Supporting information

Facile and green production of aqueous graphene dispersions for biomedical applications

Samad Ahadian[a], Mehdi Estili[b,*], Velappa Jayaraman Surya[c], Javier Ramón-Azcón[a], Xiaobin Liang[a], Hitoshi Shiku[d], Murugan Ramalingam[a,e], Tomokazu Matsue[a,d], Yoshio Sakka[f], Hojae Bae[g], Ken Nakajima[a], Yoshiyuki Kawazoe[c,h], and Ali Khademhosseini[a,g,i,*]

[a]WPI-Advanced Institute for Materials Research, Tohoku University, Sendai 980-8577, Japan

[b]International Center for Young Scientists (ICYS), National Institute for Materials Science (NIMS), Tsukuba 305-0047, Japan

[c]New Industry Creation Hatchery Center, Tohoku University, Sendai 980-8579, Japan

[d]Graduate School of Environmental Studies, Tohoku University, Sendai 980-8579, Japan

[e]Centre for Stem Cell Research, A unit of the Institute for Stem Cell Biology and Regenerative Medicine, Christian Medical College Campus, Vellore 632002, India; Institut National de la Santé Et de la Recherche Médicale U977, Faculté de Chirurgie Dentaire, Université de Strasbourg, Strasbourg 67085, France

[f]Materials Processing Unit, NIMS, Tsukuba 305-0047, Japan

[g]College of Animal Bioscience and Technology, Department of Bioindustrial Technologies, Konkuk University, Hwayang-dong, Kwangjin-gu, Seoul 143-701, Republic of Korea

[h]Institute of Thermophysics, Siberian Branch of Russian Academy of Sciences, Novosibirsk 630090, Russia

[i]Department of Medicine, Biomaterials Innovation Research Center, Brigham and Women's Hospital, Harvard Medical School, Cambridge, Massachusetts 02139, USA; Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA; Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts 02115, USA; Department of Physics, Faculty of Science, King Abdulaziz University, Jeddah 21569, Saudi Arabia

[*]Corresponding authors: ICYS, NIMS, Tsukuba 305-0047, Japan. Fax: +81 29 859 2200 (M. Estili). Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA. Fax: +1 617 768 8477 (A. Khademhosseini).
E-mail addresses: estili.mehdi@nims.go.jp (M. Estili) and alik@rics.bwh.harvard.edu (A. Khademhosseini).

Table S1. Atomic concentrations of elemental C, N, and O and chemical groups in graphite and graphene measured by XPS.

Sample	Element	Concentration (%)	Chemical group [binding energy (eV), concentration (%)] [1]
Graphite	C	100	C-C [286.00, 100]
	N	0	n.a
	O	0	n.a
Graphene	C	63.3	C-C [285.20, 62.2] C-O [287.10, 23.7] C=O, C-N [288.91, 12.5] C-C=O [290.60, 1.6]
	N	13.8	N-sp^2C [400.80, 94.2] N-O [402.70, 3.4] N-sp^3C [398.91, 2.4]
	O	22.9	C=O [532.60, 92.8] C-O-H [533.90, 4.4] O-N [534.80, 2.8]

Table S2. Binding energies of amino acid moieties on graphene.

Amino acid	Binding energy (meV)	Intermolecular distance (Å)
Ala	-30.00	3.92
Cys	-33.10	2.73
Gly	-46.70	3.09
ILeu	-37.50	3.38
Leu	-54.60	3.08
Met	-40.20	3.55

Phe	-35.70	3.75
Pro	-56.20	3.10
Tryp	-49.00	3.84
Tyr	-41.20	3.5
Val	-29.60	3.25

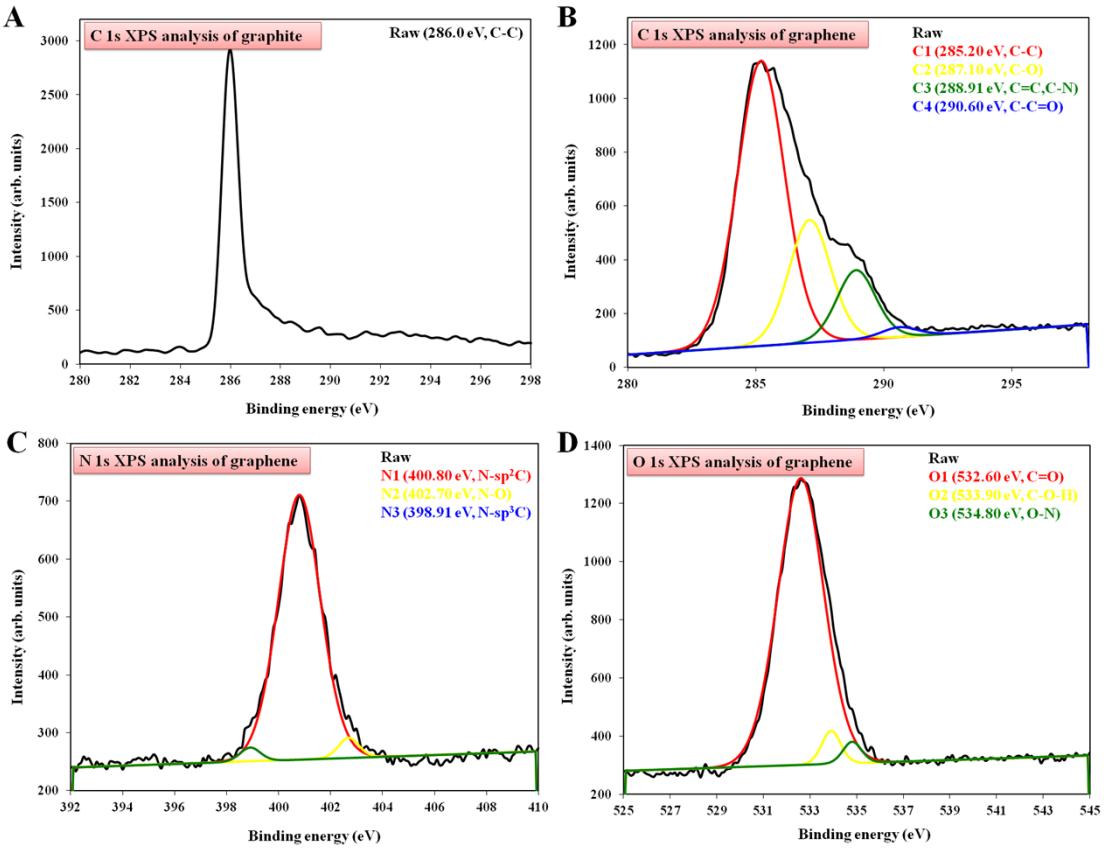

Figure S1. Detailed C 1s, N 1s, and O 1s XPS analyses of graphite and graphene. Atomic concentrations of elemental C (A and B), N (C), and O (D) and chemical groups in graphite and graphene obtained by XPS analysis.

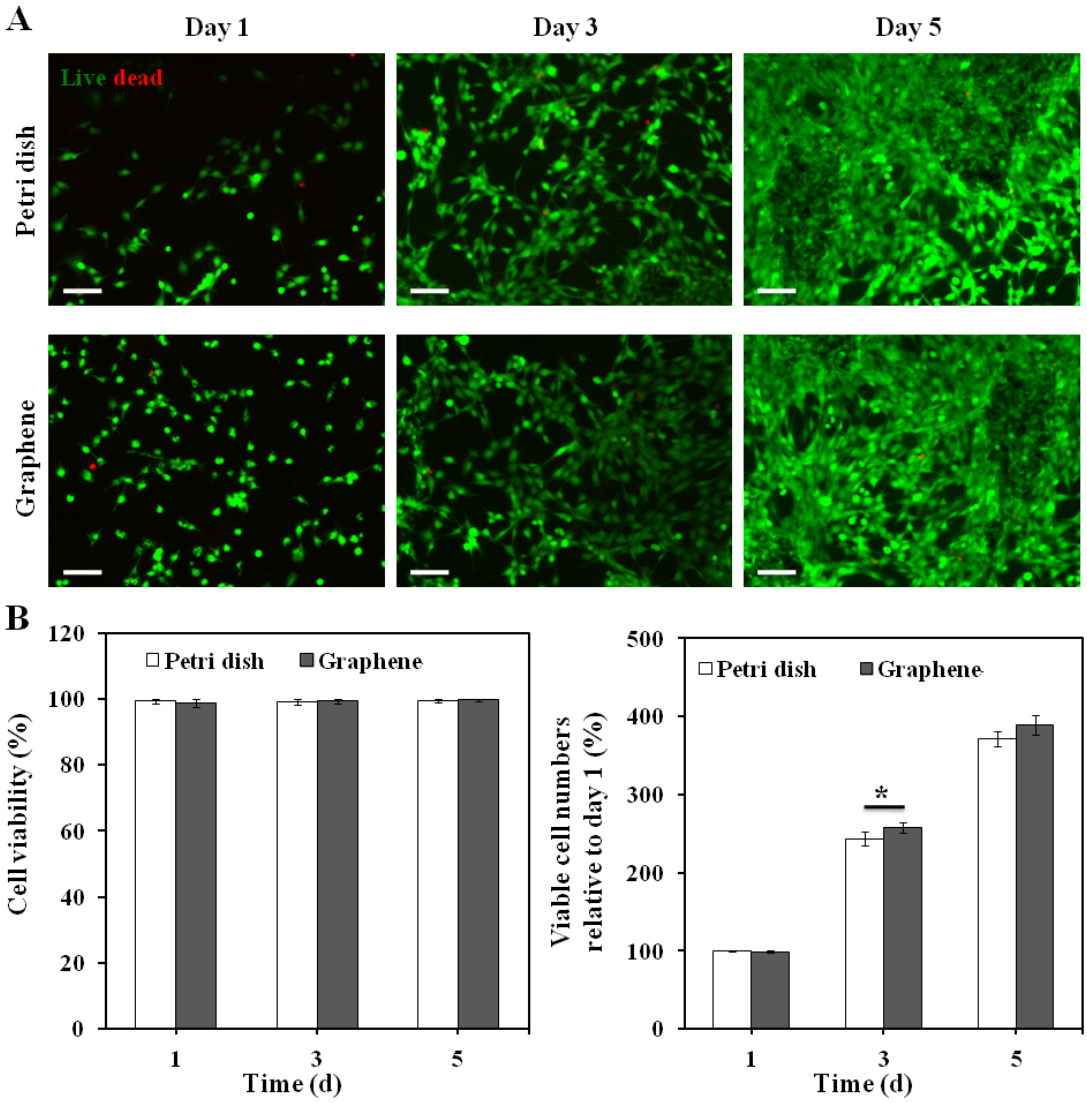

Figure S2. Biocompatibility and proliferation of C2C12 myoblasts on Petri dish and graphene substrates. (A) Quantification of live cells and proliferation rate at different days of culture using live/dead assay. The dead and live cells were labeled as red and green, respectively. (B) There were no significant differences between the substrates with regard to cell viability during the different culture periods, and the cells proliferated well on all of the substrates. Note that the proliferation rate of the cells on graphene was significantly higher than that on the Petri dish substrate at day 3 of culture. The scale bars correspond to 100 μm (*$p < 0.05$).

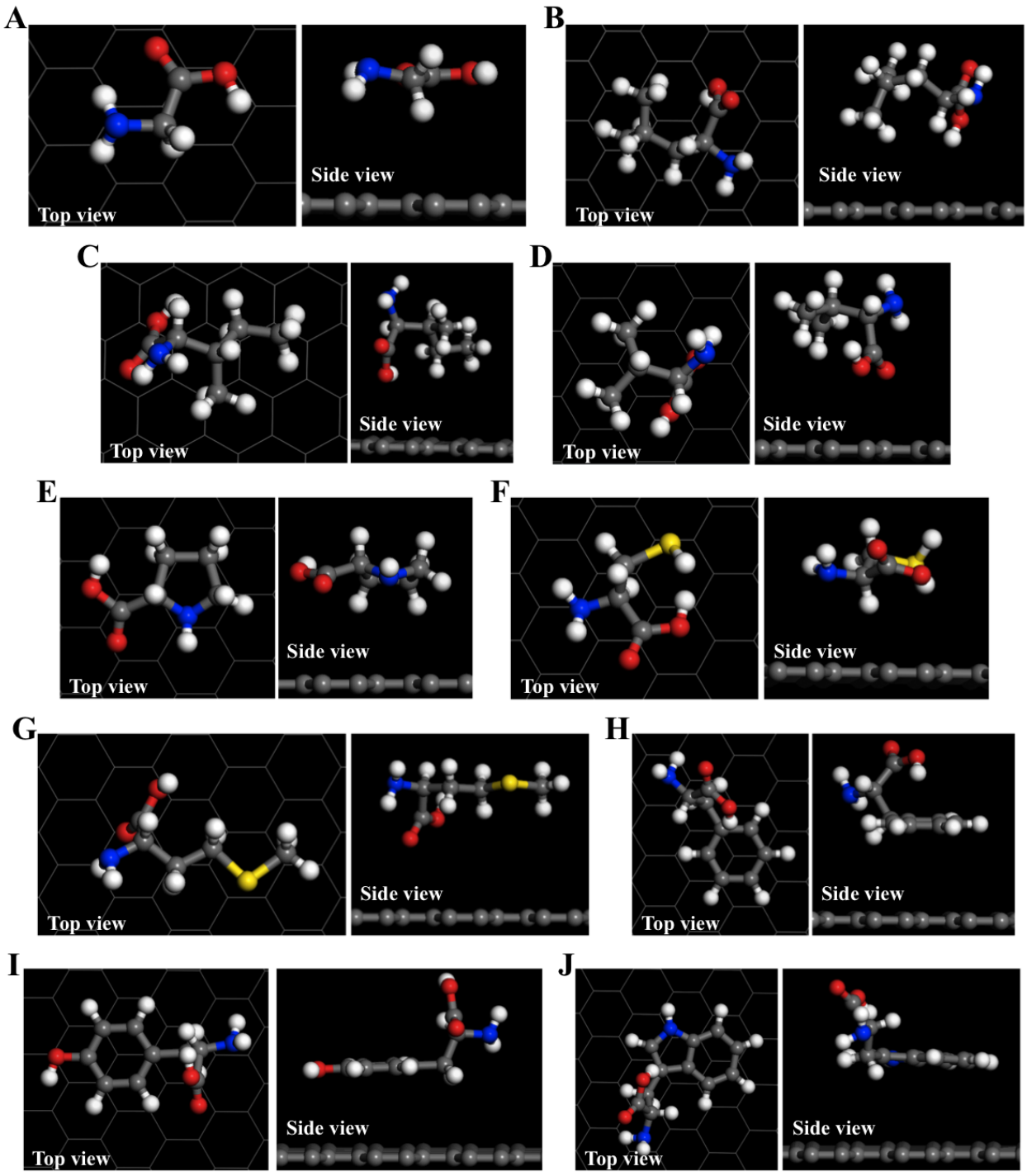

Figure S3. Optimized structures of graphene with Gly (A), Leu (B), ILeu (C), Val (D), Pro (E), Cys (F), Met (G), Phe (H), Tyr (I), and Tryp (J) from top and side views. White, grey, blue, red, and yellow balls represent H, C, N, O, and S atoms, respectively.

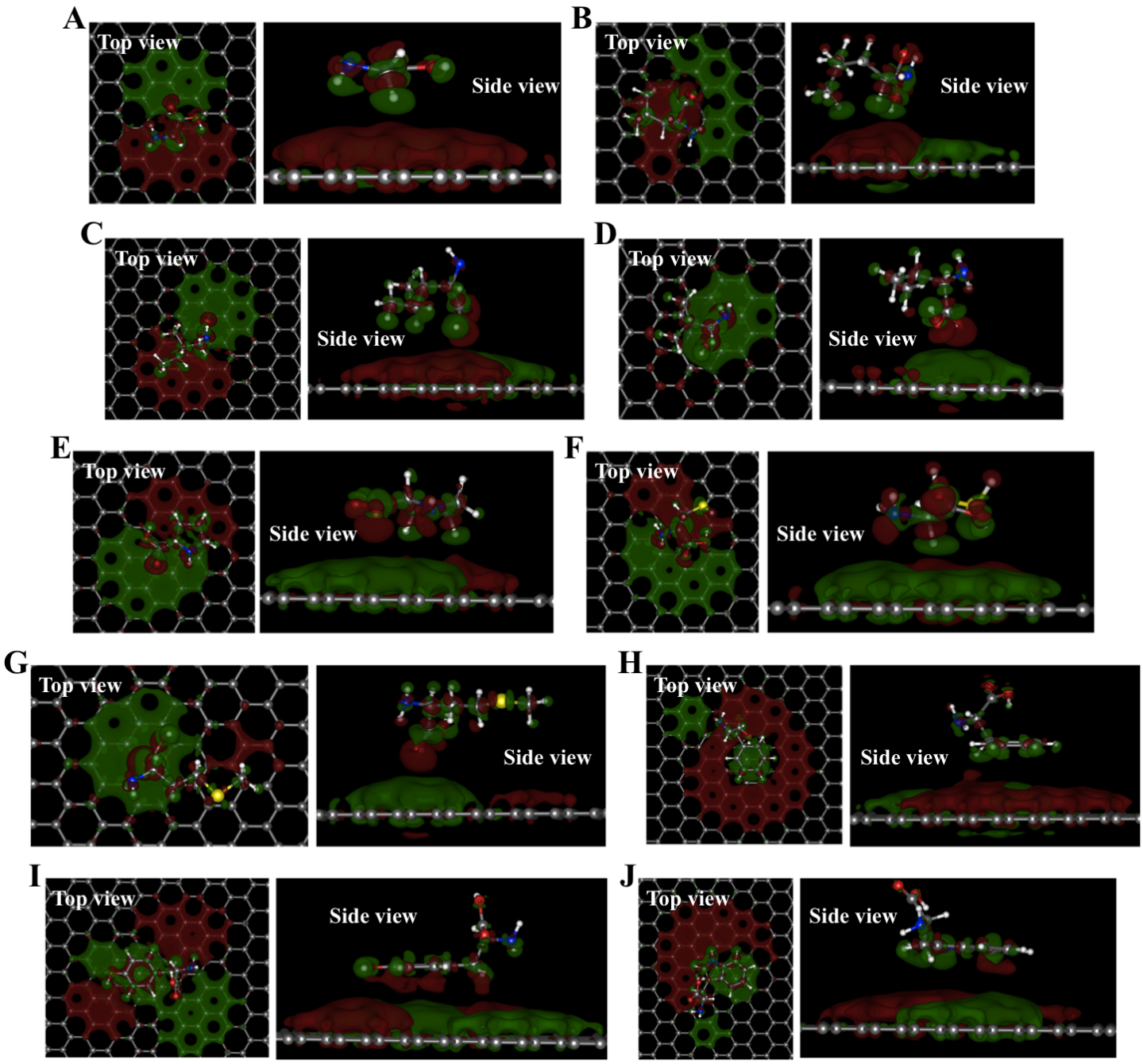

Figure S4. Isosurface charge density plots of graphene with Gly (A), Leu (B), ILeu (C), Val (D), Pro (E), Cys (F), Met (G), Phe (H), Tyr (I), and Tryp (J) from top and side views. White, grey, blue, red, and yellow balls represent H, C, N, O, and S atoms, respectively.

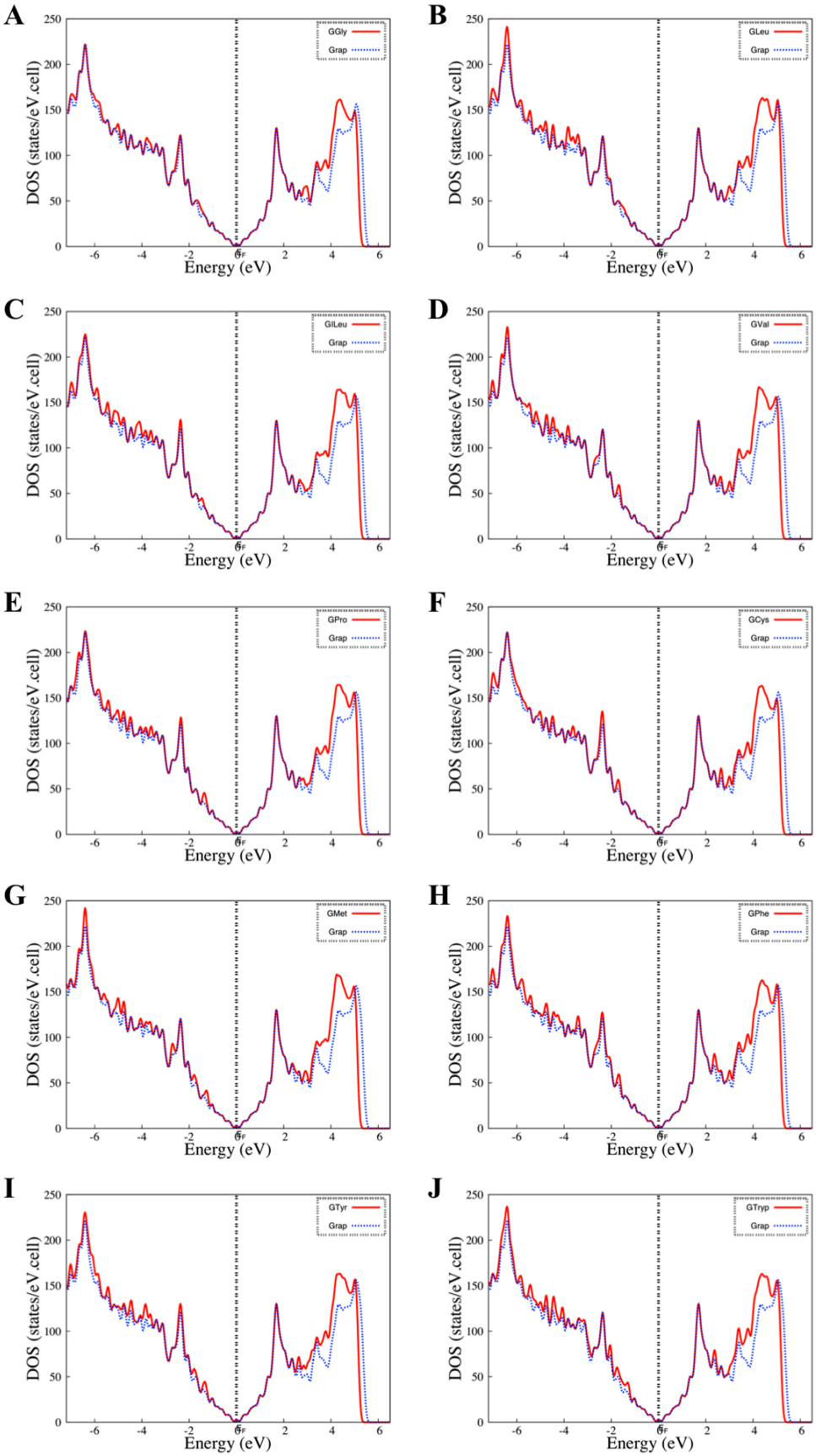

Figure S5. Total DOS plots of graphene with Gly (A), Leu (B), ILeu (C), Val (D), Pro (E), Cys (F), Met (G), Phe (H), Tyr (I), and Tryp (J). Red and dashed blue lines indicate the DOS plots of the graphene-amino acid complex and of the pristine graphene, respectively.

Experimental section

Materials

The materials used in this work and their suppliers are as follows: Graphite (200 mesh, 99.9995% purity) (Alfa Aesar, USA); 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), methacrylic anhydride, penicillin/streptomycin (P/S), gelatin, and BSA (Sigma-Aldrich Chemical Co., USA); fetal bovine serum (FBS) (Bioserum, Japan); 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) (Ciba Chemicals, Osaka, Japan); and trypsin/EDTA, Dulbecco's Modified Eagle's Medium (DMEM), and DPBS (Invitrogen, USA).

Characterization

TEM images were recorded using a TEM (JEM-2100, JEOL, Japan) at 200 kV. For the TEM measurements, a drop of the diluted dispersion was dried in air over a carbon-coated Cu TEM mesh grid. SEM images were recorded using a field emission SEM (FE-SEM) (JSM-6500F, JEOL, Japan) operated at 15 kV. For the SEM observations, a drop of the diluted dispersion was dried in air over a silicone substrate. AFM height images were analyzed using NanoScope Analysis software, version 1.40r1 (Veeco Instruments Inc.). Zeta potential measurements were done using a Zetasizer Nano-Z (Malvern Instruments Ltd., UK). Raman spectra were measured using a micro-Raman spectrometer (Horiba Jobin-Yvon T64000) with a 514.532 nm laser beam. X-ray photoelectron spectra (XPS) were collected using a MultiLab 2000 spectrometer (Thermo Electron Co., USA) employing monochromatic X-ray AlK (15 keV) radiation, and the atomic concentrations were calculated using the Avantage software package. Ultraviolet-visible (UV-Vis) absorption spectra were collected over the range of 220-800 nm using a JASCO V-650 spectrophotometer.

The I-V characteristics of gels were measured at room temperature (Keithley 4200-SCS). Electrochemical impedance spectroscopy (EIS) was performed using a CompactStat potentiostat (CompactStat; Ivium Technologies, Netherlands) and the results were analyzed using IviumSoft software.

Preparation of stable aqueous graphene dispersion

100 mg of BSA powder was mixed with 1800 mL of water at ~50 ºC for ~ 12 h. The pH of the aqueous BSA solution was adjusted to ~ 3.6 with HCl according to the zeta potential measurements. Then, 1 g of graphite powder was dispersed in 200 mL of the aqueous BSA solution. The mixture was then probe-sonicated for 3 h while being mixed with a magnetic stirrer. The sonicated mixture was allowed to stand for ~ 24 h to allow some of the large graphene aggregates and graphite particles to settle prior to separation of the dark supernatant from the sediment. The mixture was centrifuged at 3000 rpm for 30 min. The supernatant was composed of 0.2 mg/mL graphene in water. Adding 400 and 600 mL of the aqueous BSA solution to 1 g of graphite (Ratios of BSA to graphite 22.22 and 33.33 mg/g, respectively) yielded 0.5 and 0.8 mg/mL graphene in water, respectively. The graphene concentration was calculated after drying and weighing a specific volume of graphene dispersion. The average production yield of graphene dispersions was 85%.

Fabrication of graphene substrates

100 µl of a graphene-containing (0.2 mg/mL) aqueous dispersion was spun or drop-casted on a glass slide with a surface area of ~1 cm × 1 cm. The dispersion deposited on the glass slide was left untouched to obtain a dry and homogenous graphene substrate.

GelMA hydrogel synthesis

Adding 12 mL of methacrylic anhydride to 6 g of gelatin in DPBS at 50°C for 1 hr resulted in ~80% methacrylation of the gelatin. A dialysis membrane (12-14 kDa) was used to dialyze the mixture against distilled water at 40°C for one week. The product was then lyophilized for one week. 10% (w/v) GelMA hydrogel and 1% (w/v) Irgacure 2959 were dissolved in distilled water at 60°C.

Fabrication of GelMA and GelMA-graphene gels

Plasma oxygen treatment was done for a glass slide followed by the TMSPMA treatment for 1 hr under vacuum. A chamber was fabricated between two glass slides (one untreated and one treated with the plasma oxygen and TMSPMA) using two spacers (thickness 35 μm). A 10% GelMA prepolymer was mixed with graphene and the mixture was sonicated for 15 min. 20 μL of the hydrogel mixture (pristine GelMA or hybrid GelMA-graphene hydrogels) was pipetted between the glass slides to fill the chamber volume (8 mm × 12 mm × 0.035 mm). The GelMA prepolymer was then crosslinked using a UV light (Hayashi UL-410UV-1; Hayashi Electronic Shenzen Co., Ltd., Japan) for 300 s. The untreated glass slide was then removed and the uniform hydrogel was obtained on another glass slide.

Mechanical characterizations of GelMA and GelMA-graphene hydrogels

The AFM technique, described elsewhere [2], was employed to investigate the stiffness of GelMA and GelMA-graphene hydrogels. A MultiMode 8 AFM (Bruker Co., USA) having a colloidal probe with a radius of 1.0 μm (PT.GS, Novascan Technologies, USA) was used to quantify the Young's moduli (~ kPa) of underlying hydrogels. The

force-distance curves were analyzed using the Derjaguin-Muller-Toporov (DMT) theory [3] to map the Young's moduli on the surface of the hydrogels. The frequency of the indentation tests was 5 Hz.

Cell culture

C2C12 cells were cultured in DMEM involving 1% P/S and 10% FBS. 0.25% trypsin/0.1% EDTA was used to trypsinize muscle cells at ~80% confluence.

Analysis of cell viability and proliferation on Petri dish and graphene

For cell culture on Petri dish and graphene substrates, muscle cells were trypsinized and resuspended in the culture medium. 100 μL of the cell suspension was poured on the substrates at a cell density of 2×10^4 cells/cm^2 and was kept in incubator for 20 min for the cell attachment. After the cell seeding, the cell culture dishes were kept in the incubator upon adding a sufficient culture medium. Live cells on both substrates were detected using a live/dead assay (Invitrogen, USA). The NIH ImageJ software package was used to quantify the cell viability from at least five 10x magnified images from two replicate experiments. The proliferation rate on each day of culture was the ratio of live cells on that day compared with that on day 1 of culture.

DFT calculations

The first principles calculations were performed using the DFT method in the Vienna *ab initio* simulation package (VASP) [4]. The generalized gradient approximation (GGA) was used with Perdew-Burke-Ernzernof (PBE) functional [5]. The projector augmented wave (PAW) potentials were employed for the core part and valence electrons. The Materials Studio's visualization tool was used to create the initial

structures of graphene and amino acids. A super cell with the lattice parameters of a = 34.08 Å, b = 29.51 Å, and a = b = c = 90° including a slab of graphene with 384 carbon atoms was used. A distance (22 Å) was considered between two neighboring graphene slabs along the z-axis to neglect their interactions. The gamma-centered 2 × 2 × 1 k-point grid was employed for the structure optimization. The individual amino acids were taken in a large cubic cell with a 16 Å vacuum spacing, and a single gamma point was used for the relaxation. The geometric structures were optimized using the conjugate gradient algorithm. The criterion for energy convergence was defined as 1×10^{-4} eV. The criterion for force convergence on each atom was defined as 0.01 eV/Å. The DOS analysis was performed using a 4 × 4 × 1 k-point grid and a 0.1 eV smearing width. The charge transfer analysis was conducted using the Bader charge analysis method [6]. The charge densities were plotted using the VESTA program [7]. The charge density difference between graphene and amino acids (Δρ) was calculated using the following formula:

$$\Delta\rho = \rho_{graphene+amino\ acid} - \rho_{graphene} - \rho_{amino\ acid},$$

where $\rho_{graphene+amino\ acid}$, $\rho_{graphene}$, and $\rho_{amino\ acid}$ represent the charge densities of graphene+amino acid, graphene, and the amino acids, respectively. The binding energy (E_B) of amino acids on graphene was obtained by the following formula:

$$E_B(amino\ acid) = E_T(graphene+amino\ acid) - E_T(graphene) - E_T(amino\ acid),$$

where E_T represents the total energy of the respective systems.

Statistical analysis

Significant differences between 2 groups of data were calculated using an independent Student's *t*-test. All repeated data were shown as the mean ± standard deviation, and p-values<0.05 were deemed significant.

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