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Electronic Supplementary Information

Injectable and mechanically robust 4-arm PPO-PEO/graphene oxide composite hydrogels for biomedical applications

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Materials and Methods

Materials

4-arm polypropylene oxide (PPO)−polyethylene oxide (PEO) (molecular weight = 18,000 g/mol; Tetronic 1307) was supplied from BASF Korea (Seoul, Korea). Graphite powder (< 20 μm), horseradish peroxidase (250–330 U/mg; HRP), hydrogen peroxide (30 wt% in H_2O; H_2O_2), 4-dimethylaminoprydine (DMAP), *p*-nitrophenylchloroformate (PNC), tyramine (TA), potassium permanganate ($KMnO_4$), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Triethylamine (TEA) and dimethylsulfoxide (DMSO) were purchased from Kanto Chemical Co. (Tokyo, Japan). Sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) were purchased from Daejung Chemicals and Metals Co. (Seoul, Korea). All chemicals and solvents were used without further purification.

For the *in vitro* cell study, α-modified Eagle's minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin−streptomycin (P/S), trypsin−EDTA, and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY, USA).

Synthesis of Tet−TA conjugate

Tet−TA was synthesized through a two-step conjugative reaction as previously described[1]: 1) Terminal hydroxyl groups of Tetronic were modified with PNC in the presence of DMAP and TEA to obtain amine-reactive Tet-PNC; (2) TA was then added into Tet−PNC dissolved in DMSO, and the resulting solution was precipitated, filtered, and dried under vacuum to yield the Tet−TA. The chemical structure of Tet−TA was characterized by ^{1}H-NMR spectroscopy (AS400, OXFORD instrument, UK) and UV/VIS spectrophotometer (V-750, Jasco, Japan). The ^{1}H-NMR ($CDCl_3$) spectrum of Tet−TA: δ 1.12 and 3.2−4.4 (m, protons of Tetronic), δ 6.7−7.1 (m, aromatic protons of TA). The phenol content of Tet−TA was determined to be 210 μmol per 1g of polymer.

Oxidation of graphite

For synthesis of GOs with different oxidation degrees, graphite powder was oxidized according to the modified Hummers' method by varying the amount of $KMnO_4$.[2] Briefly, graphite powder (2 g) was stirred

in 45 mL of H_2SO_4 (98%) for 2 h, and $KMnO_4$ (3 g for GO I, 9 g for GO II) was added dropwise while the temperature was kept at 20 °C. The mixture was then heated to 35 °C, and stirred vigorously for 2 h. The resulting solution was diluted with 90 mL of distilled water, and further treated by adding 10 mL of H_2O_2 (30%) and 150 mL of distilled water. The suspension of graphite oxide was washed repeatedly by centrifugation, first with 5% HCl aqueous solution and then with distilled water until a neutral pH was reached. Subsequently, the obtained precipitates were subjected to lyophilization. Finally, graphite oxide powder (20 mg) was dispersed in 1 mL of PBS, and the exfoliation to GO was achieved by an intense ultrasonic treatment for 1 h using an ultrasonicatior (UW 2070, Bandelin, Germany). As a control, graphite powder was also exfoliated to prepare a graphene (GP) suspension using the same procedure as described above. The characterization of the GOs with different oxidation levels was carried out using X-ray photoelectron spectra (K-Alpha, Thermo Electron, USA; XPS) and flourier transform infrared spectroscopy (NICOLET 6700, Thermo Scientific, USA; FT-IR).

Preparation of Tet−TA/GO composite hydrogels

Tet−TA/GO composite hydrogels were prepared by simply mixing exfoliated a GO suspension and Tet−TA solution in the presence of HRP and H_2O_2. GP, GO I or GO II suspension (20 mg/mL) was mixed with Tet−TA (14.3 wt%) dissolved in PBS, and then the mixture was divided into two aliquots including either HRP (0.05 mg/mL) or H_2O_2 (1 wt%) (volume ratio of Tet−TA : GP/GOs : HRP/H_2O_2 = 7 : 2 : 1). The composite hydrogels were formed after homogeneous mixing of two components at room temperature. The detailed conditions are given in Table S1.

Characterization of Tet−TA/GO composite hydrogels

Gelation time

The gelation time of composite hydrogels was determined using a vial tilting method.[3] After mixing 100 μL of each Tet−TA/GP or GO solution containing either HRP or H_2O_2 in a vial, the time at which the gel did not flow was recorded as the gelation time. The experiments were performed with different amounts of HRP

(0.005-0.02 μg/mL) while the concentrations of H_2O_2, GP/GOs and Tet−TA were fixed at 0.1 wt%, 0.4 wt% and 10 wt%, respectively.

Swelling ratio

To measure the swelling ratio of hydrogels, 400 μL of Tet−TA composite hydrogels were prepared in a Teflon mold (10 × 10 × 10 mm^3). After incubation in 5 mL of PBS for 3 days, the degree of swelling of each hydrogel specimen was determined by measuring the weight ratio of swollen gels (W_s) with respect to initially formed gels (W_i). The mass swelling ratio (Q_m) of hydrogels was then calculated using following equation: $Q_m\ (\%) = (W_s - W_i)/W_i \times 100$.

Stability test

The stability of hydrogels was examined by monitoring changes in the weight of hydrogels over time. 300 μL of Tet−TA and Tet−TA/GO II hydrogels prepared in microtubes were incubated in 1mL of PBS at 37 °C. At appropriate time intervals, PBS was removed and the weight of each hydrogel was measured. After that, fresh PBS was added to the microtube for the next time point.

Surface morphology

The surface morphology of dehydrated hydrogels was analyzed using a scanning electron microscope (SEM; JSM-6380, JEOL, Japan). The hydrogels prepared in a Teflon mold (10 × 10 × 7 mm^3) were freeze-dried under vacuum for 3 days. The dried samples were sputter-coated with gold, and their surface morphologies were observed using the SEM.

In vitro protein release test

For *the* test, 200 μL of hydrogels containing 100 μg of BSA were formed in 24-well plate, and they were incubated with 1 mL of PBS. The release experiments were performed at 37 °C and 100 rpm for 7 days. At predetermined time intervals, the media were removed and then replaced with the same volume of fresh PBS.

The amount of released BSA from hydrogels was quantitatively measured using a micro BCA protein assay kit (Pierce, USA) according to the manufacturer instruction.

Compressive and tensile strength

The mechanical properties of hydrogels were evaluated by measuring the compressive and tensile strength using a universal testing machine (Instron 3343, Instron, USA; UTM) equipped with a 100 N load cell. For the compressive strength test, the cube-shaped hydrogels were fabricated in Teflon molds (10 × 10 × 10 mm^3) and then were incubated in 10 mL of PBS for 3 days. The swollen samples were placed between the self-leveling plates, and subsequently compressed at a loading rate of 1.3 mm/min.

Similar to the compressive test, the tensile strength of composite hydrogels was also measured in a fully swollen state. The hydrogel films prepared in Teflon molds (10 × 35 × 3 mm^3) were assessed using the UTM at a rate of 10 mm/min. The failure point for both the compressive and tensile strength tests was determined when the slope of the stress-strain curve dropped or started to decrease. Each measurement was repeated 5 times.

In vitro cell viability assay

The cytotoxicity of hydrogels against MC3T3-E1 cells was examined on the surface of a gel matrix using a Live/Dead Viability/Cytotoxicity Assay Kit (Invitrogen, USA). To evaluate cell adhesion to Tet−TA hydrogels, a synthetic cell-adhesive peptide (GRGDGGGGGY; RGD−Y) was conjugated to Tet−TA/GOs hydrogels as described previously.[4] Briefly, two types of Tet−TA polymer solutions containing 0.4 wt% GOs were prepared in (1) PBS with 200 μg/mL HRP, and (2) PBS with 0.1 wt% H_2O_2 and 4 mg/mL RGD−Y peptide, which were separately loaded in a dual syringe kit. After injection into the Teflon molds, the formed hydrogel disks (13 mm in diameter) were washed with PBS, and placed in a 24-well plate. MC3T3-E1 cells were seeded on the surface of hydrogels at a density of 4 × 10^4 cells/cm^2, and were cultured in α-MEM supplemented with 10% FBS and 1% P/S under standard culture conditions (37 °C and 5% CO_2). After 24 h incubation, the cells were incubated with a mixture consisting of 2 mM calcein AM

and 4 mM ethidium homodimer-1 at 37 °C for 30 min, and then identification of live/dead cells was performed using a fluorescence microscope (TE-2000, Nikon, Japan).

The amount of RGD−Y on the surface of hydrogels was quantitatively determined using a fluorescamine assay.[1] Briefly, the prepared hydrogel discs were thoroughly washed using PBS to remove unconjugated peptide, and they were immersed in a mixture of PBS (375 μL) and fluorescamine (125 μL, 4 mg/mL in acetone). After the reaction for 1 min, the fluorescence of the solution was measured at an excitation wavelength of 390 nm and an emission wavelength of 475 nm.

Statistical analysis

All results are expressed as mean ± standard deviation. Statistical analysis was performed using a Student's t-test and the significance of the results was set to a P-value < 0.005.

Characterization of Tet-TA/GO composite hydrogels

In an attempt to correlate mechanical properties with morphological properties, we first characterized the surface morphology of the composite hydrogels using SEM (Fig.S3). The images showed unexpected morphological changes of hydrogels after lyophilization: the Tet−TA/GOs composite hydrogels had noodle-like sinuous surfaces while the Tet−TA and Tet−TA/GP hydrogels had small pores. Inner structures of hydrogels showed similar patterns. Based on the obtained results, however, we could not make correlation between mechanical and morphological properties. In some cases (depending on a material or crosslinking type), SEM images do not seem adequately to represent a real gel structure because a lyophilization process may greatly alter both original swollen gel shape and structure.[5] Indeed, we found that Tet−TA gels underwent significant volume reduction during lyophilization.

The composite hydrogels were further characterized in terms of the stability and drug delivery capability. As shown in Fig. S4a, the Tet-TA and Tet-TA/GO II hydrogels appeared to be stable for 2 weeks though they exhibited an initial weight loss due to shrinkage of thermosensitive Tet−TA. This result demonstrates that Tet-TA with urethane linkages is highly resistant to hydrolysis, and thus their hydrogels can be useful for hard tissue replacement and long-term drug delivery. For *in vitro* drug release test, BSA as

a model protein drug was physically entrapped into hydrogel matrices and their release behaviors were examined as a function of time (Fig. S4b). Tet−TA/GOs hydrogels showed a sustained release of 25-35% of BSA for 7 days while about 65% of BSA was released from Tet−TA. The Tet−TA/GP hydrogel exhibited a similar release profile. We assume that the delayed release of BSA from Tet−TA/GOs is resulted from a decrease in the swelling ratio, robust cross-linking network and the permeability barrier of GO sheets in the gel matrix. In addition, it was found that Tet-TA/GO I showed a more sustained drug release than Tet-TA/GO II, due to the electrostatic repulsion between negatively charged carboxyl groups on GO II and negatively charged BSA.

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Table S1. The experimental conditions for the preparation of composite hydrogels

Sample	Tet−TA (wt%)	HRP (mg/mL)	H_2O_2 (wt%)	GP or GOs (wt%)	O/C ratio[a]
Control	10	0.005	0.1	−	−
GP	10	0.005	0.1	0.4	0.08
GO I	10	0.005	0.1	0.4	0.33
GO II	10	0.005	0.1	0.4	0.59

[a] The result of O/C ratios (GP, GO I and GO II) were obtained from the quantitative analysis of wide-scan XPS spectra.

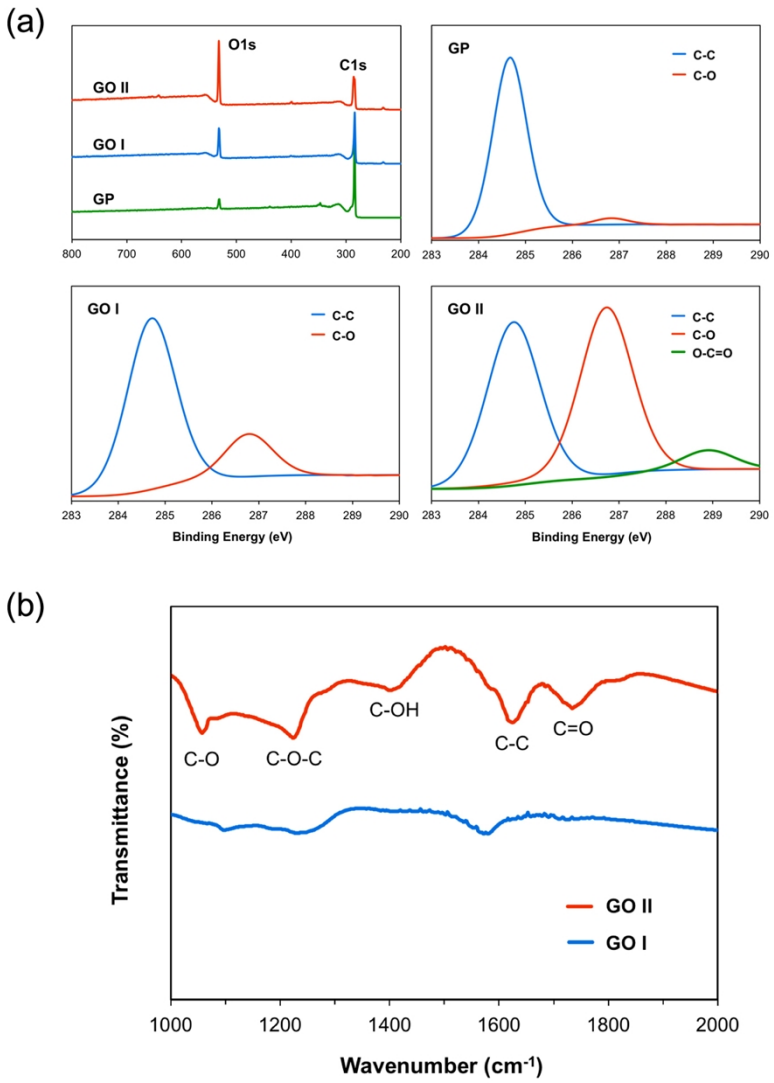

Fig. S1. XPS spectra (wide scan and high-resolution C1s spectra) (a) and FT-IR spectra (b) of graphene-based nanomaterials with different degrees of oxidation.

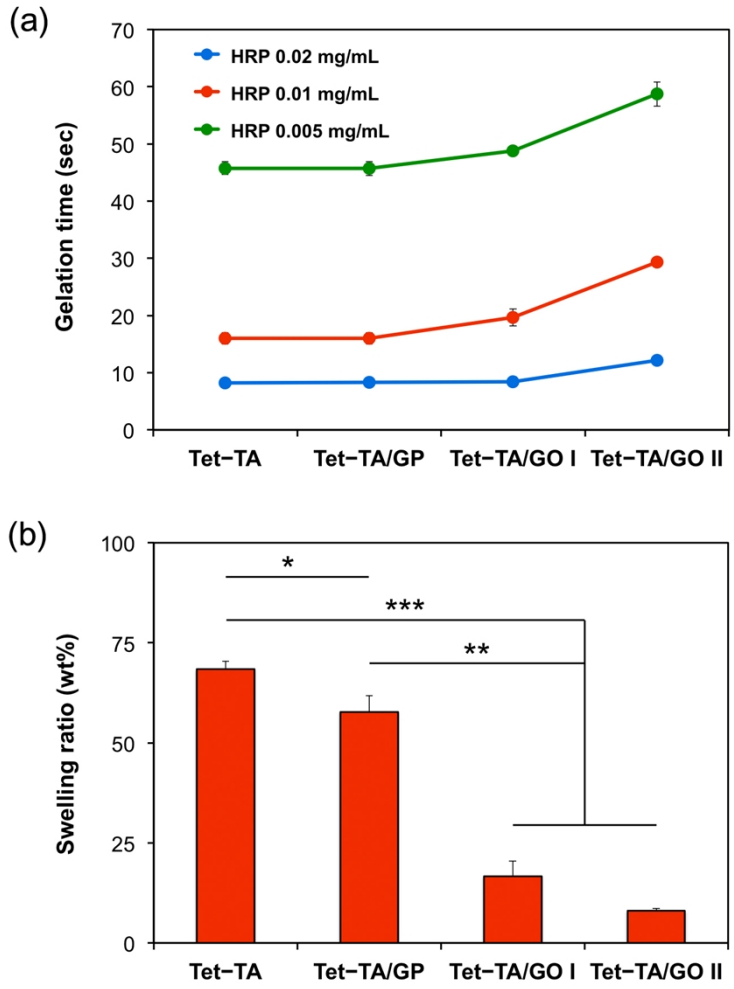

Fig. S2 Gelation time of composite hydrogels at varying HRP concentrations (n=3) (a). Swelling ratio of composite hydrogels, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (n=3) (b).

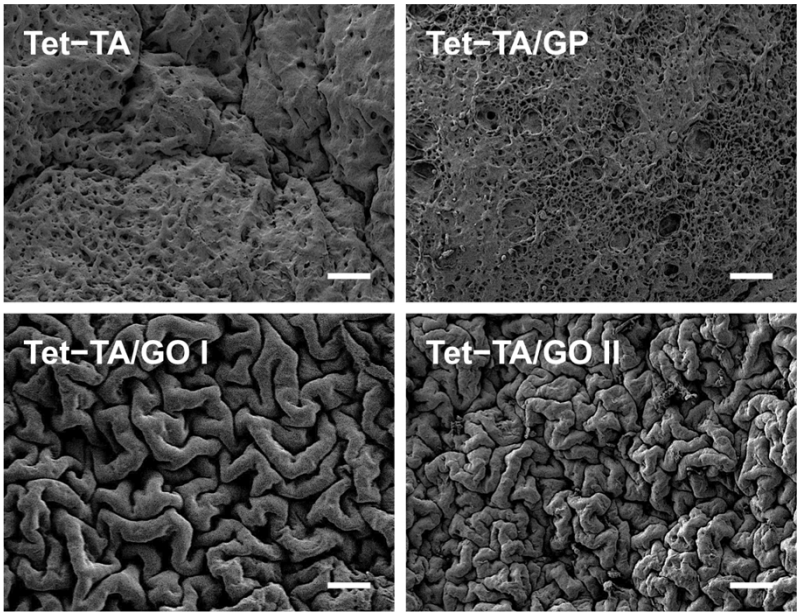

Fig. S3 SEM images of the surfaces of Tet−TA composite hydrogels. The scale bars indicate 100 μm.

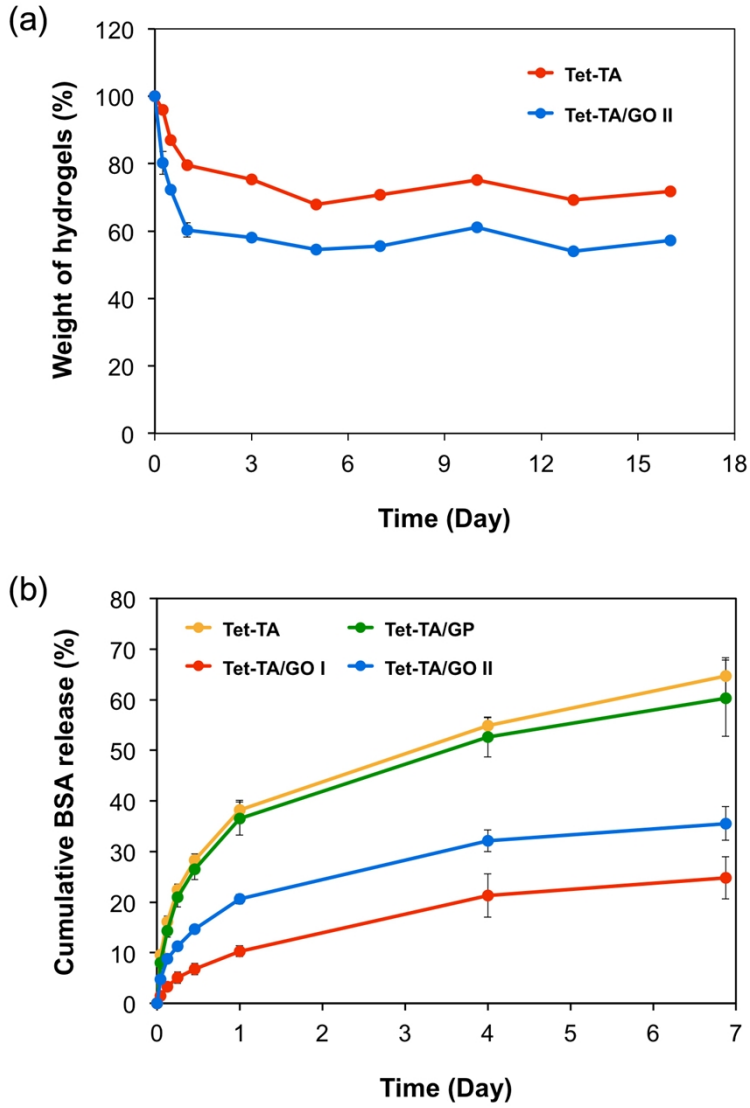

Fig. S4 The stability of Tet−TA and Tet−TA/GO II composite hydrogels for 15 days (n=3) (a) and the cumulative release profile of BSA from composite hydrogels (n=3) (b).

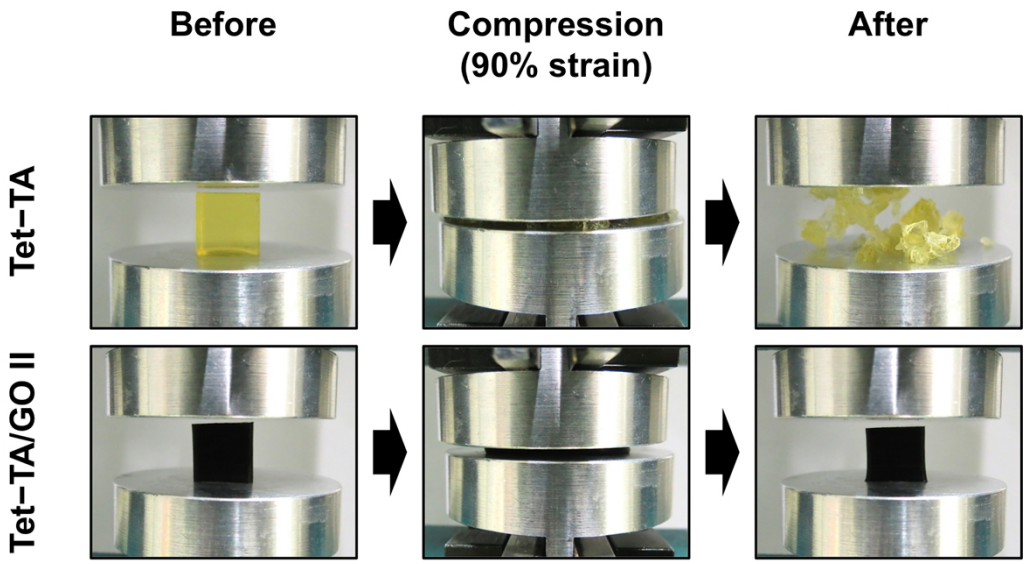

Fig. S5 Digital images of Tet-TA hydrogel and Tet−TA/GO II composite hydrogel subjected to a compression test up to strain of 90%.