Engineering of a novel pluronic F127/graphene nanohybrid for pH responsive drug delivery

Haiqing Hu,1 Jinhai Yu,1 Yongyong Li,2 Jian Zhao,1 Haiqing Dong2

1Key Laboratory of Rubber-Plastics Ministry of Education/Shandong Provincial Key Laboratory of Rubber-plastics, School of Polymer Science and Engineering, Qingdao University of Science and Technology, Qingdao 266042, China
2The Institute for Advanced Materials and Nano Biomedicine, Tongji University, Shanghai 200092, China

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Abstract: Herein, a novel Pluronic F127/graphene nanosheet (PF127/GN) hybrid was prepared via an one-pot process including the simultaneous reduction of graphene oxide and assembly of PF127 and GN. The nanohybrid exhibits high water dispersibility and stability in physiological environment with the hydrophilic chains of PF127 extending to the solution while the hydrophobic segments anchoring at the surface of graphene via hydrophobic interaction. The PF127/GN nanohybrid is found to be capable of effectively encapsulating doxorubicin (DOX) with ultrahigh drug-loading efficiency (DLE; 289%, w/w) and exhibits a pH responsive drug release behavior. The superb DLE of the PF127/GN nanohybrid relies on the introduction of GN which is structurally compatible with DOX. Cellular toxicity assays performed on human breast cancer MCF-7 cells demonstrated that the PF127/GN nanohybrid displays no obvious cytotoxicity, whereas the PF127/GN-loaded DOX (PF127/GN/DOX) shows remarkable cytotoxicity to the MCF-7. Cell internalization study reveals that PF127/GN nanohybrid facilitates the transfer of DOX into MCF-7 cells, evidenced by the image of confocal laser scanning microscopy. The above results indicate the potential application of this novel nanocarrier in biomedicine.


INTRODUCTION

Nanomaterials-based drug vehicles have appeared as a bridge linking up nanotechnology with advanced drug delivery system, and their formulations mainly include nanoparticles,1 liposomes,2 polymeric microspheres,3 carbon nanotubes,4 and so on. The pharmaceuticals can be loaded on these nanoscaled materials by diverse mechanisms, such as surface absorption, hydrogen bonding, embedding, and other kinds of noncovalent interactions. To attain good therapeutic effect with the loaded pharmaceuticals, excellent biocompatibility, prolonged blood circulation time, adequate-loading efficiency, and controlled release capability are all essential issues to be taken into consideration for the drug carriers.

Graphene nanosheet (GN), a novel planar nanomaterial reported for the first time in 2004,5 has generated great attentions due to its distinct physical properties and potential applications on nanoelectronic devices,6–8 transparent conductors,9,10 nanocomposites,11,12 and so on. In this context, extensive researches are being pursued on single-, bi- and few-layers graphene13–15 as well as graphene oxide (GO).16,17 More recently, it is found that the planar structure of GN imparts it excellent capability for the immobilization of a large number of substances, including biomolecules,18 metals,19 drugs,20 fluorescent molecules,21 and so forth. Meanwhile, compared with other carbon nanomaterials, GN and its derivatives display longer blood circulation time, lower uptake in reticuloendothelial system,22 and ultrahigh in vivo tumor uptake.23 These properties make them optimal candidate for biomedical applications, such as drug-loading and delivery,24 photodynamic therapy,25 cell imaging, and diagnosis.26 Dai et al.27 did the pioneering work on utilizing PEGylated GO as a nanocarrier to load water-insoluble anticancer drugs by physisorption and evaluated its cytotoxicity to human colon cancer cell. Subsequently, Chen and coworkers28 explored the loading and controlled release behavior of drug loaded on GO. In Chen’s work, the drug-loading efficiency (DLE) of GO could reach as high as 235%, much higher than that of conventional polymeric drug carriers usually possessing a DLE lower than 100%.29

Compared with GO, which can be well-dispersed in aqueous solution due to its abundant hydrophilic groups such as carboxyl acid, hydroxyl groups, GN will easily precipitate in water attributed to the hydrophobic nature and...
the huge specific surface area. Consequently, for GN there are several preconditions for its biological application. First, rational functionalization is needed to offer GN with better aqueous solubility and biocompatiblity in the physiological environment. Second, GN with reasonable size is necessary. Dimensional control on various length scales and individual separation are necessary for graphene to suitably interact with biological systems in vitro or in vivo. The dimension of GN or GO available thus far is normally microns or larger in size, which is inappropriate for the biomedical application. Lastly, the dosage of GN used for biological systems must be as less as better. Although with no obvious toxicity, GN will also deposit in some organs for a long time due to its tough degradation. However, some development works have used the graphene for in vivo application and greatly encouraged further researches of graphene for biomedical applications.

In this article, we fabricate a new graphene-based nanohybrid with potential biomedical applications via an one-pot process including the simultaneous reduction of GO and assembly of Pluronic F127 (PF127) and GN. The presence of PF127 renders the GN high aqueous solubility and stability in physiological environment. The loading efficiency of the PF127/GN nanohybrid for doxorubicin (DOX) can be as high as 289% (w/w), which is much higher than that samples using PF127 only, and the release behavior of DOX from PF127/GN observably relies on pH value. Cellular toxicity assays and image of confocal laser scanning microscopy (CLSM) confirm that the DOX-loaded nanohybrid shows remarkable toxicity to MCF-7 cells and the DOX can be easily internalized into the MCF-7 cells mediated by PF127/GN nanohybrid.

EXPERIMENTAL
Materials and reagents
Flake graphite was purchased from Shanghai Yifan Graphite Co., with the average particle diameter of 25 μm (purity >99.9%). A 98% H2SO4 (AR), 30% H2O2 (AR), NaNO3 (AR), triethylamine (Et3N) (AR), and KMnO4 (AR) were obtained from Spectrum Laboratories with the average particle diameter of 25 μm (purity >99.9%). A 98% H2SO4 (AR), 30% H2O2 (AR), NaNO3 (AR), triethylamine (Et3N) (AR), and KMnO4 (AR) were obtained from Spectrum Laboratories (diameter ¼ 36 mm), which has a molecular weight cutoff of 8000–15,000 g/mol. All other chemicals were obtained from Sinopharm Chemical Reagent Company with analytical grade and were used as received.

Preparation of PF127 stabilized GN
In a typical experiment, GO was first prepared according to the modified Hummers’ method using natural flake graphite. The dimension of the obtained GO was about 80 nm, and the small size provided the possibility application in biological systems. For the preparation of PF127 functionalized graphene nanohybrid, 400 mg of PF127 was added to 15 mL of GO aqueous suspension (1.0 mg/mL) and the mixture was stirred for 30 min at room temperature. Then, 400 μL of hydrazine monohydrate was added to the mixture to reduce the GO to GN. The mixture stirred continuously at 40°C for 24 h under the sonication, then dialyzed against deionized water to remove the hydrazine monohydrate and unreacted copolymer. After reaction, a stable homogeneous black solution of PF127/GN nanohybrid was obtained.

Drug loading of DOX onto PF127/GN nanohybrid
The loading of DOX onto PF127/GN was performed by simply mixing PF127/GN with the given DOX. Briefly, 10 mL of 0.05 mg/mL PF127/GN in water was first sonicated with a certain concentration (0.1 mg/mL, 0.3 mg/mL, 0.5 mg/mL, 0.7 mg/mL, 0.9 mg/mL) of DOX in N,N-dimethylformamide for 0.5 h, respectively, and then stirred overnight at room temperature in the dark. After reaction, the mixture was ultracentrifuged at 13,000 rpm for 1 h so as to calculate the DLE. The amount of DOX loaded on the nanohybrid was calculated as follow. The DOX concentration in the supernatant was measured using a UV/Vis spectrophotometer by the absorbance at 480 nm and estimated by a standard DOX concentration curve generated from a series of DOX solutions with various concentrations. The amount of loaded DOX was harvested by subtracting the amount of supernatant from the initial amount of DOX. The DLE was calculated from the following formula:

\[
\text{DLE (w/w%)} = \left( \frac{\text{weight of loaded drug}}{\text{weight of nanohybrid}} \right) \times 100\%
\]

Release behavior of DOX from PF127/GN
The release behavior of DOX from the PF127/GN nanohybrid was carried out at 37°C in the saline buffer solution with different pH. Briefly, 2 mL of 1 mg/mL PF127/GN/DOX samples used for release experiments were placed into the dialysis chambers, which were dialyzed in 100 mL of saline buffer solution with pH 5, 7, and 9, respectively. The release reservoirs were placed in a shaking bed at 37°C with a rotation speed of 150 rpm, and 3 mL release media in the reservoir was taken out for characterization at desired time intervals and replenished with an equal volume of fresh media. The amount of DOX released from PF127/GN was quantified by the absorbance at 480 nm using UV/Vis spectroscopy.

Cytotoxicity of the PF127/GN-loaded DOX
WST assay was used to evaluate the cytotoxicity of PF127/GN and PF127/GN/DOX at different concentrations. Briefly, MCF-7 cells were seeded into 96-well plates (5000 cells/well) using 200 μL DMEM medium and incubated at 37°C for 24 h. Then, the medium in each well was replaced with 200 μL of culture medium containing PF127/GN and PF127/GN/DOX with different concentrations, respectively. After incubated at 37°C for 24 h, the medium in each well was replaced with 100 μL of fresh medium and 10 μL of WST-1 solution. After continuous incubation for 2 h, the relative cell viability was checked by the WST assay.
CLSM observation
MCF-7 cells were seeded in a 6-well plate at a density of 1 × 10^5 cells/well with complete medium. The cells were incubated for 24 h in a humidified atmosphere with 5% CO₂ at 37°C. Cells were washed by PBS and incubated at 37°C for 24 h with PF127/GN-loaded DOX hybrid in complete DMEM and then were washed with PBS twice and fixed with 4% formaldehyde. Then, using the DAPI to stain the cell nuclear and the cell were washed with PBS after 10 min. The slides were mounted and observed with a laser scanning confocal microscope (Olympus, FV300, IX71, Tokyo, Japan) equipment.

Characterization of PF127/GN and DOX-loaded PF127/GN
To characterize the resulting nanohybrid, Fourier transform infrared (FTIR) spectra of powdered samples were recorded on a Tensor 27 FT-IR spectrometer (Bruker AXS, China). UV-Vis detection and fluorescence spectra were obtained with an ultraviolet-visible spectrophotometer (Varian, Hong Kong) and a Hitachi F2500 luminescence spectrometer (Hitachi, Hong Kong), respectively. Thermogravimetric analyses (TGAs) was performed on a Pyris Diamond TG/DTA with the freeze-dried sample and the thermograms covered the temperature range from room temperature to 800°C at a scanning of 5°C/min. X-ray diffraction (XRD) was performed on a D/max2550VB3+/PC X-ray diffractometer using CuKa radiation (40 kV, 100 mA) with a scanning rate of 4°/min at 20°C. Atomic force microscope (AFM) images of samples deposited on a freshly cleaved silicon surface were observed in the tapping mode with a SPA-300HV instrument.

RESULTS AND DISCUSSION
GO is first prepared according to the modified Hummers’ method using natural flake graphite, whose size was carefully controlled down to sub-100 nm via adjusting both the oxidation condition and the power of subsequent ultrasound. The nanohybrid was obtained by a facile one-pot reduction of GO in aqueous solution in the presence of hydrazine and PF127, where hydrazine acted as the reduction agent and the PF127 as stabilizing agent. PF127 is chosen due to its amphiphilic nature and excellent biocompatibility, where the former property can be applied to stabilize the reduced GO through hydrophobic interaction and the latter will be favorable for the biomedical application. To confirm the successful reduction, the color change of the reactant is traced. The obvious color change take place as shown in Figure 1, the brown GO aqueous solution [Fig. 1(a)] change to dark after reduction [Fig. 1(c)], indicating that the GO has been changed chemically. To prove the change in the aspect of microstructure, the FTIR test is firstly obtained. As shown in Figure 2, a strong absorption band at 3403 cm⁻¹ corresponding to the H–O stretching vibration in GO dramatically decrease in PF127/GN nanohybrid combining with the disappearance of characteristic

FIGURE 1. Photographs of color change and the stability in cell medium between GO and PF127/GN, (a) GO in aqueous solution, (b) GO in cell medium, (c) PF127/GN in aqueous solution, and (d) PF127/GN in cell medium. The concentrations of all samples were kept at 0.2 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
peak for C=O absorption at 1723 cm$^{-1}$, indicating that the oxygen-containing functional groups on GO have been removed. In addition, two new characteristic absorption bands of PF127/GN appeared at 2983 cm$^{-1}$ and 2843 cm$^{-1}$, indicating the presence of $-\text{CH}_3$ and $-\text{CH}_2-$, which demonstrate the GN is modified by PF127. Interestingly, the characteristic peak of $-\text{CH}_2-$ for PF127/GN/DOX shows a little red shift compared with PF127, which may be ascribed to the hydrophobic interaction. To further illustrate the reduction of GO, XRD is used. The XRD patterns of GO and PF127/GN are shown in Figure 3 and the characteristic diffraction peak of exfoliated GO emerges at 9.4° with a interlayer distance of 0.87 nm [Fig. 3(a)], which is larger than that of the pristine graphite. This may be due to the formation of epoxy, hydroxyl, and carboxyl group on the surface of GO. However, for he resulting PF127/GN nanohybrid, the interlayer distance decline to 0.37 nm due to the removal of most of the oxygen-containing functional groups. Meanwhile, the diffraction peak located at 9.4° disappear, suggesting that the destroyed sp$^2$-hybridized carbon network are successfully restored by reduction.

TGA, which characterizes the decomposition behavior of the samples during heating, is used to analyze the content of graphene in the PF127/GN nanohybrid. Figure 4 shows the decomposition behavior of PF127/GN nanohybrid and GO. The TGA curve of GO exhibits three major mass loss as the increasing temperature. There is an about 10% weight loss below 100°C corresponding to the evaporation of water and about 42% around 200°C, presumably due to pyrolysis of the labile oxygen-containing functional groups to yield CO, CO$_2$ and steam. The mass loss at around 600°C is attributed to the bulk pyrolysis of carbon skeleton [Fig. 4(b)]. Compared with GO, the TGA curve reveals that the weight loss of the hybrid is also divided into three phases. The initial 10 wt% weight loss at around 250°C is ascribed to the removal of the labile oxygen-containing functional groups, which indicates the reduction is incomplete. A further 85 wt% weight loss is observed at around 360°C, which is arising from the decomposition of copolymer PF127. Finally, a loss of GN in mass 5 wt% happen at around 500°C due to the bulk pyrolysis of carbon skeleton [Fig. 4(a)]. Thus, the content of graphene in the nanohybrid is calculated to be around 15 wt%, which indicates a predominant weight ratio of PF127 in the nanohybrid.

The morphology of GO and PF127/GN nanohybrid are characterized with AFM in tapping mode. As shown in Figure 5, the AFM image of GO (left) dispersed in water on silicon surface shows the average thickness is about 0.876 nm which is about two layers and the lateral size is around 80 nm. Compared with the GO, the average thickness of the PF127-coated GN (right) is about 9.646 nm, which is much thicker than that of the GO. Obviously, it is reasonable to conclude that this phenomenon may be ascribed to the abundant copolymer PF127 immobilized on the surface of GN. From the AFM image, we can also see that GN remains separated in the dispersion with the aid of PF127.
The nanostructures should possess high stability in physiological condition, which is a prerequisite to meet the practicality for biomedical application, thus cell medium is used to preliminarily characterize the stability of PF127/GN nanohybrid in physiological environment. As shown in Figure 1(d), the resulting PF127/GN nanohybrid is found to be steady in cell medium and can maintain for more than one week, which is attributed to the hydrophobic poly(propylene oxide) (PPO) segments binding to the hydrophobic surface of graphene via the hydrophobic effect and the hydrophilic poly(ethylene oxide) (PEO) chains extending into water. This unique structure would suppress the appearance of aggregation phenomenon. In contrast, obvious aggregation for GO is observed in cell medium once GO aqueous solution being added [Fig. 1(b)]. Consequently, the stability property in the cell medium of GN modified by copolymer PF127 provides a fundamental basis for the drug delivery in the physiological environment. This approach also leads to a copolymer functionalized GN that is stable in the aqueous solution.

DOX, an antitumor drug, is used as a model drug to evaluate the drug-loading capability of the PF127/GN nanohybrid. The loading capacity of PF127/GN nanohybrid is measured by UV-Vis spectrum at absorbance of 480 nm, which is calculated by the difference of DOX concentration between the initial DOX solution and the supernatant solution after loading. To study the correlation between the concentration of DOX and the DOX-loading efficiency of PF127/GN, the loading experiment is carried out under the condition of different initial DOX concentrations with respect to the same concentration of PF127/GN (0.5 mg/mL). As shown in Figure 6, it is found that the loading efficiency of the PF127/

**FIGURE 5.** AFM images of GO (left) and PF127/GN (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
and PF127/GN both play important roles in the high loading of DOX onto PF127/GN. To elucidate this issue, fluorescence and UV tests are carried out. For free DOX, fluorescence emission maximum appears at 593 nm with an excited source at 480 nm. However, on excitation at the same wavelength, significant fluorescence quenching of its emission band is observed for the PF127/GN/DOX containing the same concentration of DOX (Fig. 7), which is mainly ascribed to the π–π stacking between DOX and PF127/GN and results in a photoinduced electron-transfer process or efficient energy transfer. Figure 8 shows the UV-Vis spectrum of PF127/GN, GO, DOX, and PF127/GN/DOX. It is found that GO displays a single broad absorption peak at 226 nm, whereas the peak red shifts to 244 nm after reduction for the PF127/GN, which proves the structure of GN is partially restored. Free DOX solution exhibits strong absorption at 233, 253, 291, and 480 nm, and the stacking of DOX onto PF127/GN is demonstrated from the UV–Vis spectrum of the PF127/GN/DOX solution, which shows the characteristic absorption peaks of DOX clearly. In addition, after DOX loaded onto the PF127/GN nanohybrid, the absorption peaks of DOX show a little red shift. For example, the absorption peaks at 233 and 480 nm shift to 247 and 488 nm, which are generally assumed due to the ground-state electron donor–acceptor interaction between the two components.43

pH alteration may lead to the change of hydrogen bonding and different solubility of DOX resulting in different release rate, so the release behavior of DOX from the PF127/GN is investigated in different pH medium. As shown in Figure 9, it is found that DOX releases rapidly from PF127/GN at the initial stage, and the release rate gradually remains unchanged after 6 h in pH ≥7. Under basic and acidic conditions, the releasing amount of DOX in the first 90 h is much higher than that at neutral conditions. Specifically, about 56% and 25% of the total loaded DOX is released from the nanohybrid after 90 h at pH 5 and 9, respectively, whereas only 15% of DOX is released under...
neutral conditions (pH = 7) in the same period. The more retarded release at neutral condition may be due to that the hydrogen-bonding interaction at neutral condition is the strongest, resulting in the inefficient release. In addition, the release of DOX from the nanohybrid under acid condition is much higher than that under basic condition, which is attributable to the much higher solubility of DOX under acid condition than that under basic condition. This pH-dependent drug release mechanism plays an important role in the clinical setting, because the microenvironments in extracellular tissues of tumor, intracellular lysosomes, and endosomes are acidic.

On the basis of the above experimental attempts, we investigate the cellular cytotoxicity of PF127/GN and PF127/GN/DOX to the human breast cancer MCF-7 cells. All the samples are incubated with MCF-7 cells for 24 h, respectively, and WST assay is performed. As shown in Figure 10, no obvious toxicity is detected for PF127/GN with different concentrations even under the high concentration up to 1000 mg/mL, and MCF-7 cells still maintain high cellular viability. Compared with free PF127/GN, the cytotoxicity of PF127/GN-loaded DOX against MCF-7 is obvious higher than that without DOX. Furthermore, the cellular viability gradually reduce as the enhancement of concentration, and it is found that the PF127/GN/DOX with a concentration of up to 1000 mg/mL shows the most remarkable cytotoxicity to the MCF-7 cells line, which indicates that PF127/GN/DOX has the potential for killing cancer cells in vitro.

Finally, we use CLSM to investigate the cellular uptake behaviors of the PF127/DOX-loaded DOX against MCF-7 cells. As shown in Figure 11, obvious red fluorescence of DOX is detected inside the MCF-7 cells after incubation of the MCF-7 cells with PF127/GN-loaded DOX, suggesting the successful internalization of the DOX. Meanwhile, we can observe that the fluorescence of DOX is detected in the whole cell.

CONCLUSIONS

In this article, Pluronic copolymer PF127 is utilized as the solubilizing agent for GN formed through in situ reduction by hydrazine from chemically exfoliated GO. The obtained copolymer PF127-coated graphene aqueous solution is stable in the cell medium, which is due to the hydrophobic PPO segments binding to the hydrophobic surface of graphene via hydrophobic effect, whereas the hydrophilic PEO chains extending into water. Furthermore, utilizing the hydrophobic interactions and π-π stacking between DOX and PF127/GN, the high-loading efficiency and pH-dependent release of DOX on PF127/GN are investigated. The loading efficiency of PF127/GN increases with the concentration of DOX and can reach as high as 289% (w/w) at the initial DOX concentration of 0.9 mg/mL. The release behavior of DOX from PF127/GN with the change of pH shows that the release amount at acid condition is higher than that at neutral and basic conditions. From the cytotoxicity assay and the image of CLSM, we can see that the PF127/GN/DOX shows remarkable toxicity and the DOX can be easily internalized into the MCF-7 cells mediated by PF127/GN nanohybrid. This graphene-based nanohybrid, with good solubility and biocompatibility, is expected to find new practical applications in biomedicine, biomaterials separation, and biodiagnosis.

REFERENCES


