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Carbohydrate Functionalization of Few-Layer Graphene through Microwave-Assisted Reaction of Perfluorophenyl Azide

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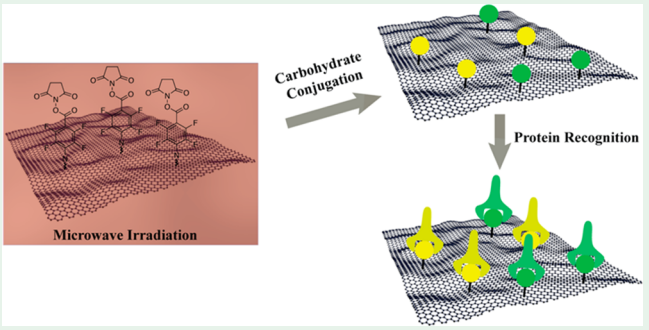
Supporting Information

ABSTRACT: The excellent physical and chemical properties of graphene make it an attractive nanomaterial and a component in high-performance nanocomposite materials. To prepare graphene-based nanocomposite materials, chemical functionalization is often necessary. Water-soluble ligands such as carbohydrates not only make the functionalized graphene compatible with aqueous media, but also introduce biorecognition, which is important for graphene to be used in biotechnology. In this study, we report the derivatization of few-layer graphene (FLG) with carbohydrates through microwave-assisted reaction of perfluorophenyl azide (PFPA). FLG was first treated with PFPA under microwave radiation. Subsequent conjugation with glycosyl amine gave carbohydrate-presenting FLG. Thermogravimetric analysis showed that microwave radiation gave a higher degree of functionalization compared to conventional heating, with higher weight losses for both PFPA and Man ligands. The carbohydrates (mannose and galactose) retained their bioactivity, as demonstrated by the lectin binding assays. Higher degree of binding toward lectins was obtained for the carbohydrate-functionalized FLG prepared by microwave radiation than the conventional heating.



KEYWORDS: *graphene, carbohydrate, perfluorophenyl azide, microwave radiation, lectin*

INTRODUCTION

Graphene and graphene-based nanomaterials have attracted intense research interest since graphene became experimentally available[1] not only for their exceptional properties, but also the ability to improve the performance of a wide range of other materials.[2−5] To realize these potentials, modification of graphene is often necessary to facilitate the fabrication and processing. Pristine graphene, like other carbon nanomaterials, has poor solubility in most solvents and lacks reactive functionalities. One of the widely used modification methods is the oxidation of graphene under harsh conditions, such as the Hummer method,[6,7] to produce graphene oxide (GO) that possesses hydroxy, epoxy, and carboxyl groups. These functionalities render GO soluble in water, and they can also further react with amine- or hydroxy-containing (bio)-molecules via amidation or esterification reaction.[8] One disadvantage of this method is that the oxidation process disrupts the conjugated structure of graphene. Even followed by reduction of GO (to give rGO), the sp^2 hybridization cannot be completely restored. The remaining O-containing species and defects caused by the oxidation/reduction process contribute to much worsened properties of GO and rGO compared to pristine graphene.[9−13]

An alternative approach is the direct functionalization of pristine graphene. Several chemical functionalization methods have been developed to modify pristine graphene,[14−18] which mostly involves the use of a reactive intermediate.[17] One such example is perfluorophenyl azide (PFPA),[19] which has been used for conjugation of a variety of molecules to nanomaterials[20−25] to various substrates.[26−31] Upon light or thermal activation, PFPA is converted to highly reactive singlet perfluorophenyl nitrene, which can react with C=C bonds in the graphite structure through (1 + 2) cycloaddition.[29,32−34]

Since the initial adoption in the 1980s,[35] microwave radiation has become a standard technique in organic synthesis.[36,37] In conventional thermal reactions, heat is transferred from the heating source, such as oil bath or heating mantle, to the reagents through the reaction vessel. The heat transfer can be slow, depending on a number of factors including the thermal conductivity, shape, and surface area of the reaction vessel. In addition, temperature gradient can develop within the sample, which results in uneven heating as

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well as local overheating. In microwave radiation, the relatively high frequency electromagnetic radiation passes through the reaction vessel to induce molecular collisions of the reagents inside. The reagents can reach the desired temperature quickly, and the heating is uniform throughout the sample. The efficient heating and milder reaction conditions usually result in faster kinetics, higher or comparable product yield, and fewer side products as compared to the traditional heating protocol.[38−42]

Graphene functionalization often requires lengthy reaction time and elevated temperature due to the low reactivity of pristine graphene. For example, while the reaction of PFPA with organic molecules or polymers only takes a few minutes of UV radiation,[43] the reaction of few-layer graphene (FLG) flakes with PFPA under UV radiation require over 60 min.[32] The same reaction carried out in an oil bath at 90 °C takes at least 3 days to complete.[32] We conducted a pilot study on the reaction between PFPA and single-wall carbon nanotubes (SWNTs) in an oil bath and also in a microwave reactor.[44] The results were highly conspicuous; while a reaction time of over 3 days at 130 °C was required when applying conventional heating, the reaction was completed in 6 h under microwave radiation. The reaction time was further reduced to 1 h when the reaction was carried out at 150 °C in the microwave reactor.[44] Thus, the microwave condition resulted in an over 10-fold acceleration of the functionalization reaction. The microwave-assisted approach is furthermore easy to scale up, thus providing an efficient means for the modification of carbon nanomaterials.[45−47] Graphene-based nanocomposites have also been synthesized under microwave radiation for high-performance supercapacitors[48,49] and lithium-ion batteries.[50,51] In addition, pristine graphene is particularly suitable to microwave radiation owing to its high thermal conductivity and high microwave adsorption capacity.[52] For example, microwave radiation allowed for rapid and simultaneous oxidation of entire graphene sheets to give low-oxygen graphene with high conductivity.[53]

Carbohydrates constitute one of the most abundant and structurally complex classes of biomolecules. They play vital roles in a variety of biological processes through carbohydrate−protein interactions such as immune response, bacterial/viral infection, and cell apoptosis. Carbohydrate-functionalized nanomaterials have demonstrated potentials in bioimaging, biosensing, and drug delivery.[54−58] However, for graphene-based materials, most of these carbohydrate-conjugated structures still used GO as the scaffold,[59−61] and the study of covalent functionalization of pristine graphene with carbohydrates and their biological recognition properties has only been reported for FLG functionalized with mannosyl dendrons.[42]

Herein, we report the covalent functionalization of pristine FLG by PFPA using microwave radiation. The reaction efficiency was investigated in comparison to the reaction performed under conventional heating. D-Mannose and D-galactose were subsequently conjugated, and the binding affinity and selectivity of the resulting glyco-FLG toward lectins concanavalin A and *Ricinus communis* agglutinin I were evaluated.

EXPERIMENTAL SECTION

Materials. The graphite flakes were obtained from Sigma-Aldrich. Fluorescein-labeled concanavalin A (FITC-Con A) and *Ricinus communis* agglutinin I (FITC-RCA I) were purchased from Vector Laboratories. All other chemicals used were purchased from Alfa Aesar, Fluka, or Sigma-Aldrich and were used as received without further purification. Water used was from a Milli-Q ultrapure water purification system. *N*-Succinimidyl 4-azidotetrafluorobenzoate (PFPA-NHS),[28] 2-[2-(2-aminoethoxy)ethoxy]ethyl α-D-mannopyranoside (Man-NH_2), and 2-[2-(2-aminoethoxy)ethoxy]ethyl β-D-galactopyranoside (Gal-NH_2) were synthesized following previously reported procedures.[44]

Instrumentation. Microwave reactions were carried out using a Biotage Initiator (Biotage Sweden AB). Fourier transform infrared (FTIR) spectra were collected on a PerkinElmer FTIR spectrometer Spectrum 2000 and a Mettler Toledo ReactIR iC 10 (Columbia, MD). Fluorescence spectroscopy was conducted on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). Transmission electron microscopy (TEM) images were obtained on a JEOL 100CX transmission electron microscope operating at an accelerating bias voltage of 100 kV. Thermogravimetric analysis (TGA) was carried out on a TA Instruments (Q-50 series). Fluorescence microscopy images were obtained on a Nikon Ellipse E600 Epi-fluorescence microscope.

Preparation of Few-Layer Graphene (FLG). Graphite was exfoliated by sonicating a suspension of graphite powder (1 g) in *N*-methyl-2-pyrrolidone (NMP, 500 mL) for 2 h using a sonication probe (VCX750, SONICS). The mixture was centrifuged at 500 rpm for 45 min, after which the supernatant was collected, diluted to 0.5 mg/mL, and sonicated for another 1 h. The resulting sample was used for the subsequent functionalization studies.

Synthesis of PFPA-Functionalized FLG (PFPA-FLG) by Microwave Radiation. PFPA-NHS (275 mg, 0.83 mmol) was added to a suspension of FLG flakes in NMP (20 mL, 0.5 mg/mL) in a 20 mL Biotage microwave vial and sonicated for 20 min. The reaction mixture was then subjected to microwave radiation at 150 °C for 0.5 h. The mixture was centrifuged, and the solid was washed sequentially with chloroform and acetone followed by centrifugation (11 000 rpm, 30 min) to remove excess reagents until no brown color was observed in the solution. The precipitate was finally dried under reduced pressure to give PFPA-FLG (8.6 mg).

Synthesis of PFPA-FLG by Conventional Heating. In a 100 mL round-bottle flask, a suspension of FLG flakes in NMP (40 mL, 0.5 mg/mL) and PFPA-NHS (550 mg, 1.66 mmol) was mixed, sonicated for 20 min, and heated at 150 °C for 1.5 h. The reaction mixture was subsequently centrifuged, after which the solid was washed sequentially with chloroform and acetone followed by centrifugation (11 000 rpm, 30 min) to remove excess reagents. The precipitate was dried under reduced pressure to give PFPA-FLG (22 mg).

Conjugation of Man-NH_2 or Gal-NH_2 to PFPA-FLG. Man-NH_2 or Gal-NH_2 (11 mg, 0.035 mmol) in 0.5 mL of H_2O was added to a dispersion of PFPA-FLG (14 mg) in DMF (4 mL), and the mixture was stirred at room temperature for 48 h. The resulting mixture was subsequently centrifuged (11 000 rpm, 30 min), and the solid was washed three times with DMF/H_2O (v/v = 1:4) and once with acetone, each followed by centrifugation (11 000 rpm, 30 min). The precipitate was dried under reduced pressure to give Man-FLG or Gal-FLG (∼11.5 mg).

TGA Experiments. Tested samples include PFPA-FLG and Man-FLG prepared by conventional heating (CH) and microwave radiation (MW) as well as FLG. All samples were dried for 3 days in a vacuum oven before the experiment. Measurements were carried out under nitrogen atmosphere at a constant heating rate of 10 °C/min from 20 to 1000 °C. The data were normalized against the initial sample weight and are presented as percent weight (%) versus heating temperature in the TGA curves.

Interaction of Man-FLG and Gal-FLG with Lectin. FITC-labeled lectins, FITC-Con A and FITC-RCA I, were used to evaluate the binding. To minimize nonspecific protein binding, Man-FLG or Gal-FLG was first treated with BSA[24,25,62−65] by incubating the sample (3.0 mg) in a solution of BSA (3%) in pH 7.4 PBS buffer (1.0 mL, 10 mM) for 30 min, and subsequently centrifuged. The precipitate was collected and washed with pH 7.4 PBS buffer for

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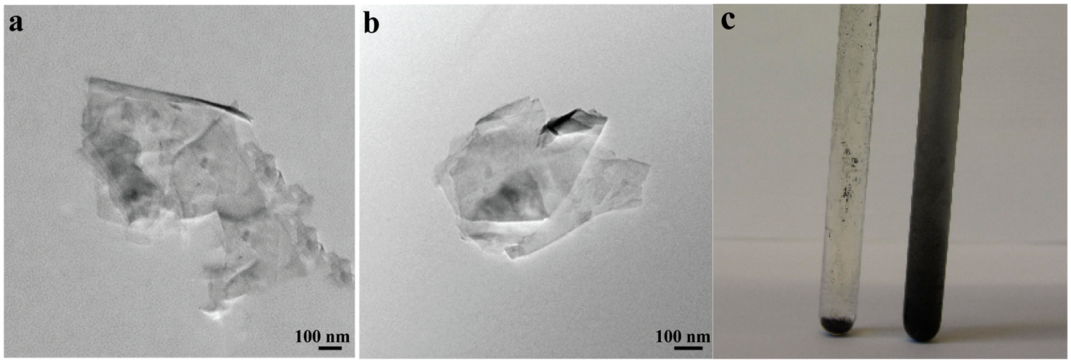
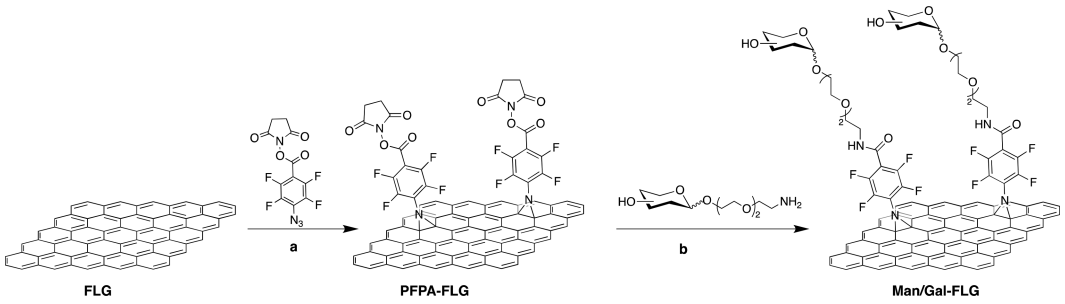


Figure 1. TEM images of (a) FLG and (b) Man-FLG. (c) Dispersions of PFPA-FLG (left) and Man-FLG (right) in DMSO.

Scheme 1. Synthesis of Man-FLG or Gal-FLG[1]



[1](a) PFPA-NHS, NMP, microwave radiation (150 °C, 0.5 h), or conventional heating (150 °C, 1.5 h). (b) Man-NH_2 or Gal-NH_2, DMF, r.t., 48 h.

another 20 min to remove excess BSA, followed by centrifugation. The resulting sample was mixed with a solution of FITC-Con A in pH 7.4 PBS buffer (5 μg/mL, 2.5 mL) containing $MnCl_2$ (1.0 mM) and $CaCl_2$ (1.0 mM) for 1 h, or FITC-RCA I in pH 7.4 PBS buffer (5 μg/mL, 2.5 mL) for 4 h under ambient condition while shaking. The mixture was centrifuged at 11 000 rpm for 15 min to separate the supernatant, and lectin concentration in the supernatant was measured by fluorescence spectroscopy. The precipitate was washed with water to remove physically adsorbed lectin and was imaged by fluorescence microscopy.

RESULTS AND DISCUSSION

FLG was prepared by liquid-phase exfoliation of graphite in *N*-methyl-2-pyrrolidinone (NMP) by sonication. After centrifugation and removal of the precipitate, the supernatant was collected and further sonicated to give FLG, which are the typical flakes of sub-to-few microns in size (Figure 1a).[66,67] The FLG flakes were then treated with *N*-succinimidyl 4-azidotetrafluorobenzoate (PFPA-NHS) under microwave radiation or conventional heating to give PFPA-FLG (Scheme 1). Reactions were carried out at 150 °C for different times and the reaction progress was monitored by FTIR. As the reaction proceeded, the intensity of the characteristic azide peak at 2145 cm^{-1} in the IR spectra decreased (Figure S2). The results showed that most PFPA-NHS was consumed under microwave radiation for 0.5 h at 150 °C. In contrast, 1.5 h reaction time was required to obtain similar conversion in the case of conventional heating. Increasing the reaction time resulted in no additional change for either method (Figure S2). Thus, microwave radiation at 150 °C for 0.5 h and conventional heating at 150 °C for 1.5 h were chosen for the subsequent studies. After functionalization with PFPA-NHS, the samples were subsequently allowed to react with Man-NH_2 (Scheme 1b) to afford Man-FLG (Figure 1b). While PFPA-FLG without the conjugated sugar precipitated out from the solution, the product Man-FLG dispersed well in dimethyl sulfoxide (DMSO) and the dispersion remained stable, demonstrating successful conjugation of Man on FLG (Figure 1c). After functionalization of FLG with PFPA, intense absorption bands at 1740 and 1368 cm^{-1} were observed in the FTIR spectrum of PFPA-FLG, attributed to the stretching vibrations of C=O and C−N in PFPA-NHS (Figure 2). Man-FLG showed the typical C−O stretching vibration of carbohydrate.

Thermogravimetric analysis (TGA) was subsequently used to analyze the degree of functionalization on FLG. PFPA-FLG

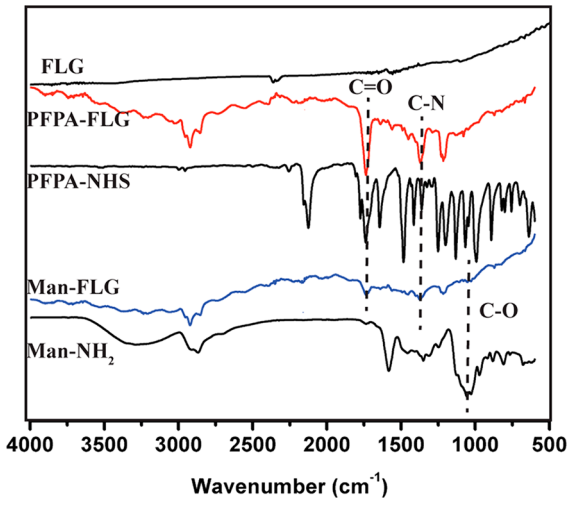


Figure 2. FTIR spectra of FLG, PFPA-NHS, PFPA-FLG, Man-FLG, and Man-NH_2. PFPA-FLG was prepared using microwave radiation.

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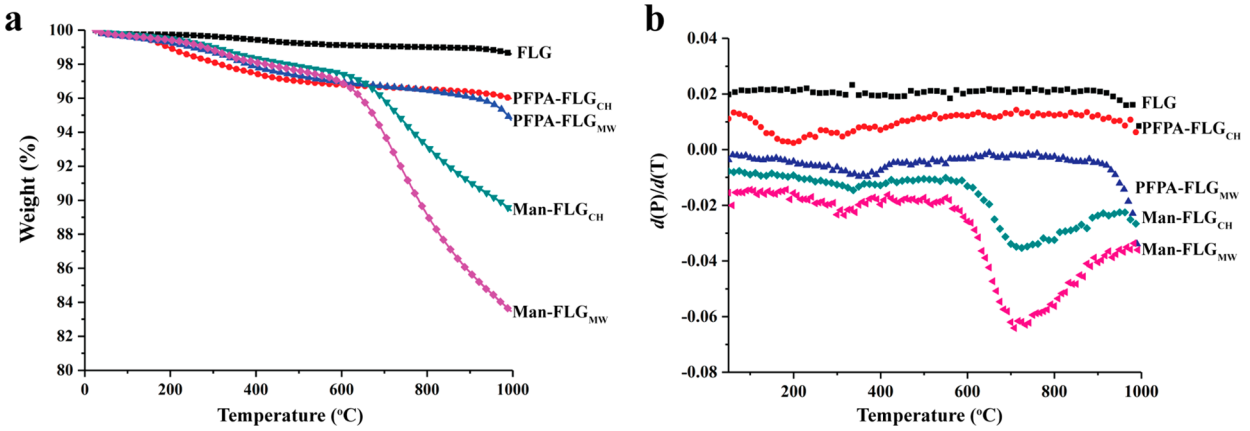

Figure 3. (a) TGA and (b) DTG curves of FLG, PFPA-FLG, and Man-FLG prepared from microwave radiation (PFPA-FLG$_{MW}$, Man-FLG$_{MW}$) or conventional heating (PFPA-FLG$_{CH}$, Man-FLG$_{CH}$).

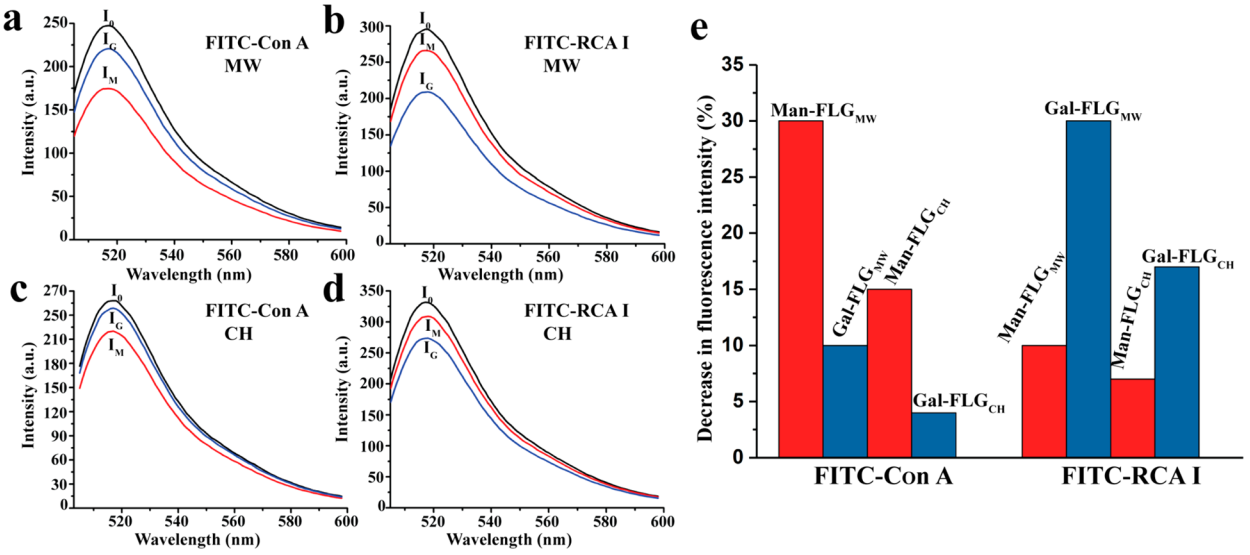

Figure 4. Fluorescence spectra of FITC-Con A and FITC-RCA I before (I_C and I_R) and after incubation with Man-FLG (I_M) and Gal-FLG (I_G) from microwave radiation (MW, a and b) and conventional heating (CH, c and d). (e) Percent decrease in fluorescence intensity after glyco-FLG was treated with FITC-lectin.

and Man-FLG prepared by conventional heating (CH) and microwave radiation (MW) as well as FLG were dried in vacuum for 3 days. TGA experiments were carried out by heating the samples from 20 to 1000 °C at a rate of 10 °C/min under the nitrogen atmosphere. The results were recorded as the weight loss of the sample (%) versus the heating temperature (Figure 3a), and the DTG derivative curves that displayed the temperatures at which the onset, end, and maximum weight-loss occurred (Figure 3b). As expected, FLG is fairly stable, showing no significant weight loss and only ~1.3% at 1000 °C (Table S1), likely due to the physisorbed organic matters. PFPA-functionalized FLG prepared by conventional heating (PFPA-FLG$_{CH}$) exhibited a weight loss between 100−200 °C from the trapped solvent, followed by another weak peak between 300 and 400 °C (red, Figure 3b). PFPA-functionalized FLG prepared by microwave radiation (PFPA-FLG$_{MW}$) showed a more distinct peak between ~300 and 400 °C (blue, Figure 3b), reflecting a higher weight loss. PFPA-FLG$_{MW}$ also exhibited higher weight loss (5.3 ± 0.6) than PFPA-FLG$_{CH}$ (4.0 ± 1.0) at 1000 °C (Table S1). After carbohydrate conjugation, a large weight loss starting from ~600 °C and peaking at ~700 °C was seen for both Man-FLG$_{CH}$ (green) and Man-FLG$_{MW}$ (pink) in addition to the one from ~300−400 °C (Figure 3b). Similar to PFPA-FLG, higher weight loss was obtained for Man-FLG$_{MW}$ (16.5 ± 1.4) than Man-FLG$_{CH}$ (10.6 ± 4.8) at 1000 °C (Table S1). These results support higher degrees of functionalization for samples prepared by microwave radiation than by conventional heating. The high efficiency of microwave-assistant reactions can be attributed to the fast and uniform heating from microwave radiation, which led to more efficient functionalization while at the same time reducing side products.

To further characterize the synthesized materials, Man-FLG$_{MW}$ was subjected to lectin binding. Fluorescein-labeled concanavalin A (FITC-Con A) was used in this study to facilitate the characterization by fluorescence microscopy in addition to electron microscopy. Con A is a tetrameric lectin having specific affinity toward α-D-mannopyranoside derivatives.[68] When mannose or its derivatives are conjugated to a nanomaterial scaffold, the resulting multivalent glyconanomaterials form agglomerates with Con A, which can be characterized by microscopy.[26,30,43,63−65,69] In the binding

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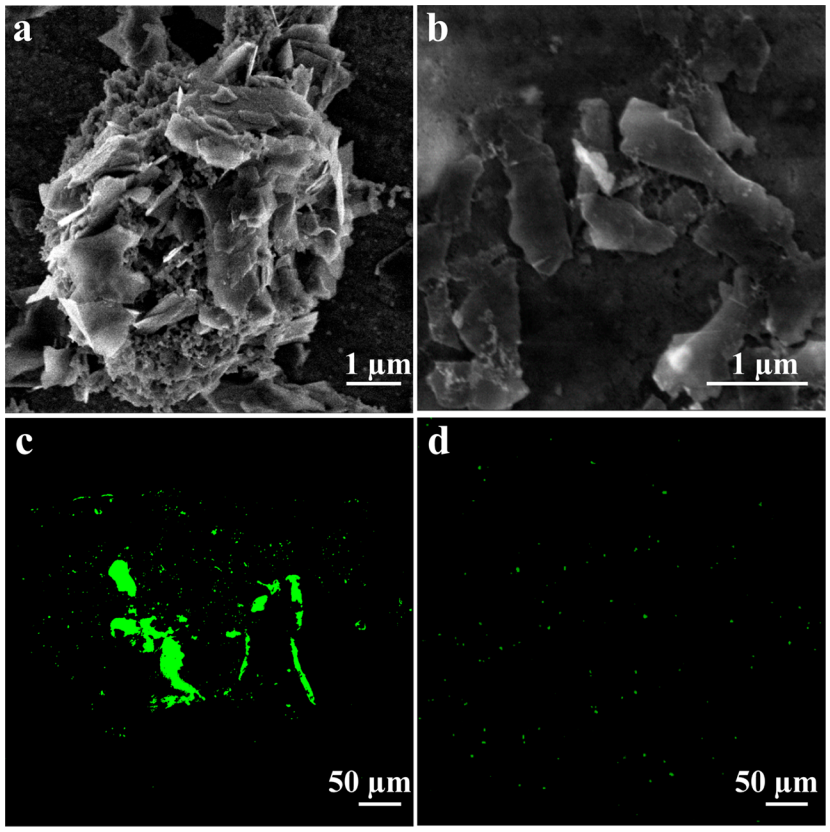

Figure 5. SEM images of (a) Man-FLG$_{MW}$ or (b) Gal-FLG$_{MW}$, and fluorescence microscopy images of (c) Man-FLG$_{MW}$ and (d) Gal-FLG$_{MW}$ after treating with FITC-Con A.

assay, 12.5 μg of lectin (5 μg/mL, 2.5 mL) was added to 3 mg of Man-FLG or Gal-FLG. On the basis of the density estimation from TGA, the total number of carbohydrate in 3 mg of Man-FLG was about 9.5×10^{20} and 8.4×10^{20}mol for Man-FLG$_{MW}$ and Man-FLG$_{CH}$, respectively. The amount of Con A added was $\sim 7.2 \times 10^{13}$ mol (molecular weight of Con A is 104−112 kDa).

When Man-FLG$_{MW}$ was incubated with FITC-Con A for 1 h, the fluorescence intensity of the solution decreased (I_M, Figure 4a). This is due to the interaction between the multivalent Man-FLG$_{MW}$ with the tetravalent Con A, resulting in cross-linking and formation of agglomerates. The mixture was centrifuged and the precipitate was examined under scanning electron microscopy (SEM). Indeed, agglomerates were observed in the precipitate (Figure 5a). To confirm the binding selectivity of Man-FLG toward Con A, galactose-presenting FLG (Gal-FLG$_{MW}$) were synthesized following the same procedure (Scheme 1b). Gal does not bind to Con A, and thus, it was anticipated that Gal-FLG$_{MW}$ would not form agglomerates with Con A. After Gal-FLG$_{MW}$ was incubated with FITC-Con A in the same manner, a slight decrease in fluorescence intensity was observed (I_G, Figure 4a), likely due to the nonspecific adsorption of Con A to FLG. No significant agglomeration was observed under SEM (Figure 5b). Bright fluorescence was also observed for the agglomerates of Man-FLG$_{MW}$ with Con A by fluorescence microscopy (Figure 5c), in contrast to the results for Gal-FLG$_{MW}$ where very little fluorescence was observed (Figure 5d). To further confirm the binding selectivity, a β-D-galactopyranoside-specific lectin, *Ricinus communis* agglutinin I,[70] was treated with the Man-FLG$_{MW}$ and Gal-FLG$_{MW}$, respectively, following the same protocol as with FITC-Con A. For Gal-FLG$_{MW}$, the decrease in fluorescence intensity was more pronounced (I_G, Figure 4b) than in the case of Man-FLG$_{MW}$ (I_M, Figure 4b). These results demonstrated that the carbohydrate moieties on the glyco-FLG$_{MW}$ retained their binding specificity.

The fluorescence-based lectin binding assay was repeated on glyco-FLG$_{CH}$ samples obtained by conventional heating. Similar to the samples obtained from microwave radiation, specific interactions were observed between Man-FLG$_{CH}$ and FITC-Con A (I_M, Figure 4c), and between Gal-FLG$_{CH}$ and FITC-RCA I (I_G, Figure 4d). These results confirmed that the glyco-FLG prepared by both microwave radiation and conventional heating could yield multivalent carbohydrate-presenting FLG for lectin recognition. In the case of microwave radiation, the extent of binding measured by the percent decrease in fluorescence intensity was higher than that by conventional heating (Figure 4e). Again, this is consistent with the previous data (Figure 3b) and can be attributed to the higher degree of PFPA functionalization by microwave radiation.

CONCLUSIONS

In conclusion, carbohydrate-presenting FLG were synthesized from PFPA-functionalized FLG prepared under microwave radiation, which resulted in increased reaction rate and higher degree of PFPA functionalization compared to conventional heating, owing to the efficient and uniform heating by microwave radiation. The conjugated carbohydrate ligands on glyco-FLG retained their binding affinity and specificity toward cognate lectins. Higher binding capacities were obtained with the glyco-FLG synthesized by microwave

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radiation, which resulted in higher carbohydrate density. Note that the developed method makes use of pristine FLG rather than the oxidized form of graphene, thereby reducing the introduction of oxygen-containing impurities and functionalities. The covalent conjugation of Man and Gal gave stable and multivalent glyco-FLG that retained the binding affinity and specificity toward lectins. The PFPA-NHS-functionalized FLG can be used to covalently attach any amine-containing (bio)molecules or materials to FLG, opening doors to the synthesis of new graphene-based nanocomposites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.8b00597.

FLG characterization, IR spectra, ligand density calculation (PDF)

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Notes

The authors declare no competing financial interest.

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