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Surface Functionalization of Chemically Reduced Graphene Oxide for Targeted Photodynamic Therapy

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Abstract

In this study, using chemically reduced graphene oxide (GO) as a model nanocarbon, we successfully developed a facile surface-functionalization strategy of nanocarbons to allow both biocompatibility and receptor targeted drug delivery. Polyvinylpyrrolidone (PVP) coating improves aqueous dispersibility and biocompatibility of GO, and provides anchoring sites for ACDCRGDCFCG peptide (RGD4C). Aromatic photosensitizer chlorin e6 (Ce6) can be effectively loaded into the rGO-PVP-RGD system *via* hydrophobic interactions and π - π stacking. The nanodelivery system can significantly increase the accumulation of Ce6 in tumor cells and lead to an improved photodynamic therapy (PDT) efficacy as compared to Ce6 alone. The facile surface functionalization strategy can be applied to other nanomaterials such as carbon nanotubes, and inorganic nanomaterials.

Graphical Abstract

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Keywords

Graphene Oxide; Polyvinylpyrrolidone; Chlorin e6; RGD; Photodynamic Therapy

INTRODUCTION

Graphene, a two-dimensional sp^2 -hybridized carbon sheet with exceptional electrical, mechanical, optical, and chemical properties, has been explored extensively for biomedical applications, such as novel biosensors, tissue engineering scaffolds, drug/gene delivery carriers, and phototherapeutic agents.^{1–9} Particularly, it has poly-aromatic surface structure with large specific surface area, which is efficient for aromatic drug loading *via* π - π stacking.^{10–13} Yet, three major issues of pristine graphene-poor dispersibility, inadequate selectivity, and inherent cytotoxicity-limit its further biomedical applications.^{13–15}

So far, a number of studies have uncovered that pristine graphene and graphene oxide (GO) could induce toxicity in biological systems, strongly depending on their surface chemistry.^{16–19} Suitable surface functionalization can improve their aqueous dispersibility while reducing aggregation, minimizing toxicity, and enhancing selectivity. The development of facile surface-functionalization strategies of modifying graphene with various biocompatible coatings and targeting ligands is paving the avenue towards the goal of effective biomedical applications of graphene. Currently, two main strategies have been employed to fabricate surface functionalized graphene: i) noncovalent adsorption,^{18, 20, 21} in which graphene can be wrapped with aromatic compounds, surfactants, polymers, or biomolecules *via* van der Waals forces, π - π stacking, or hydrophobic interactions; ii) covalent conjugation,^{1, 12, 22, 23} in which graphene can be conjugated with molecules of interest *via* covalent bonds. It is well known that non-covalent adsorption has the risk of the surface coatings being replaced by serum proteins under physiological condition, leading to undesirable accumulation and poor pharmacokinetics.

Herein, we report a facile surface-functionalization strategy of modifying chemically reduced graphene oxide (rGO) for targeted delivery of photosensitizer (PS) and

photodynamic therapy (PDT). GO sheets with abundant oxygen-containing surface groups were chemically reduced using *L*-ascorbic acid (*L*-AA).²⁴ With the formation of rGO, polyvinylpyrrolidone (PVP) was covalently grafted on the atomically flat surface of rGO.^{25, 26} Afterwards, integrin specific ACDCRGDCFCG peptide (RGD4C) molecules were anchored onto the backbone of PVP via hydrogen bonding.²⁷ The as-prepared RGD-anchored PVP-modified rGO (rGO-PVP-RGD) was used as a nanocarrier for targeted PS chlorin e6 (Ce6) delivery. Ce6 was effectively loaded into the rGO-PVP-RGD system *via* hydrophobic interactions and π - π stacking.¹⁰ The efficient loading of Ce6 on both accessible sides of rGO and the anchored RGD on the surface of rGO significantly increases the accumulation of Ce6 in cancer cells and leads to an improved PDT efficacy as compared to Ce6 alone.

MATERIALS AND METHODS

Materials

Graphite powder (500 mesh) was obtained from Beijing Chemical Reagents (Beijing, China). KMnO_4 , NaNO_3 , NaOH , concentrated H_2SO_4 , concentrated HCl , H_2O_2 (30%), and PVP (MW, 40 kg mol^{-1}) were all of analytical grade and purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The dicyclic peptide ACDCRGDCFCG (RGD-4C) was synthesized by GL Biochem Ltd. (Shanghai, China). Chlorin e6 (Ce6) was purchased from Frontier Scientific, Inc. (Logan, UT). Singlet oxygen sensor green (SOSG) was purchased from Invitrogen (Grand Island, NY). All the above chemicals were used without further purification. In all experiments, aqueous solutions were prepared with ultrapure water (18 M Ω).

MGC803 gastric cancer cell line was obtained from the cell store of Chinese Academy of Sciences (Shanghai, China). Cell culture products and reagents, unless mentioned otherwise, were purchased from Gibco (USA). MGC803 cells were kept at 37 °C and under 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen).

Preparation of GO and rGO-PVP-RGD

GO was prepared using natural graphite powder as the raw material by a modified Hummers method and our previous reports.^{24, 28} For the preparation of PVP coated rGO, 0.05 g of PVP (MW, 40 kg mol^{-1}) and 0.1 g of *L*-ascorbic acid (*L*-AA) were added to 200 mL of GO aqueous solution (0.25 mg mL^{-1}). The mixture was sonicated for ~10 min, and then stirred at 80 °C for 4 h. A stable homogeneous black suspension was obtained. The black rGO-PVP product was purified through filtration, washed three times with DD water, and finally redispersed in pH 7.4 phosphate buffered saline (PBS). Subsequently, 100 μL of RGD-4C (10 mg mL^{-1}) was added to the above rGO-PVP in PBS (20 mL, 0.5 mg mL^{-1}). The mixture was stirred for 12 h at 70 °C. Finally, the black rGO-PVP-RGD product was purified through filtration, washed three times with DD water, and finally redispersed in pH 7.4 PBS.

Photosensitizer Ce6 Loaded by rGO-PVP-RGD

Ce6 (1 mL, 1 mg mL⁻¹) was added to rGO-PVP or rGO-PVP-RGD in PBS (20 mL, 0.5 mg mL⁻¹ rGO) at room temperature and the mixture was stirred for 24 h. Then the mixture was purified through filtration, washed three times with pH 7.4 PBS, and finally redispersed in pH 7.4 PBS for further use. The encapsulation and loading efficiency of Ce6 was calculated by using Ce6 UV calibration curve at 663 nm (in PBS solution). Each experiment was repeated three times.

Characterization

Atomic force microscope (AFM) images of rGO-PVP and rGO-PVP-RGD were taken on a MultiMode Nanoscope V scanning probe microscopy (SPM) system (Veeco, USA). Commercially available AFM cantilever tips with a force constant of ~48 N m⁻¹ and resonance vibration frequency of 330 kHz were used. The scanning rate was set at 0.7 to 1 Hz. Transmission electron microscopy (TEM) images were acquired using a JEM-2010 (JEOL Ltd., Japan) operated at an accelerating voltage of 200 kV. UV-Vis spectra were measured at 20 °C using a UV-2550 UV-Visible spectrophotometer (Shimadzu, Japan) equipped with a 10-mm quartz cell, where the light path length was 1 cm. Fluorescence spectra were recorded on a FL-4600 spectrofluorimeter (Hitachi, Japan). The Fourier transform infrared (FTIR) spectra were recorded on a Bruker EQUINOX 55 FTIR Spectrometer.

Cellular Uptake *in vitro*

MGC803 cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum and incubated at 37 °C in a humidified 5% CO₂ atmosphere. For subcellular localization, cells (1 × 10⁴ cells per well) were seeded in 8-well Lab-Tek chamber slide and incubated overnight. Then the cells were incubated with rGO-PVP-Ce6 (10 μM Ce6), RGD plus rGO-PVP-RGD-Ce6 (10 μM Ce6), and rGO-PVP-RGD-Ce6 (10 μM Ce6), respectively, in the dark. After 2 h incubation, cells were rinsed with PBS (pH 7.4) for three times and monitored by fluorescence microscopy (Olympus X71) and CRi Nuance multispectral imaging systems (Cambridge Research & Instrumentation, Inc., Woburn, MA, USA). The spectral cubes for cells, which contain the complete spectral information at 10-nm wavelength intervals from 520 to 720 nm, were collected by CRi Nuance systems.

For cellular uptake efficiency study, the cells (5 × 10⁴ cells per well) were seeded in 6-well plates and incubated overnight, then incubated with rGO-PVP-Ce6 (10 μM Ce6), RGD plus rGO-PVP-RGD-Ce6 (10 μM Ce6), and rGO-PVP-RGD-Ce6 (10 μM Ce6), respectively. After 2 h incubation, cells were rinsed with PBS for three times, trypsinized, and resuspended with medium. Afterwards, cells were analyzed by Accuri C6 flow cytometer using a C Flow Plus software (BD Biosciences, USA).

Cytotoxicity Assessment

MGC803 cells (3 × 10³ cells per well) were seeded in 96-well plates and incubated overnight at 37 °C in a humidified 5% CO₂ atmosphere. After being rinsed with PBS (pH 7.4), the cells were incubated with rGO-PVP and rGO-PVP-RGD in the concentration range of 0~500 μg mL⁻¹ for 24 h at 37 °C in the dark. Then the cells were rinsed with PBS and

incubated for another 48 h. Cell viability was determined by a MTT assay. Cells were rinsed with PBS and then incubated with culture medium containing 0.5 mg mL^{-1} of MTT reagent for 3 h. The medium was then removed and the formazan crystals formed were dissolved in $100 \mu\text{L}$ of DMSO. The absorbance at 492 nm for each well was recorded by a microplate reader.

***In vitro* PDT Effect**

Four 96-well plates were set up as rGO-PVP-RGD-Ce6 without laser irradiation (control group), free Ce6 with laser irradiation, rGO-PVP-Ce6 with laser irradiation, and rGO-PVP-RGD-Ce6 with laser irradiation. MGC803 cells (3×10^3 cells per well) were seeded in the above plates and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. After being rinsed with PBS (pH 7.4), the cells were incubated with free Ce6, rGO-PVP-Ce6, and rGO-PVP-RGD-Ce6 in the concentration range of 0~50 μM (Ce6) for 24 h at 37°C in the dark.

The cells in the irradiation group were then rinsed again with PBS and immersed in $100 \mu\text{L}$ of fresh culture medium before being irradiated using a 671 nm laser with energy density of $\sim 30 \text{ mW cm}^{-2}$ for 3 min. After irradiation, cells were incubated for 48 h in a 5% CO_2 , 95% air humidified incubator at 37°C . The dark control group keeps identical to the irradiation groups except for the lack of laser irradiation. Cell viability was also determined by a MTT assay. Meanwhile, the PDT effect was also determined by propidium iodide (PI) and 0.4% trypan blue staining. The cells were also observed using a fluorescence microscopy (Olympus X71) and a CRi Nuance multispectral imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA, USA).

RESULTS AND DISCUSSION

Preparation of rGO-PVP-RGD

The synthetic route of Ce6 loaded rGO-PVP-RGD is illustrated in Scheme 1. GO was prepared using graphite powder as the raw material by a modified Hummers method.²⁸ At the beginning of the reduction, some of the *tert*-amide groups in the pyrrolidones of PVP were protonated by the L-AA, activating the amide group of PVP. Subsequently, the activated *tert*-amide groups of PVP were attacked by the epoxide or hydroxyl groups of GO, resulting in the break of the amide bond and the formation of an ester bond. After incubation with RGD-4C at 70°C for 12 h, the noncovalent peptide-polymer interactions will take place between pyrrolidones of PVP and N-terminus of RGD-4C. Finally, the Ce6 was loaded onto the surface of rGO-PVP-RGD *via* hydrophobic interactions and π - π stacking.

Characterization of rGO-PVP-RGD-Ce6

PVP has been extensively employed as a basic building material for the surface modification of various nanomaterials, such as magnetic nanoparticles²⁹ and gold nanoparticles,³⁰ etc. PVP has also been used as a polymeric nanocarrier for drug delivery. In our case, PVP was coupled to GO *via* pyrrolidine ring opening and ester bond formation.²⁵ The size and morphologies of the as-prepared GO and rGO-PVP were characterized by atomic force microscope (AFM). Fig. 1a and b showed the AFM images of GO and rGO-PVP. The

morphologies of GO and rGO-PVP existed in sheet-like shapes. The average thicknesses of rGO and rGO-PVP are 0.96 ± 0.05 and 2.81 ± 0.18 nm (Fig. 1c), respectively. The difference in thickness, ~ 0.85 nm, is attributed from the PVP layers covering both sides of rGO.

For all the nanomaterials, although they can partly accumulate into tumor tissues based on EPR (enhanced permeability and retention) effect,^{31, 32} the passive accumulation efficiency of nanomaterials into tumor tissues is typically low. Therefore, special biotarget molecules were called for to improve the accumulation efficiency of nanomaterials into tumor tissues by active targeting mechanism.^{33, 34} Here we chose $\alpha_v\beta_3$ integrin as the target because of its high expression in gastric carcinoma.³⁵ RGD-4C peptide is an ideal integrin ligand for targeting tumor angiogenesis (i.e., new blood vessel formation) and various tumor cells, such as MGC803 cells.^{35, 36} After PVP coating of rGO, the RGD-4C peptide was anchored on the chains of PVP *via* hydrogen bonding. Afterwards, Ce6 was loaded on both sides of rGO-PVP-RGD *via* hydrophobic interactions and π - π stacking. Successful decoration of RGD-4C on rGO-PVP was evidenced by Ellman's test and loading of Ce6 on rGO-PVP-RGD was confirmed by UV-Vis, FT-IR, and fluorescence emission spectra. After loading Ce6, the absorption of rGO-PVP-RGD-Ce6 displays the characteristic Soret peak of Ce6 at 404 nm, and the Q-bands at 651 and 706 nm (Fig. 2a).¹⁰ The encapsulation efficiency is 80% based on the added Ce6 ($m_{\text{rGO-PVP-RGD}} : m_{\text{Ce6}} = 10 : 1$), calculated by using Ce6 UV calibration curve at 663 nm. The corresponding Ce6 loading efficiency of rGO-PVP-RGD-Ce6 is $\sim 7.41\text{wt}\%$.

Fig. 2b shows FT-IR spectra of rGO-PVP, rGO-PVP-RGD, and rGO-PVP-RGD-Ce6. All samples exhibit a broad absorption band at around 3450 cm^{-1} , which is attributed to the stretching vibration of hydroxyl group. In the case of rGO-PVP, the IR signals at 1624, 1540, and 1406 cm^{-1} are assigned to the stretching vibrations of tertiary amide group of PVP (mainly C=O stretching vibrations), phenyl ring and bending vibrations of methylene group, respectively. Obviously, in both rGO-PVP-RGD and rGO-PVP-RGD-Ce6, there are newly generated peaks at around 2961 and 2855 cm^{-1} , which are assigned to $\nu_{\text{as}}(\text{CH}_2)$ and $\nu_{\text{s}}(\text{CH}_2)$ of the alkyl chain of RGD, respectively. For rGO-PVP-RGD, the band at around 1636 cm^{-1} results from secondary amide group of RGD, which is higher than the stretching vibration of tertiary amide group of PVP. Due to the formation of H-bond between pyrrolidones of PVP and N-terminus of RGD-4C, the band of amide group of PVP shifted from 1624 to 1564 cm^{-1} , overlaps with the vibrations of phenyl group, resulting in the enhancement of IR signal at 1564 cm^{-1} . The band at around 1100 cm^{-1} is assigned to the C-O stretching vibration mode of RGD-4C. These results suggest that RGD-4C was successfully anchored on the chains of PVP *via* hydrogen bonding. Comparing the IR spectrum of Ce6 with those of rGO-PVP-RGD-Ce6 (Fig. 2c and d), both of them have three markedly similar bands sited at 1708, 1233, and 733 cm^{-1} , which cannot be found in the IR spectrum of rGO-PVP-RGD.

The band at around 1708 cm^{-1} can be assigned to the C=O stretching vibrations of three carboxyl groups of Ce6. The NH deformation vibration of the Ce6 also makes a contribution to the band of 1233 cm^{-1} and the band observed at around 733 cm^{-1} is assigned to the maximal amplitude of NH out-of-plane bending. These results suggest that Ce6 was

successfully loaded by rGO-PVP-RGD. To further understand the interaction between rGO-PVP-RGD and Ce6, fluorescence spectra of Ce6, rGO-PVP, rGO-PVP-RGD, and rGO-PVP-RGD-Ce6 are shown in Fig. 2e. The fluorescence spectra of Ce6 and rGO-PVP-RGD-Ce6 are similar with emission peak at ~652 nm. The results also show drastic fluorescence quenching of Ce6 in rGO-PVP-RGD-Ce6, most likely due to the direct contact between Ce6 and aromatic regions of the rGO sheets.³ The generation of singlet oxygen ($^1\text{O}_2$) from rGO-PVP-RGD-Ce6 was detected by measuring the fluorescence signal of singlet oxygen sensor green (SOSG) which reacts with $^1\text{O}_2$.³⁷ The fluorescence intensity of SOSG at 528 nm increases with prolonged irradiation (Fig. 2f). For SOSG alone or a mixture of SOSG and rGO-PVP-RGD, the fluorescence intensities remain unaltered upon irradiation, indicating rGO-PVP-RGD alone cannot generate $^1\text{O}_2$.

Cellular Uptake *in vitro*

To investigate the selective targeting ability of rGO-PVP-RGD-Ce6, MGC803 cell uptake efficiency of rGO-PVP-Ce6, RGD plus rGO-PVP-RGD-Ce6, and rGO-PVP-RGD-Ce6 were evaluated by flow cytometry (Fig. 3a). After 2 h incubation, the fluorescence intensity of Ce6 in the rGO-PVP-RGD-Ce6 group was significantly higher than that in the rGO-PVP-Ce6 group. RGD-4C peptide can effectively block the binding of rGO-PVP-RGD-Ce6 with $\alpha_v\beta_3$ integrin. Since the wrapping time is affected by many factors, including available amount of free ligands on cell membrane,^{38, 39} the uptake of rGO-PVP-RGD-Ce6 may be slowed down due to the occupation of free receptors. The corresponding fluorescence images (Fig. 3b–d) are in a good agreement with the flow cytometry result, confirming that more rapid and improved MGC803 cell uptake of rGO-PVP-RGD-Ce6 over rGO-PVP-Ce6 was due to integrin mediated endocytosis.

Cytotoxicity Assessment

To evaluate the cytotoxicity of rGO-PVP and rGO-PVP-RGD, an MTT assay was performed on MGC803 cells (Fig. 4a). Cell viability was normalized by the control group of untreated cells. The results indicate no cytotoxicity of rGO-PVP and rGO-PVP-RGD in the concentration range of 0~500 $\mu\text{g mL}^{-1}$, with cell viability more than 95%. PDT effect of rGO-PVP-RGD-Ce6 was investigated in the concentration range of 0~50 μM (0.6~30 $\mu\text{g mL}^{-1}$ Ce6) on cells (Fig. 4b), irradiation induced a concentration-dependent cytotoxicity to cells for Ce6, rGO-PVP-Ce6, and rGO-PVP-RGD-Ce6. The PDT effect of rGO-PVP-RGD-Ce6 is much stronger than that of free Ce6 and rGO-PVP-Ce6 at all tested concentrations, which is ascribed to the active targeting ability of RGD.

In vitro PDT Effect

Laser-triggered PDT effect of rGO-PVP-RGD-Ce6 was also observed using propidium iodide (PI) and trypan blue staining.³² Fig. 5a showed the MGC803 cells at the center of laser spot. The overlay of Ce6 and PI fluorescence suggested effective cell killing by rGO-PVP-RGD-Ce6 upon irradiation. The cells after irradiation and PI staining exhibited two emission peaks at 635 and 670 nm, which are attributed to PI and Ce6, respectively (Fig. 5b). As shown in Fig. 5c, all the cells were killed and displayed a blue color on the laser spot. At the boundary of laser spot, cells without irradiation displayed good physiological state. Intense red signal around the nucleus was also observed, indicating that most Ce6

molecules remain after irradiation, which implied that the irradiation treatment can be repeated.

CONCLUSIONS

In this study, using rGO as a model nanocarbon, we successfully developed a facile surface-functionalization strategy to allow both biocompatibility and receptor targeted drug delivery. PVP coating improves aqueous dispersibility and biocompatibility of rGO, and provides anchoring sites for RGD peptide. Aromatic PS Ce6 can be effectively loaded into the rGO-PVP-RGD system via hydrophobic interactions and π - π stacking. The nanodelivery system can significantly increase the accumulation of Ce6 in tumor cells and lead to an improved PDT efficacy as compared to Ce6 alone. Our results lay the foundation for surface-functionalization of nanocarbons and inorganic nanomaterials for further biomedical applications, particularly targeted drug delivery.

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Fig. 1. Atomic force microscopy (AFM) images of (a) GO and (b) rGO-PVP on mica substrates, and (c) height of GO and rGO-PVP. The scale bar equals to 100 nm.

Fig. 2.

(a) UV-vis spectra, (b–d) FT-IR spectra, (e) fluorescence emission spectra. (f) Fluorescence emission spectra of rGO-PVP-RGD-Ce6 in SOSG solution with increased laser irradiation time.

Fig. 3.
 (a) Fluorescence intensity by flow cytometry and (b–d) fluorescence images of MGC803 cells after incubation with rGO-PVP-Ce6, RGD-4C plus rGO-PVP-RGD-Ce6, and rGO-PVP-RGD-Ce6 ($10 \mu\text{M}$ Ce6) for 2 h at 37°C . DAPI (4',6-diamidino-2-phenylindole) staining was used to visualize nuclei.

Fig. 4.

(a) Dark toxicity of rGO-PVP and rGO-PVP-RGD on MGC803 cells. (b) MGC803 cell viability at different concentrations of Ce6, rGO-PVP-Ce6 or rGO-PVP-RGD-Ce6 for 24 h at 37 °C with or without irradiation using a 671 nm laser (30 mW cm⁻²). Asterisk denote the phototoxicity of rGO-PVP-RGD-Ce6 is significantly higher than other three groups at that concentrations. ($P < 0.05$).

Fig. 5. (a) Propidium iodide (PI) and (c) trypan blue staining images of MGC803 cells incubated with rGO-PVP-RGD-Ce6 ($10 \mu\text{M}$ Ce6) for 2 h at 37°C after laser irradiation. (b) Fluorescence emission spectra of Ce6 and PI in the treated MGC803 cells.

Scheme 1.

The synthetic route of Ce6 loaded rGO-PVP-RGD. GO sheets with abundant oxygen-containing surface groups were chemically reduced using *L*-ascorbic acid (*L*-AA). With the formation of rGO, PVP was covalently grafted on the atomically flat surface of rGO. Afterwards, integrin specific RGD-4C peptide molecules were anchored onto the backbone of PVP via hydrogen bonding. Ce6 was effectively loaded into the rGO-PVP-RGD system via hydrophobic interactions and π - π stacking.