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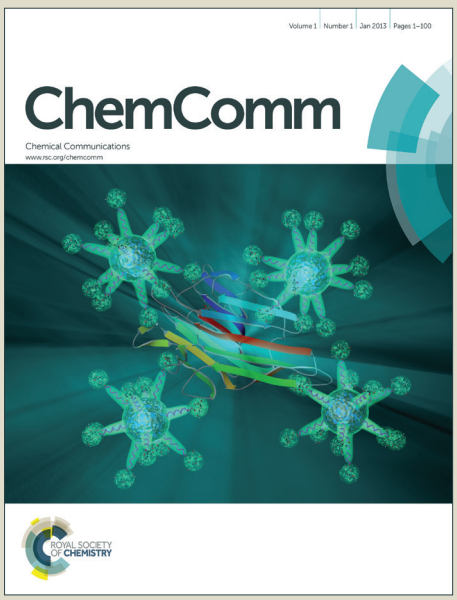

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Injectable and mechanically robust 4-arm PPO-PEO/graphene oxide composite hydrogels for biomedical applications

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Yunki Lee[a], Jin Woo Bae[a], Thai Thanh Hoang Thi[a], Kyung Min Park[b] and Ki Dong Park[a,*]

Graphene-based nanomaterials with different oxidation degrees were incorporated into Tetronic-tyramine (Tet−TA) hydrogels *via* enzymatic cross-linking. The molecular oxidation of graphene in combination with amphiphilic Tet−TA significantly improved water dispersibility of graphene oxide (GO), resulting in a significant reinforcement of Tet−TA/GO composite hydrogels that can be used as an injectable biomaterial platform.

Hydrogels, hydrophilic polymer networks with a three-dimensional configuration, have received substantial attention as promising implantable biomaterials for a broad range of biomedical applications due to their structural properties that are similar to the native soft tissues.[1, 2] In particular, *in situ* cross-linkable hydrogel materials have been widely explored in injectable applications *via* minimally invasive procedures, which can provide more efficient and effective treatment options for patients as well a reduction in health care costs.[3, 4] They undergo a sol-gel phase transition upon physical or chemical stimuli and typically have a jelly-like softness. Although the soft nature of *in situ* cross-linkable hydrogels facilitate notable advances in tissue engineering and drug delivery applications, their inherent weak mechanical properties remain challenging to open up new minimally invasive biomedical applications, such as their use as functional substitutes of hard tissues and long-term drug delivery.[5, 6]

So far, various approaches have been proposed to improve the mechanical properties of hydrogels (*e.g.* interpenetrated network (IPN) formation, 3D architecture control, and the generation fo composite hydrogels).[7] Among composite hydrogels made from a homogenous mixing of multiple constituents, recent studies demonstrated that the incorporation of graphene oxide (GO) could induce strong interfacial interactions between GO and polymer chains through hydrogen bonding, ionic interaction and physical adsorption.[8] For examples, simple addition of GO into gel-forming materials (*e.g.* polyacrylamide, polyvinyl alcohol, chitosan and gelatin) increased the toughness of the resulting composite hydrogels.[9-12] However, despite the successful improvement of the mechanical properties of hydrogels, there are still some issues that need to be resolved for injectable biomedical applications. These include the heterogeneous gelation caused by the low dispersibility of GO in aqueous polymer solutions as well as complicated and toxic processes.[13]

To address these issues, the present study reports a new facile approach to prepare *in situ* cross-linkable tough and elastomeric composite hydrogels composed of 4-arm polypropylene oxide (PPO)−polyethylene oxide (PEO)−tyramine (Tet−TA) and GO *via* horseradish peroxidase (HRP)-catalyzed cross-linking. It was demonstrated that the dispersibility of GO in aqueous solutions was highly improved by further oxidation of GO and favorable interactions with Tet−TA block copolymers. Furthermore, we confirmed that well-dispersed GO sheets within hydrogel matrices significantly improved the mechanical properties (over 0.65 MPa) of composite hydrogels, showing good cytocompatibility. To our knowledge, this is the first study that investigates the effects of GO oxidation on the mechanical properties of enzymatic *in situ* cross-linkable composite hydrogels.

We hypothesized that, by increasing the oxidation level of GO, the water dispersibility of GO might be improved and that further stabilization of the GO dispersion by amphiphilic Tet−TA molecules might improve the mechanical properties of composite hydrogels formed *in situ via* enzymatic cross-linking. To prepare GO with different oxidation levels, chemical modification of graphite was carried out according to the modified Hummers' method (See details in ESI†).[14] After adding a varied amount of $KMnO_4$ as oxidizing agent, the solution mixture was sonicated to exfoliate GO with different degrees of oxidation to obtain single-layer GO dispersions (GO I and GO II). As a control of GO, the non-oxidized graphite powder was also exfoliated to prepare a

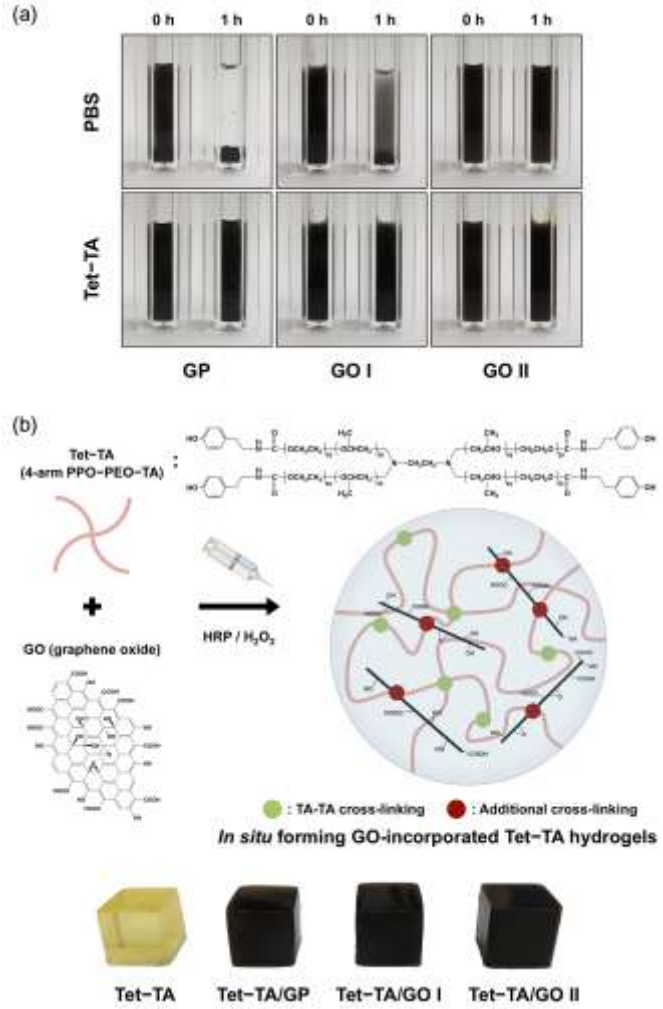

Fig. 1 Dispersibility of GP, GO I and GO II in PBS and Tet−TA solutions at a concentration of 4 mg/mL (a). Schematic illustration and digital images of Tet−TA/GO composite hydrogels formed *via* HRP-catalyzed cross-linking (b).

graphene (GP) dispersion. The structural and chemical compositions of GO were characterized by X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FT-IR) to confirm the oxygenated groups on the GO sheets. The wide-scan XPS spectrum clearly showed an increase of the oxidation degree, where a larger increase of the signal corresponding to the O1s peak was observed with increased atomic O/C ratios from 0.08 (GP) to 0.56 (GO II) (Table S1). The O/C ratio of GO II was comparable to that of fully oxidized GO, as reported previously.[15] In the high-resolution C1s spectra, two prominent peaks corresponding to C−O (286.6 eV) and O−C=O (289.0 eV) gradually increased with increasing the amount of the oxidizing agent.[16] The FT-IR results also showed the relative increases in the intensity of oxygen-containing groups, *i.e.*, hydroxyl (1403 cm^{-1}), carboxyl (1720 cm^{-1}) and epoxy (1250 cm^{-1}) groups (Fig. S1b). These results demonstrated that the oxygen-containing groups were successfully introduced onto the GO sheets and that the oxidation level of GO was clearly different between the groups.

In general, the improvement of the mechanical properties of composite materials is closely associated with the dispersibility of reinforcing additives in a hybrid state.[17] In addition, molecular interactions occurring at the interfaces of the composites contribute to the mechanical reinforcement. To investigate water dispersibility of graphene-based nanomaterials and the influence of amphiphilic Tet−TA conjugates on the water solubility, graphene-based nanomaterials (GP, GO I and GO II) were simply dispersed in PBS or a Tet−TA solution (pre-gel solution). As shown in Fig. 1a, GO II with the highest oxidation degree exhibited a relatively stable aqueous dispersion for 1 h, whereas most of the GP and GO I quickly or readily sedimented over time. In the Tet−TA solution, a significant improvement in the dispersibility of all suspensions was observed. This result can be explained by the complementary role of two domains in Tetronic, where hydrophobic PPO segments strongly interact with graphitic basal planes and hydrophilic PEO chains face the surrounding aqueous environment.[18, 19] Thus, it can be postulated that the use of Tet−TA as well as the molecular oxidation of GP would improve the mechanical properties of composite materials.

To prepare Tet−TA-based composite hydrogels, a graphene-based nanomaterial was pre-mixed with a Tet−TA solution and the mixture was subjected to the HRP-catalyzed oxidative reaction (Fig. 1b). This reaction catalyzes chemical cross-linking between phenol molecules of polymers, resulting in a hydrogel network. This emerging approach has been widely utilized to produce *in situ* cross-linkable hydrogels owing to easy manipulation of the cross-linking rate and degree, both affecting hydrogel properties such as gelation time and mechanical properties.[20-22] We first investigated the effect of graphene-based nanomaterials on the gelation kinetics of composite hydrogels by varying the HRP concentration (Fig. S2a). It is worth noting that 0.4 wt% graphene-based nanomaterials were used because of their strong tendency to aggregate and their rapid increase in viscosity above 20 mg/mL (mixing volume ratio of Tet−TA : GO : HRP/H_2O_2 = 7 : 2 : 1). At a fixed H_2O_2 concentration (0.1 wt%), increasing the HRP concentration led to a faster gelation ranging from 8 to 58 sec. In particular, the incorporation of GO II resulted in a slightly slower gelation (4−13 sec). This is probably due to a scavenging effect of GO by electrostatic interactions between carboxyl groups on the GO sheets and arginine residues of HRP during the gelation, thereby partially inactivating HRP.[23, 24] Although the gelation rate was retarded by the incorporation of GO, the gelation time was still controllable within a reasonable range for injectable applications.

It is known that the swelling capacity of hydrogels greatly affects their mechanical toughness. Hence, we assessed the swelling ratio of composite hydrogels by measuring the mass ratio of swollen gels (W_s) to initially formed gels (W_i). As shown in Fig. S2b, the swelling ratios of composite hydrogels were highly dependent on the oxidation level of the graphene-based nanomaterials. The Tet−TA hydrogels containing GO I or GO II exhibited a significant decrease in the swelling ratio compared to the pure Tet−TA hydrogel (68.4 ± 1.9%). The swelling ratio of the Tet−TA/GP hydrogel (57.8 ± 4.1%) also decreased, but its extent was not as great as that of the Tet−TA/GOs, indicating that

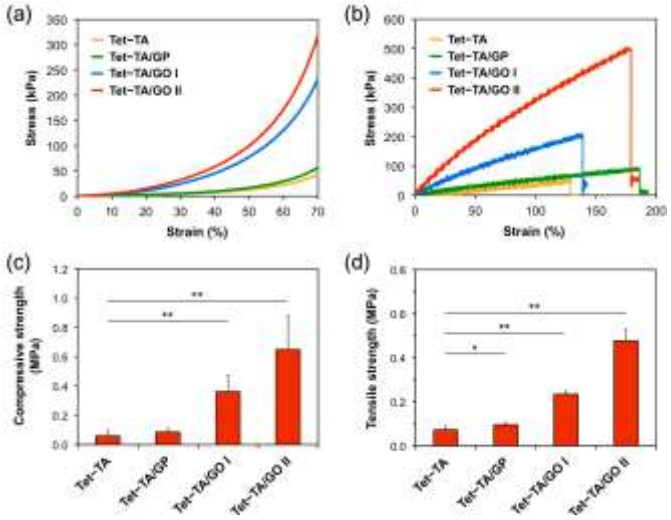

Fig. 3 Stress−strain curves for 10 wt% Tet−TA hydrogels containing 0.4 wt% GP, GO I and GO II under uniaxial compression (a) and tensile force (b). The Tet−TA hydrogel was used as a control. The failure compressive (n=3) (c) and tensile strength (n=5) (d) of composite hydrogels, * $P < 0.05$ and ** $P < 0.0001$.

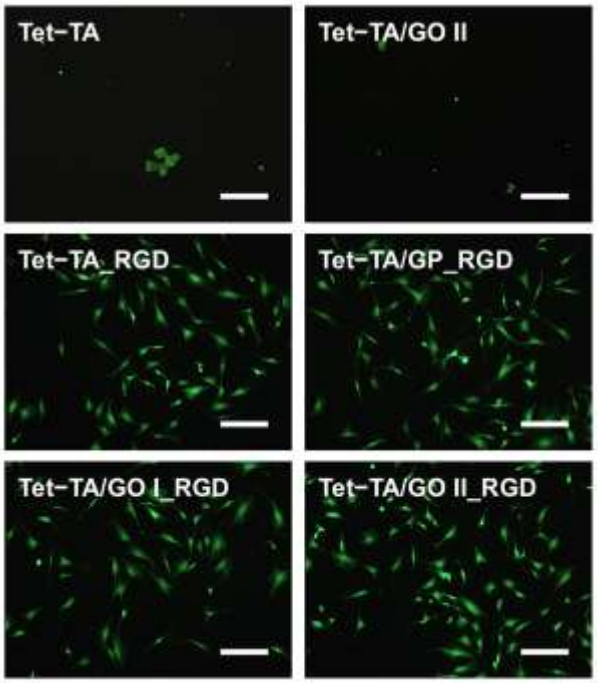

Fig. 2 Live/dead staining images of MC3T3-E1 cells adhered on Tet−TA hydrogels with or without graphene-based nanomaterials after 24 h of culture. The scale bars indicate 200 μm.

oxygenated hydrophilic groups on the GO sheets predominantly interact with Tet−TA in the hydrogel network rather than hydrophobic bonding between GOs and Tet−TA. We also investigated the morphological properties of hydrogels but could not make good correlation between samples due to gel distortion under lyophilisation (Fig. S3). In addition, the composite hydrogels were further characterized in terms of the stability and drug delivery capability (Fig. S4).

We next investigated the effects of GO incorporation on the mechanical properties of the composite hydrogels by measuring the compressive and tensile strength of composite hydrogels. Fig. 2a and 2b show the representative strain−strain curves of fully swollen composite hydrogels measured under compressive and tensile forces, respectively. The rigidity of Tet−TA hydrogels was considerably improved when GO I or GO II were mixed, showing a tendency that corresponds to the oxidation level of GP. The Tet−TA hydrogels containing 0.4% GOs exhibited a 6− to 11−fold increase in compressive strength (from 0.06 to 0.36−0.65 MPa; Fig. 2c) and a 4− to 8−fold increase in tensile strength (from 0.06 to 0.23−0.48 MPa; Fig. 2d) compared to the pure Tet−TA hydrogel. A negligible change was observed between Tet−TA/GP and Tet−TA hydrogels. In fact, there have been several attempts to use GO for mechanical reinforcement of composite hydrogels, as previously mentioned.[9, 10, 25, 26] They also demonstrated a significant increase in the mechanical properties of the composite hydrogels. However, the differences are somewhat less notable, and the systems employed appear unsuitable for injectable biomedical use.

The results obtained so far demonstrated two major aspects of the composite hydrogels: 1) the oxidation of graphene-based nanomaterials enhanced the water dispersibility, and 2) the amphiphilic nature of Tet−TA facilitated a better dispersion of GOs, which in turn led to a significant enhancement of the mechanical performances of the composite hydrogels (Fig. S5). As shown in Fig. 1, the stabilities of all aqueous dispersions were improved after mixing with Tet−TA conjugates. However, GP having extremely hydrophobic surfaces was eventually aggregated and precipitated within a few hours, while the more hydrophilic GOs remained stable. We thus expect that the resulting GP agglomerations created structural defects in the composite hydrogel, thereby possibly causing a deterioration of the reinforcing effect. More importantly, it was found that the improved rigidity and toughness of composite hydrogels could be attributed to the extent of oxygenated groups present on the GOs. Although a limited number of samples were tested due to the difficulty of preparing GOs with distinctly different oxidation levels, other research groups also demonstrated that GO could interact more effectively with amphiphilic polymers through the assembled morphology at the interface.[27-29] Therefore, it is likely that the significant improvement of the mechanical properties of Tet−TA/GOs composite hydrogels is achieved by a synergistic effect of both a homogeneous GO dispersion and strong interfacial interactions between GO and Tet−TA.

The cytotoxicity of composite hydrogels was evaluated using *in vitro* 2D culture of MC3T3-E1 cells. We have previously shown that various cell types (MC3T3-E1, C2C12, HUVECs, and hMSCs) were highly viable when cultured on RGD-conjugated Tet−TA hydrogels.[30-32] Similarly, the RGD peptides ranging from 0.73 to 0.81 nmol/cm^2 were introduced to enhance cell adhesion on the hydrogel surfaces. As shown in Fig. 3, the adhered cells were only observed on the surfaces of RGD-conjugated hydrogels while barely adhering on the Tet−TA and Tet−TA/GO II hydrogels prepared without the RGD treatment. The majority of cells adhered on the hydrogels appeared to be

highly viable (stained green, > 99%) with only few dead cells (stained red) (Fig. 3). In fact, it has been reported that various parameters, including concentration, size, and surface functionality, are relevant for the cytotoxicity of graphene-based nanomaterials.[33, 34] The oxidative stress of GO, which is altered by individual or combinatorial factors, is believed to be an important cytotoxic pathway. However, in our composite hydrogel system, the GO concentration was very low compared to studies that specifically focused on the cytotoxicity of GO itself. Hence, the surface-exposed GO seems to barely affect the cytotoxicity of the composite hydrogels. Similar results can be found elsewhere.[11, 25, 35]

This study presents tough and elastomeric composite hydrogels composed of GO and Tet−TA for expanded applications in biomedical fields. The molecular oxidation of GP, in combination with amphiphilic Tet−TA, improved the water dispersibility of GO. Consequently, the mechanical properties of Tet−TA/GO composite hydrogels prepared *via* HRP-catalyzed cross-linking were significantly ameliorated. In addition, we were able to manipulate the swelling and mechanical properties of composite hydrogels by varying the oxidation degree of GO. The cytotoxicity of the composite hydrogels appeared to be very low. This biocompatible, tough and elastomeric Tet−TA/GO composite hydrogel can be used as an injectable *in situ*-forming implant for hard tissue replacement and long-term drug delivery.

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[a] *Department of Molecular Science and Technology, Ajou University, San 5, Woncheon, Yeongtong, Suwon 443-749, Republic of Korea. E-mail: kdp@ajou.ac.kr; Fax: +82 31-219-1592; Tel: +82 31-219-1846*

[b] *Division of Bioengineering, Incheon National University, Incheon 406-772, Republic of Korea*

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