli-Q water was generated with a Millipore Simplicity 185 system. Flake graphite (Cat# 32461), sulfuric acid (ACS reagent, 95-89%), potassium permanganate (puriss, >99.0%), hydrogen peroxide solution (50wt.% in H_2O, stabilized), hydrazine (anhydrous, 98%), phosphorus pentoxide (>99.0%), potassium persulfate (ACS reagent, >99.0%), copper(II) bromide (99.999% trace metals basis), N,N,N', N'',N''-pentamethyldiethylenetriamine (PMDETA, 99%), tin(II) 2-ethylhexanoate (> 92.5%), ascorbic acid (reagent grade, crystalline), 2-hydroxyethyl methacrylate (97%), 2-carboxyethyl acrylate (abbr. CA, 99%), poly (2-hydroxyethyl methacrylate) (BioReagent, powder), methanol (for HPLC ≥99%), ethylenediaminetetraacetic acid (EDTA, anhydrous, crystalline, BioReagent), triethylamine (abbr. Et_3N, ⩾99%), 2-(4-aminophenyl)ethanol (98%), isoamyl nitrite (98%), α-bromoisobutyryl bromide (abbr. BiBB, 98%), N,N-dimethylformamide (abbr. DMF, anhydrous, 99.8%), ethanol (abbr. EtOH, 200 proof, anhydrous, >99.5%), and anisole (anhydrous, 99.7%) were purchased from Sigma-Aldrich (Milwaukee, WI). Dulbecco's Phosphate-Buffered Saline (PBS) was purchased from Life Technologies. HEMA and CA monomers were purified with basic Al_2O_3 columns to remove the inhibitor. All other materials and reagents that do not have a source denotation were purchased from Fisher and used as received.

Graphene Oxide Nanosheets (GO) Preparation and Purification. Graphite oxide was prepared from natural flake graphite powder by a modified Hummer's method with a pre-oxidization procedure[1]. The graphite powder (20 g) was put into an 80°C concentrated solution of H_2SO_4 (30 mL), $K_2S_2O_8$ (10 g), and P_2O_5 (10 g). The resultant dark blue mixture was thermally isolated and allowed to cool to room temperature over a period of 6 h. The mixture was then carefully diluted with Milli-Q water, filtered, and washed on the filter until the rinse water pH became neutral. The product was dried in air at ambient temperature overnight. This pre-oxidized graphite was then subjected to oxidation by modified Hummers' method. The oxidized graphite powder (20 g) was put into cold (0°C) concentrated H_2SO_4 (460 mL). $KMnO_4$ (60 g) was added gradually with stirring and cooling so that the temperature of the mixture was not allowed to reach 5°C. The mixture was stirred at 35°C for 2 h, followed by Milli-Q water (920 mL) addition and hydrolysis at 90°C for 45 min. The reaction was terminated by adding Milli-Q water (2.8 L) and 30% H_2O_2 solution (50 mL), leading to a color change to bright yellow. The mixture was then filtered and washed with 1:10 HCl solution (5 L) to remove metal ions. The muddy product was re-suspended in Milli-Q water to give a viscous, brown, 2% dispersion, and it was subjected to dialysis for 48 h to completely remove metal ions and acids. After a freeze-drying procedure, the resulting brown GO powder was stored in a vacuum desiccator.

Hydroxyl Functionalized Graphene Nanosheets (G-OH). GO was converted to G-OH by following the method[2]. GO (1 g) was dispersed in Milli-Q water (400 mL) in a three-necked round bottom flask with stirring and sonication. The suspended GO was reduced with 10 mL hydrazine hydrate at 100°C for 4 h. Then 2-(4-aminophenyl) ethanol (4 g) and isoamyl nitrite (3 mL) were added into the flask. The mixture was vigorously stirred at 80°C for 12 h and filtered through a 0.45 μm PVDF membrane. The filter cake was washed with Milli-Q water, absolute ethanol, and DMF repeatedly until the filtrate was colorless. The black solid obtained was dried under vacuum at 60°C for 24 h to give the final product.

Initiator Functionalized Graphene Nanosheets (G-Initiator). G-OH (200 mg) was dispersed in anhydrous DMF (40 mL) through sonication. After an addition of trimethylamine (2 mL), the suspension was cooled to 0°C in an ice bath. α-bromoisobutyryl bromide (2.46ml) in 10 mL anhydrous DMF was added dropwise into the flask under vigorous stirring. The mixture was stirred at room temperature for 24 h under a nitrogen atmosphere. The solid was separated by filtration through a 0.45 μm PVDF membrane, and washed with an excessive amount of Milli-Q water to remove the salts formed during the reaction. The crude product was then dispersed in a small amount of DMF, filtered, and then thoroughly washed with DMF and acetone. The obtained filter cake was dried under vacuum at 60°C for 48 h.

Graphene Nanosheets Grafted with Poly(HEMA) Brushes (G-HEMA). G-Initiator (200 mg, about 0.59 mmol of initiator groups) was firstly dispersed in DMF (20 ml) by sonication. The suspension was transferred into a 100 ml Schlenk flask containing $CuBr_2$ (2.7 mg, 0.012 mmol). After adding PMDETA (7.6 μL,0.036 mmol) and 2-hydroxyethyl methacrylate (7.01 g, 60 mmol) in turn, the mixture was stirred and degassed under nitrogen flow for 3 h. Then the sealed flake was placed in an oil bath at 30°C under constant stirring. The ARGET ATRP reaction was trigged by adding $Sn(EH)_2$ (388.2 μL, 1.2 mmol) or ascorbic acid (103.8 mg, 0.6

mmol). After 16 h, the polymerization was stopped by dilution with 5 mM EDTA DMF solution, and the product was separated by filtration through a 0.45 μm PVDF membrane, then thoroughly washed with DMF to remove any unattached polymer or unreacted monomer. The filter cake was redispersed in DMF by sonication, and dialyzed in Milli-Q water for 48 h to remove the Cu/PMDETA complex and unreacted monomer, followed by another filtration through a 0.45 μm PVDF membrane. The purified product was obtained after drying the filter cake under vacuum at 60°C for 8 h. G-$HEMA_T$ and G-$HEMA_A$ were applied to denote the samples prepared with $Sn(EH)_2$ and ascorbic acid as reducing agents.

Graphene Nanosheets Grafted with Poly(HEMA-CA) Brushes (G-HECA). Random copolymer poly (2-hydroxyethyl methacrylate)-(2-carboxyethyl acrylate) (poly(HEMA-CA)) brushes grafted graphene nanosheets (G-HECA) was achieved following the protocol described above, except for the addition of 2-carboxyethyl acrylate (CA) as comonomer. Different reaction system compositions result in different carboxyl group contents in the copolymer brushes, as shown in Figure1. When m/n=4, it is denoted as G-$HECA_1$; when m/n=2, it is denoted as G-$HECA_2$.

Scaffold Substrates Preparation. Typical spray coating protocol was applied to make thin graphene film on coverslips as scaffold substrates for cell viability assays. The different graphene substrates were sonicated individually in 20 mL of 1:1 v/v DMF/EtOH solution to obtain saturated suspensions. The suspension was sprayed with an air brush gun (Image®) onto 10 × 10 mm square glass coverslips (cut from Fisher brand microscope cover glass slides 24 × 50 #1 with a thickness of about 110 μm), which had been previously heated up to 160°C to allow the fabrication of uniform films. For each coverslip, 4 ml suspension was spray coated with another 20 min baking treatment on the hotplate (120°C). The coated coverslips were allowed to dry under N_2 flow and then used for cell culture after a sterilization of absolute ethanol immersion for 3h. Then the sterilized coverslips were transferred into 24-well tissue culture-treated polystyrene plates pre-coated with 0.1% poly(HEMA) to prevent cell attachment. Three additional phosphate-buffered saline (PBS) rinses were conducted before further use.

Protein Absorption Assay. The protein absorption assay was carried out on the spray-coated coverslips for 6 and 12 h in PBS containing 10% FBS (WiCell). The coverslips were transferred into a 12-well plate with a 0.1% poly(HEMA) pre-coating, followed by sterilization in absolute ethanol for 1 h. Then, three PBS rinses of at least 1 h each were performed to remove any impurities from the substrate's surface. 1.5 ml of PBS solution containing 10% FBS was added to the wells. After an incubation of the desired time at 37°C, three PBS rinses were conducted to remove any loose proteins that had not adhered to the samples. Then, a QuantiPro bicinchoninic acid (BCA) protein assay kit was used as instructed by the manufacturer (Sigma-Aldrich). After 30 min of incubation at 37°C, an absorbance at 560 nm was measured in a GloMax-Multi + Multiplate Reader (Promega), and a subsequent protein concentration was obtained relative to a predetermined standard curve.

Cell Culture and Seeding. Swiss mouse NIH-3T3 ECACC (European Collection of Cell Cultures) fibroblasts and human umbilical vein endothelial cells (HUVECs) were used for the biocompatibility and cell viability assays. NIH-3T3 cells were cultured in a high-glucose 20% serum medium consisting of high-glucose DMEM (Gibco), 20% FBS (WiCell), 2 mM L-glutamine (Invitrogen), and penicillin–streptomycin (Invitrogen), and passaged at a 1:40 ratio every 6 days via 5-minute EDTA treatment (Life Technologies). Maintained cultures were regularly checked for mycoplasma. Prior to seeding, NIH-3T3 cells were treated with EDTA for 5 min and washed with PBS. Then the cells were seeded at a density of 2×102 cells/cm2 in the high-glucose medium described above. Spent medium was aspirated and replaced with 1 mL of fresh medium daily. Human umbilical vein endothelial cells (HUVECs) were maintained in EGM-2 supplemented with growth factors as instructed by the manufacture (Lonza). HUVECs were seeded onto the scaffold substrates at a density of 1×104 cells/cm2, and the medium was changed every other day.

Cell Viability Assay. The growth of NIH-3T3 cells and HUVECs was examined by using the Live/Dead Viability/Cytotoxicity assay kit (Molecular Probes Life Technologies). NIH-3T3 fibroblast cell viability was determined at day 1 and day 3 after seeding. HUVEC viability was determined at day1, day 3, and day 5 after seeding. The assay kit was used as instructed by the manufacture (Life Technologies). The stained cells were fixed using a 4% paraformaldehyde PBS solution for 15 min, followed with three times PBS rinse. Fluorescent images were obtained using a Nikon A1R confocal microscope.

MTS Assay of NIH-3T3 Cells. CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used to determine the number of cells during the NIH-3T3 cell culture experiment. This assay utilizes the MTS tetrazolium compound that is bioreduced by cells into a colored formazan product. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. Standard curves were established by performing the tests on cells seeded on the cell culture wells and confirmed by comparison to hemocytometer readings prior to these experiments. Upon testing, 80 µl CellTiter 96 Aqueous One Solution reagent was pipetted into each well of the plate containing the samples in 400 µl of culture media. After incubation at 37°C for 1 hour, 100 µL of spent media was removed and added to a clear 96-well plate. The absorbance of this plate at the 450 nm wavelength was read with a GloMax-Multi + Multiplate Reader (Promega) and the subsequent cell number was determined relative to the negative control. A positive control was not necessary according to the protocol.

Image Analysis. The measurement of cell shape index (CSI) was carried out using freeware ImageJ software (NIH, http://rsb.info.nih.gov/ij/). The raw image was converted into an 8-bit file and treated with the unsharp mask feature (settings of 1:0.2) before removing the image background (rolling ball radius of 5). After smoothing, the resulting image, which appears similar to the original photomicrograph but with minimal background, was converted into a binary image by setting a threshold. The threshold values were determined empirically by selecting a setting that gave the most accurate binary image. The cell shape index (CSI) was calculated by "Analyze Particles" in ImageJ. A minimum of 200 cells per condition were analyzed.[3]

Static Analysis. Data were expressed as mean ± standard deviation and analyzed by a one-tailed Student's t-test. Statistical significance was determined with p values less than 0.05, which was specified each time.

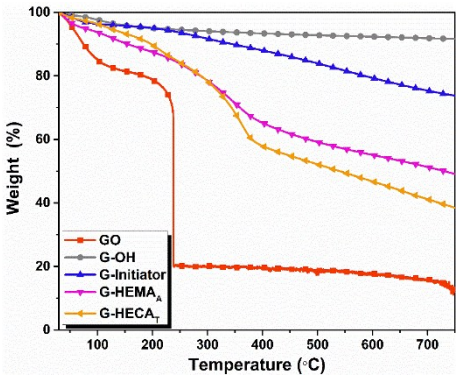

Figure S1. TGA thermograms of GO, G-OH, G-Initiator, G-HEMAA and G-HECAT.

Thermogravimetric analyses (TGA) were conducted to assess the relative composition of polymer brushes on the graphene nanosheets. As shown in Figure S1, the exfoliated GO has two obvious weight loss stages: the first stage occurs at about 20% below 180°C, while the second stage is rapid at about 60% between 200°C to 250°C. Both weight loss stages is attributed to the volatilization of absorbed water and the decomposition of oxygen-containing groups. The thermal stability of G-OH improves significantly, whose weight loss at 550°C is about 10%. The G-Initiator exhibits a continuous weight loss until 400°C due to the cleavage of the initiator moiety, followed by further weight loss upto about 35%. Similar decomposition patterns are observed on G-HECA$_A$ and G-HECA$_T$, whose thermal stability shows a reasonable decrease (about 40% weight loss at 400°C) attributing to the thermal decomposition of the poly(HEMA) brush chains. In comparison, the weight loss observed on G-HECA$_T$ around 400°C is much higher than that observed on G-HECA$_A$, which can be attributes to the higher grafting density of poly(HEMA) brush on the graphene nanosheets.

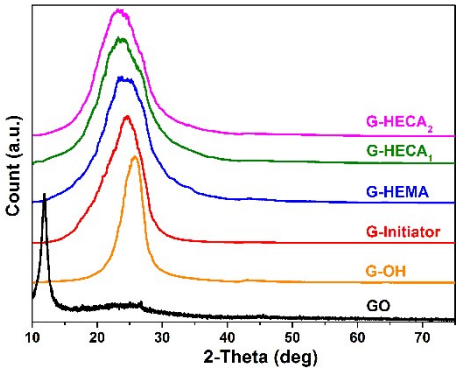

Figure S2. XRD curves of GO, G-OH, G-Initiator, G-HEMA, G-HECA1 and G-HECA2.

Figure S2 provides the X-ray diffraction patterns of GO, G-OH, G-Initiator, G-HEMA, G-HECA1 and G-HECA2. For GO, the oxidization reaction and existence of oxygen-containing groups on the graphene surface cause an interlayer distance increase corresponding to the strong diffraction peak at 11.9°. G-OH shows a diffraction peak at 25.8°, resulting from the hydrazine reduction removal of oxygen-containing groups. The covalent grafted initiator moiety and poly(HEMA) or poly(HEMA-b-CA) brushes facilitate the exfoliation of reduced graphene nanosheets. The polymerization starts from the initiator moieties and pushes the graphene nanosheets apart. However, there is no remarkable pattern difference among the G-HEMA, G-$HECA_1$ and G-$HECA_2$ samples. Broad peaks around 23.6° appearance indicates the existence to some extent of aggregation.

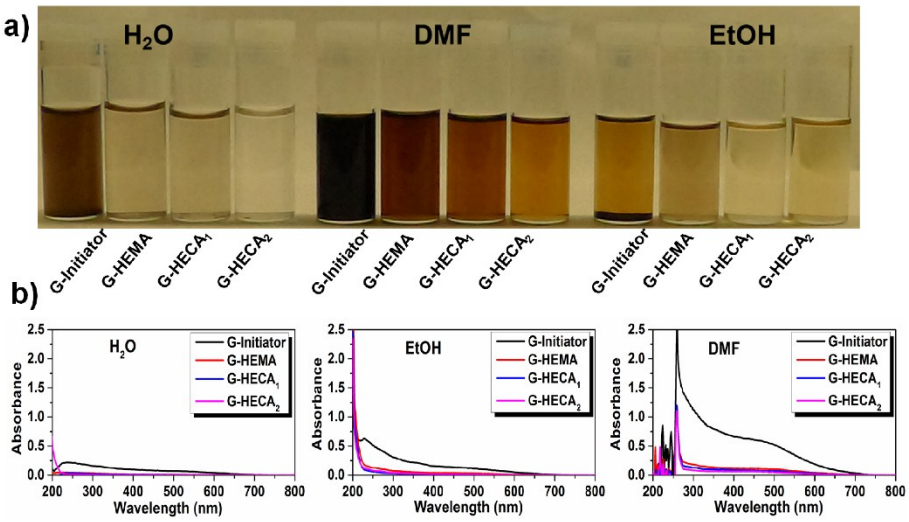

Figure S3. (a) Photographs and (b) UV-visible absorption spectra of G-HEMA, G-HECA1 and G-HECA2 suspended in EtOH, DMF, and H2O at saturated concentrations.

Grafting poly(HEMA) brushes onto the graphene surface would induce some extent change to the dispersibility of graphene in solvents. The poly(HEMA) is generally regarded hydrophilic, and only can be dissovled in extremely polar solvents, e.g. DMF, DMSO, and HMPA. As shown in Figure S3(a), the dispersibility of the G-Initiator, G-HEMA, G-HECA1 and G-HECA2 was examined in three representative polar solvents: water, DMF, and ethanol. The G-Initiator shows good dispersion in all three solvents due to the improved polarity of attached initiator moiety. However, after two weeks standing and layering, dark sediments are observed in water and ethanol solution except for the deep dark color DMF solution. G-HEMA, G-$HECA_1$ and G-$HECA_2$ present limited dispersibility in water and ethanol, but form stable yellow solution in DMF. The solution dispersibility of the G-Initiator, G-HEMA, G-$HECA_1$ and G-$HECA_2$ was further investigated by the UV-visible absorption spectra (Figure S3(b)). The UV-visible spectrum of graphene normally shows two peaks: a broad peak around 270 nm characteristic of π-π electron transition in the polyaromatic system of graphene layers, and a sharp peak around 230 nm related to π-π electron transition in the polyene-type structure from the defects of the graphene nanosheets5. For G-Initiator, medium peaks around 230 nm are observed in all three solutions, attributed to the covalent bonding of 2-(4-aminophenyl)

ethanol and α-bromoisobutyryl bromide on the graphene surfaces. In contrast, the absorbance of G-HEMA, G-HECA1 and G-$HECA_2$ in water and ethanol solution is so low that closes to zero. Only their DMF solutions show medium peaks around 230 nm and strong peaks at 270 nm. The UV-visible spectra measurement confirms the good dispersity of G-HEMA, G-$HECA_1$ and G-$HECA_2$ in DMF.

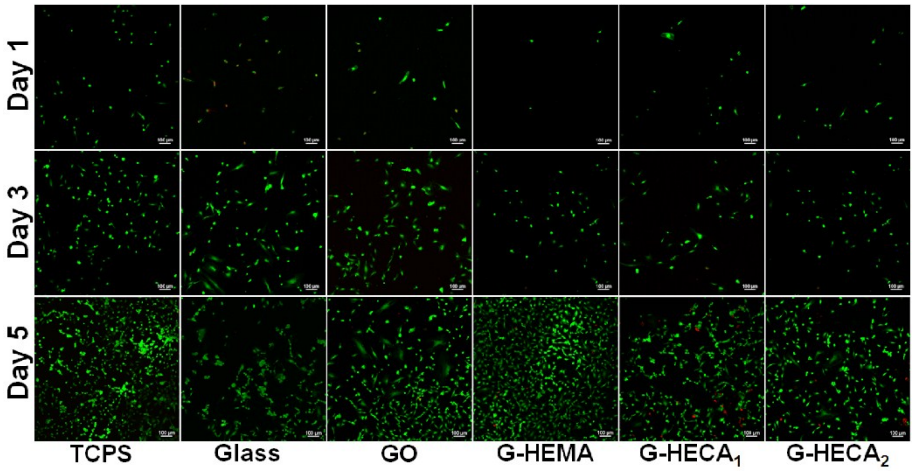

Figure S4. Confocal microscopic observation of Live/Dead stained HUVECs growing on different substrate surfaces at day 1, day 3, and day 5 of incubation (Scale bars are 100 μm).

The viability assay of human umbilical vein endothelial cells (HUVECs) was conducted and the confocal microscopy images are shown in Figure S4. At day 1 and day 3 incubation, the vital cells on G-HEMA, G-HECA1 and G-$HECA_2$ are slightly less than those on the control TCPS, glass, and GO. After 5 days incubation, HUVECs present continuous proliferation on G-HEMA, G-$HECA_1$ and G-$HECA_2$ substrate, and the vital cells on the G-$HECA_1$ and G-$HECA_2$ are less than these on G-HEMA. In general, HUVECs are prone to grow on surfaces with positively charged amine groups6. Hydrophobic surfaces are less favorable for HUVEC adhesion and proliferation7. However, the specific mechanisms that induce HUVEC adhesion and proliferation difference is still not clear. More studies are needed to reveal the influence of graphene substrates on the growth behavior of HUVECs. In the present study, the cell viability assays demonstrate that the obtained graphene nanosheets grafted with poly(HEMA) brushes are biocompatible and support HUVECs attachment and proliferation.

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