MRI-Visualized, Dual-Targeting, Combined Tumor Therapy Using Magnetic Graphene-Based Mesoporous Silica

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Targeting peptide-modified magnetic graphene-based mesoporous silica (MGMSPI) are synthesized, characterized, and developed as a multifunctional theranostic platform. This system exhibits many merits, such as biocompatibility, high near-infrared photothermal heating, facile magnetic separation, large T2 relaxation rates (r2), and a high doxorubicin (DOX) loading capacity. In vitro and in vivo results demonstrate that DOX-loaded MGMSPI (MGMSPID) can integrate magnetic resonance imaging, dual-targeting recognition (magnetic targeting and receptor-mediated active targeting), and chemo-photothermal therapy into a single system for a visualized-synergistic therapy of glioma. In addition, it is observed that the MGMSPID system has heat-stimulated, pH-responsive, sustained release properties. All of these characteristics would provide a robust multifunctional theranostic platform for visualized glioma therapy.

1. Introduction

An efficient theranostic platform for cancer requires the combination of imaging, targeting recognition, and different theranostic modalities in a single system. The main challenge for this platform was the design and synthesis of a multifunctional nanomedicine.[1] Recently, sp2 carbon nanomaterials, particularly graphene nanosheets, were found to possess extraordinary mechanical, electronic, and thermal properties and were investigated for applications in drug delivery, photothermal therapy, and biosensors.[2] In addition, to create graphene nanomedicine with additional properties and functions, such as targeting recognition or diagnosis, various biomolecules and inorganic nanoparticles have been conjugated on graphene surfaces.[3] All of these materials provided an opportunity for the preparation of a multifunctional graphene nanomedicine. However, dramatic improvements in current capabilities are still needed for practicable nanomedicine,[4] such as combining the different merits in one novel medicine and exerting their synergistic effect,[5] reducing the hydrophobicity and toxicity in biological systems of pristine graphene or reduced graphene oxide as therapeutic reagents,[6] improving the unstable release and loading capacity of anti-cancer drugs aroused by the weak π–π stacking interaction,[7] or enhancing the bonding capacity of different functional biomolecules. Few functional groups exist on the surfaces of pristine graphene or reduced graphene oxide, making them difficult to bind other molecules except via weak non-covalent adsorption.[8] As partially
overcoming these drawbacks, a therapeutic platform based on mesoporous silica-coated graphene nanosheet was developed for combined therapy of tumor in vitro in our previous work. However, it was highly desired that an image-guided chemo-photothermal theranostic platform with enhanced targeting ability should be constructed and investigated in vitro and in vivo because this is a trend of new clinical mode.

In regards to the application of multifunctional nanomedicine, glioma is the most aggressive brain tumor in humans, with a survival rate of only 5% after 5 years. The common therapy for glioma is surgery, chemotherapy, and radiotherapy. Unfortunately, the diffuse and highly invasive nature of glioma cells, as well as the undesired adverse effects to normal tissues and insufficient dosage to diseased regions with uncontrollable drug delivery, contributes to the failure of glioma treatment. Thus, novel and efficient therapeutic strategies are urgently needed to achieve significant improvement. Magnetic resonance imaging (MRI) is currently the gold standard for non-invasively diagnosing glioma. Ligand-modified superparamagnetic iron oxide nanoparticles used as sensitive MRI contrast agents have been shown to be nontoxic, biocompatible, and highly specific for imaging glioma. The intrinsic magnetic properties of nanoparticles have also been applied in magnetic separation and targeting drug delivery. Thus, magnetic-targeting delivery and MRI monitoring of the therapeutic progress would be helpful for eliminating the unintended damage or side effects of nanomedicine in healthy organs and tissues.

In this work, we developed a novel, nontoxic, and multi-functional magnetic graphene-based nanomedicine that combines pH and photothermal responsive release with specific dual-targeting chemo-photothermal therapy and MRI monitoring for glioma therapy.

2. Results and Discussion

The multifunctional nanomedicine was structured and shown in Scheme 1. Magnetic graphene (MG) was prepared by one-step solvothermal reduction of small graphene oxide (GO) nanosheets and Fe$_3$O$_4$. An amido-mesoporous silica coating was obtained by surfactant assisted copolymerization of tetraethyl orthosilicate (TEOS), aminopropyltriethoxysilane (APTES), and MG. Scanning electron microscopy (SEM) showed that the resulting magnetic graphene-based mesoporous silica (MGMS) nanosheets have a lateral size of approximately 200 nm (Figure 1A), much smaller than the added GO nanosheets due to the successive ultrasonication and solvothermal treatment. Transmission electron microscopy (TEM) of the MGMS revealed spherical nanoparticles evenly distributed on graphene nanosheets, and both were...
covered by mesopores (Figure 1B). The spherical nanoparticles were indexed as magnetic Fe3O4 nanoparticles with a size of 4–15 nm (JCPDS: 19-629) using high-resolution TEM (Figure 1C and Figure S1). The graphene content of MG and MGMS were 43 wt% and 18 wt%, respectively, according to the TG and DTG curves (Figure S2). The N2 adsorption-desorption isotherm and pore size distribution curve (Figure 1D) indicated that the MGMS had a large BET surface area (1027 m2/g), pore volume (0.8 cm3/g), and uniform pore diameter (3.1 nm). Raman spectra proved the existence of defective graphene in MGMS with the emergence of obvious signals at 1330 cm−1 (D-band), 1580 cm−1 (G-band), and 2700 cm−1 (2D band) (Figure 1E).16 IR spectra demonstrated that the oxygen-containing groups of the added GO were largely reduced during the magnetic nanoparticle growth and mesoporous silica coating (Figure S3A–C).17

To improve the biocompatibility of the MGMS and offer it another targeting function, the MGMS (–NH2) and glioma-targeting interleukin-13-based peptide (IP-SH) were covalently linked to bifunctional PEG (NHS–PEG3400–MAL) by specific reactions with –NH2–NHS and –SH–MAL, respectively, resulting in the IP-modified conjugate (MGMSPI). Successful synthesis of MGMSPI was confirmed by IR spectra (Figure S3C,D).18 The product with only PEG modification (MGMSP) was also synthesized.

Investigation of the magnetic properties of the prepared nanocomposites determined that, although the saturation magnetization values of MGMSPI were smaller than those of MG and MGMS due to the coating (Figure S4A), it was still suitable for magnetic separation (magnetic target, inset in Figure S4A). More importantly, modification of PEG-IP can markedly enhance the transverse relaxivity (r2) (Figure S4B) and improve the T2-weighted MR images (Figure 2A). These effects will benefit therapy and diagnosis.

Studying the photothermal effect demonstrated that MGMSPI had the highest NIR absorbance compared to GO, MG, and MGMS at the same concentrations (quantified according to graphene content) due to the reduction of GO (Figure S5A–D), and allowed for effective photothermal heating of solutions (Figure S6A,B). At a MGMSPI concentration above 30 mg/L, rapid photothermal heating to the photoablation limit of 50 °C occurred within 5 min irradiated using an 808 nm laser at 6 W/cm2 (Figure S6A).

DOX can be loaded into the mesopores of MGMSPI through both the adsorption of mesoporous silica and the π-π stacking interaction with graphene, forming MGMSPI. Due to the large surface area and pore volume, the loading capacity and entrapment efficiency of DOX can reach up to 0.95 ± 0.08 μg/μg (DOX/MGMS) and 43.19 ± 3.99%, respectively. MGMSPI exhibited laser-mediated and pH-dependent release behavior (Figure 2B and Figure S6C). Low pH and laser irradiation can accelerate the release of DOX, because the pH results in a decreased electrostatic interaction between DOX and mesoporous silica, and the pH/heat dissociation results in a reduced hydrophobic interaction between DOX and graphene.19 The cumulative release was less than 25% after 24 h, which achieved the sustained release properties required for a high DOX-loading capacity.

These characteristics will be useful for lethal tumors and reduce unintended damage and side effects to normal cells.

In order to evaluate the therapeutic effect of MGMSPI, the in vitro toxicity of different treatments was determined by confocal microscopy. MGMSPI had no obvious toxicity for glioma, whereas chemo-photothermal therapy (MGMSPI with NIR irradiation) resulted in the highest toxicity compared to single chemotherapy (MGMSPID) or photothermal therapy (MGMSPI with NIR irradiation) (Figure 3A–D). The CCK-8 assay was used for quantitative evaluation of cell viability (Figure 3E). The IC50 of each treatment is shown in Table S1, and the combination index (CI) of different therapies was 0.4823 (<1), demonstrating the synergistic therapeutic effect of MGMSPI nanomedicine.20

For receptor-mediated active targeting, IP was used as the glioma-targeting ligand in this study. In order to evaluate the IP-mediated targeting ability of MGMSPI, glioma cells (U251) and normal cells (1800) were treated with MGMSPID and MGMSPI. As shown in Figure 4, MGMSPI exhibited significantly higher cytotoxicity in U251 cells, but no apparent difference in 1800 cells compared to MGMSPI. This point was confirmed by cellular uptake assay (Figure S7) and in vivo fluorescent imaging (Figure S8), which showed strong cumulative signals in glioma cells after IP modification. These
results indicate that MGMSPID nanomedicine has a remarkable targeting effect to glioma.

To confirm the IP-mediated targeting function of MGMSPI in vivo and demonstrate the functions of magnetic targeting and MRI, T2-weighted MR images of differently treated glioma-bearing naked mice were acquired on a 3-T MR scanner (Figure 5). Before injection, no obvious contrast signals (dark) indexed as glioma were observed in any MR image. MGMSP and MGMSPI were injected into the mice with or without magnetic arrest. After 2 hours, all MR images exhibited a dark contrast signal in the brain, indicating the glioma. Notably, the contrast was gradually enhanced with IP modification and magnetic arrest, suggesting that IP-mediated targeting and magnetic targeting can increase glioma uptake of contrast agent (the nanomedicine) and contribute to excellent MR imaging.

Figure 3. A–D) Confocal microscopy of the different treated U251 glioma cells via LIVE-DEAD staining. Green: live cells. Red: dead cells. Bar = 75 μm. E) Cell viability profiles of glioma cells. Data were expressed as mean ± S.E.M. (n = 4).

In order to understand the mechanism underlying the cellular uptake, glioma cells were incubated with FITC-labeled MGMSPID nanomedicine. Confocal microscopy (Figure 6) revealed that little MGMSPID entered the glioma cells and DOX was nearly not released during a short incubation time (5 min). With prolonged incubation,
both the cellular uptake of MGMSPID and release of DOX from the carrier (MGMSPI) increased. When the incubation time was extended to 4 h, most of the released DOX was taken up by the cell nucleus but the carrier was left outside of the karyon for continuous release (Figure 6 and Figure S9).

3. Conclusion

In summary, a multifunctional nanomedicine based on PEGylated and IP-modified magnetic graphene-based mesoporous silica was successfully prepared and applied in MRI-visualized, dual-targeting and chemo-photothermal glioma therapy. The unique properties of this nanomedicine include: (1) water dispersibility and nontoxicity resulting from the biocompatible mesoporous silica and PEG coating, (2) strong near-infrared absorbance of the reduced graphene oxide (photothermal therapy), (3) high loading capacity and sustained release of the anti-cancer drug DOX due to the adsorption of the mesoporous silica and the π-π stacking interaction with graphene (chemotherapy), (4) large transverse relaxivity ($r_2$) aroused by the dispersed Fe$_3$O$_4$ nanoparticles (visualization), (5) high specificity achieved by receptor-mediated targeting and magnetic targeting, (6) pH and photothermal responsive release mediated by changes in the electrostatic interaction and hydrophobic interaction between DOX and MGMSPI, make it be a robust theranostic platform for cancer.

4. Experimental Section

**Materials:** Graphene oxide nanosheets (GO) were prepared by the modified Hummer’s method.$^{[21]}$ Cetyltrimethylammonium bromide (CTAB, analytical reagent) was purchased from Shanghai Mingzhi Chemical Co. Ltd (China). Tetraethyl orthosilicate (TEOS, analytical reagent) and aminopropyltriethoxysilane (APTES, analytical reagent) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Maleimidyl-N-hydroxysuccinimidyl polyethylene glycol (NHS-PEG-MAL, MW 3500) was obtained from Beijing JenKem Technology Co., Ltd (China). Doxorubicin (DOX) was purchased from Beijing Huafeng United Technology Co., Ltd (China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Japan). LIVE-DEAD Kits were purchased from Molecular Probes (Eugene, OR, U.S.A.). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Tulsa, OK, U.S.A.). Hydrated ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, analytical reagent), sodium acrylate ($\text{CH}_2 = \text{CHCOONa}$, analytical reagent), sodium acetate ($\text{NaOAc}$, analytical reagent), ethylene glycol (EG, analytical reagent), diethylene glycol (DEG, analytical reagent) and the other reagents, if not specified, were all purchased from Sinopharm Chemical Reagent Co., Ltd (China). All the chemicals were used without further purification.

**Synthesis and Preparation of Drug Delivery Systems:** Small pieces of GO nanosheets were obtained by repeated sonication (600 W) and centrifugation (16,000 rpm) of GO
solution (The followed solvothermal reaction and organic compound such as PEG assisted sonication would cut the GO into much smaller nanosheets). For the formation of superparamagnetic Fe₃O₄ nanoparticles tagged reducing graphene oxide (MG), 20 mg GO, 200 μL EG and 3.8 mL DEG were equably mixed by strong sonication before adding 300 mg CH₂ = CHCOONa, 300 mg NaOAc and 108 mg FeCl₂·6H₂O. The resulting homogeneous solution was then sealed into a Teflon-lined stainless-steel autoclave and heated at 200 °C for 10 h. After magnetic separation and hot ethanol washing, the black solid (MG) was recovered. For the synthesis of MGMS, 600 mg CTAB and 300 mL NaOH solution (1 mM) were mixed with 30 mg MG by sonication for above 4 h. Then, 3 mL APTES-TEOS ethanol (= 1:6:24, v/v, for labeling, 2.3 mL of 1 mg/mL fluorescein isothiocyanate (FITC)-ethanol solution was first mixed with APTES according to the report[22]) solution was added and stirred at 60 °C for 1 min followed by the hydrothermal reaction for 24 h. Finally, the solid product was recovered by centrifugation (or magnetic separation) and washing. To remove the surfactants from the pores, MGMS was repeatedly refluxed in a mixture of methanol and NH₄NO₃ (2 mg/mL).

For the synthesis of MGMSPI, PEGylated MGMS (MGMP) was first prepared by the reaction (sonication and stirring) of 3 mg bifunctional PEG and 1.5 mg MGMS for 2 h in phosphate-buffered solution (PBS, pH 8.0). Then, the recovered MGMP powder was dispersed into 1 mL IP-PBS solution (pH 7.0, 0.5 mg/mL) and stirred for 24 h, the resulting IP-modified conjugate (MGMP) was also purified via centrifugation. To load DOX, MGMSPI was soaked and stirred in a DOX methanol solution for 24 h, the mixture was centrifuged and the supernatant was removed. The DOX-loaded drug delivery systems (MGMPID and MGMPIDP) were dried under vacuum to remove trace methanol. Finally, they were re-suspended in PBS (pH 7.4) at proper concentration.

Characterization of Drug Delivery Systems: Scanning electron micrographs (SEM) were obtained from a Philips XL-30 scanning electron microscope. Transmission electron microscopy (TEM) and High-resolution transmission electron microscopy (HRTEM) experiments were performed on JEM-2100F instruments with an acceleration voltage of 200 kV. Nitrogen adsorption-desorption isotherms at 77 K and pore size distribution curves were measured using Tristar 3000 systems. Before the measurements, the samples were outgassed for more than 5 h at 150 °C. Raman spectra (LR) were taken on a Labram-1B (Dilor, France) Raman spectrometer with a 632.8 nm wavelength incident laser light. Magnetization curves were achieved on MPMS (SQUID) VSM system (Quantum Design Inc., USA). T2 and T2-weighted MR images were determined on a 0.55 T MRI instrument (MicroMRI, Shanghai Niumag Corp.) at 32 °C, using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. Inductively coupled plasma–atomic emission spectroscopy (ICP-AES, Thermo) was used to determine the concentration of Fe. Thermo-gravimetric analysis (TGA) of MGMS was carried out on a Pyris Diamond TG/DTA thermo-gravimetric analyzer (PerkinElmer Thermal Analysis). The sample was heated from room temperature to 900 °C at 10 °C/min under air atmosphere. Fourier transform infrared (FT-IR) spectroscopy was performed with a Nicolet-670 FT-IR spectrometer using the KBr pellet method. Ultraviolet–visible absorption spectra (UV–vis) were carried out on a UV–vis absorption spectrometer (UV759, China). Confocal images were obtained with a Leica TCs SP5 microscope and using a Leica application suite.

Loading Capacity Measurement: The loading capacity of MGMSPI was determined by measuring the DOX fluorescent signals (excitation at 488 nm and emission at 600 nm) of the original solution and the supernatant using a Cary Eclipse fluorescence spectrophotometer (Varian, USA). For the measurement, the standard curve was established.

Photothermal Heating Effect: An 808 nm NIR laser was used for photothermal heating effect evaluation. For the concentration-dependent analysis of photothermal heating effect, MGMSPI solution with a series of concentrations (calculated by MGMS weight) was irradiated, fixing the power density at 6 W/cm². For the power density-dependent analysis, different power density was applied, fixing the MGMS concentration of MGMSPI solution at 30 μg/mL. A thermometer was used for monitoring the solution temperature for up to 6 min. GO corresponding to the MGMS concentration at 30 μg/mL was used as positive control and PBS (pH 7.4) was applied as negative control.

In vitro Release: Dialysis tubes (MWCO 8–10 kDa, Spectrum-labs, U.S.A.) were applied for in vitro DOX release analysis. Briefly, 0.5 mL MGMSPI solution was loaded in the donor and 6.5 mL PBS solution with different pH (5.0, 6.0 and 7.4) was put in the acceptor. At determined time point, 0.5 mL dialysis solution was removed and replaced with fresh buffer. Released DOX was measured via a fluorescence spectrophotometer. To determine the impact of photothermal heat produced by the material, MGMSPI solution was irradiated at determined time point using an 808 nm NIR laser with the power density at 6 W/cm² for 5 min.

Cell Culture: In this study, human glioma U251 cells were exploited as the tumor cells and human astrocyte 1800 cells were representative as the normal cells. Both cells were purchased from Shanghai Cell Bank, Chinese Academy of Medical Sciences, and maintained at 37 °C in 5% CO₂, in DMEM media supplemented with 10% FBS, 1% L-glutamine, 1% penicillin, and 1% streptomycin.

Cell Death Imaging: To qualitatively determine the synergistic effect of chemotherapy and photothermal therapy, LIVE-DEAD Kits were applied for the cell death visualization. U251 cells were cultured at a density of 1 x 10⁶ cells/well in 96-well microplates for 24 h. After PBS rinsed, MGMSPI and MGMSPI solution with MGMS concentration at 30 μg/mL were added into wells for 30 min incubation. For photothermal therapy, wells were irradiated with a NIR laser (808 nm, 6 W/cm²) for 5 min. The culture media was then replaced with fresh one and cultured for another 12 h. After carefully washed, cells were stained by LIVE-DEAD Kits according to the manufacturer’s instruction. Confocal images were obtained using a Leica TCS SP5 microscope equipped with a Leica application suite. Live cells exhibited green and dead ones showed red.

Cytotoxicity Analysis: CCK-8 assay was used to investigate cytotoxicity of glioma cells under different treatments. U251 cells were cultured at a density of 5 x 10⁵ cells/well in 96-well microplates for 48 h. MGMSPI or MGMSPI solution with different MGMS concentrations were separately added into wells for 4 h incubation. For photothermal therapy, cells were irradiated with a NIR laser (808 nm, 6 W/cm²) for 5 min. The culture media was then replaced with fresh one and cultured for another 12 h. After that, CCK-8 solution was added to each well and incubated for further 2 h. Then the absorbance was determined using a microplate reader at 450 nm. Viability of treated cell wells was expressed as a percentage of the viability of untreated ones. The combination
index (CI) was calculated to evaluate the combination effect of chemotherapy and photothermal therapy.\textsuperscript{[23]} The value of CI represents different meanings, while $<1$ indicates synergism, $>1$ means antagonism, and $1$ indicates additive effects.

**Targeting Effect of MGMSPID:** U251 and 1800 cells were cultured at a density of $5 \times 10^3$ cells/well in 96-well microplates for 48 h, respectively. After rinsed, MGMSPD and MGMSPID with MGMS concentration at 30 μg/mL were incubated with cells for 0.5 h (LIVE-DAED assay) and 4 h (CCK-8 assay). The culture media was then replaced with fresh one and cultured for another 12 h. After carefully washed, LIVE-DEAD Kits for confocal microscopy was performed for qualitative observation and CCK-8 analysis was carried out for quantitative evaluation.

**Cellular Uptake:** U251 and 1800 cells were cultured at a density of $5 \times 10^3$ cells/well in 96-well microplates for 48 h, respectively. After rinsed, MGMSPD and MGMSPID with MGMS concentration at 30 μg/mL were incubated with cells for 0.5 h. After further rinsed twice carefully, confocal microscopy was performed to evaluate the targeting effect of IP.

**Intracellular Localization:** U251 cells were cultured at a density of $5 \times 10^3$ cells/well in 96-well microplates for 24 h. After PBS rinsed, MGMSP solution with MGMS concentration at 30 μg/mL was added into wells for 5 min and 4 h incubation, respectively. Then the drug solution was removed and cells were treated with LysoTracker Green for 10 min to label acid compartments including endosomes and lysosomes. Confocal microscopy was performed to track intracellular localization of MGMSPID at different time point.

**In vivo Imaging Analysis:** Nude male mice with age of about 5 weeks and weight of 20–22 g were purchased from Department of Experimental Animals, Fudan University, and maintained under standard housing conditions. To obtain glioma-bearing mice, about 5 × 10^3 U251 cells were slowly implanted into the right caudatoputamen by using a stereotactic fixation device with mouse adaptor. For in vivo fluorescence imaging, at the 20th day after implantation, the tumor-bearing mice were intravenously injected with 150 μL MGMSP and MGMSPID solution (the same MGMS concentration, 6 mg/mL). The mice were anesthetized and killed by decapitation after being maintained for 3 h. Whole brains were removed and imaged by CRI system (CRI, MA, USA). Then the brains were dissected transversely and visualized again. For in vivo MR imaging, mice bearing glioma were intravenously injected with 150 μL MGMSP and MGMSPID solution (the same MGMS concentration, 6 mg/mL). After being maintained for two hours with magnetic arrest or not, the mice were imaged under 3-T clinical MRI scanner (Siemens Trio Corporation, Germany) equipped with a special coil designed for small animal imaging.

**Intracellular Fate of the Vector and its Payload:** To investigate the intracellular fate of the vector MGMSPID and its payload DOX, MGMS was labeled with FITC as described above. U251 cells were cultured at a density of $5 \times 10^3$ cells/well in 96-well microplates for 24 h. After PBS rinsed, MGMSPID solution with MGMS concentration at 30 μg/mL was added into wells for 5 min, 30 min, 1 h and 4 h incubation, respectively. Confocal microscopy was carried out to observe the state of MGMSPID.

**Statistical Analysis:** Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post test using GraphPad InStat software.

Received: April 26, 2013
Published online: July 5, 2013