AS1411-targeted graphene oxide nano-drug delivery system for chemo-photothermal therapy of cervical cancer

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Abstract

The aim of this study is to design a novel pH and photothermal dual-responsive nanodrug delivery system with high biocompatibility and tumor targeting. Based on the high loading rate and good photothermal properties of graphene oxide (GO), we selected GO modified with chitosan (CO) and γ -polyglutamic acid (γ -PGA) as nanocarriers. CO and γ -PGA increased the dispersion of GO and improved the solubility of GO-CO- γ -PGA (G-C-P) in solution. To further improve the targeting of the system, the nucleic acid aptamer NH₂-AS1411 (APT), which targets the nucleolin (C23), was attached to G-C-P and the targeted nano-delivery system APT-GO-CO- γ -PGA (A-G-C-P) was prepared by amidation. The synthesized samples were characterized using Fourier transform infrared spectroscopy (FTIR), Ultraviolet-visible spectrophotometer (UV-Vis), X-ray diffraction (XRD), Transmission electron microscopy (TEM), Dynamic light scattering (DLS) and Zeta potential, and their good biocompatibility and stable photothermal conversion properties were demonstrated experimentally. The nanocarrier can act as a photothermal agent, generating high temperatures to induce cell damage. The lower hemolysis rate reflects the good biocompatibility of the carrier. Doxorubicin hydrochloride (DOX) was selected as the model drug with a nanocarrier loading of $37.0 \pm 0.74\%$, showing dual response drug release characteristics of pH and NIR light. The cytotoxicity and photothermal toxicity of the nanocarrier A-G-C-P and the drug delivery system APT-GO-CO- γ -PGA-DOX (A-G-C-P-D) on HeLa cells were investigated in cellular experiments. It was found that A-G-C-P had almost no toxic side effects on the cells, and the killing effect of A-G-C-P-D with the application of near-infrared light irradiation was more obvious, proving its good photothermal therapeutic effect, and further demonstrating that the combined effect of chemotherapy and photothermal treatment is significant than that of single treatment. Confocal microscopy and flow cytometry determined the distribution of DOX in the cells to different degrees after cell internalization. In vivo anti-tumor experiments were conducted to further investigate the therapeutic effects and safety of the targeted nano-drug delivery system in concert with photothermal. Compared with the weight loss and heart tissue damage caused by free DOX, A-G-C-P-D and NIR light irradiation did not cause any tissue damage and toxic side effects in nude mice, and have good biosafety; A-G-C-P-D and NIR light irradiation can inhibit tumor growth, cause damage to tumor tissue, significantly reduce Ki67 expression and increase Caspase-3 expression, all of which prove their good anti-tumor effects. This relatively non-invasive approach may provide a good direction for targeted drug delivery and chemotherapeutic photothermal treatment of tumors, greatly reducing the side effects of chemotherapy. The therapeutic effect of this nano-drug delivery system provides new ideas for clinical treatment strategies with potential applications and reference values, offering a broad prospect for biological applications.

1 Introduction

Cervical cancer, mainly caused by human papillomavirus (HPV) infection, is the third most typical cancer among women [1] and the main tumor killer among women [2], [3]. There are many different treatment options available, such as surgery, targeted therapy, chemotherapy and

immunotherapy [4]. Chemotherapy is the most typical medical treatment for cancer, and about 80 drugs are used in clinical treatment [4]. Unfortunately, these typical antineoplastic drugs are subject to various limitations, including drug resistance, nonspecific distribution, and toxic side effects [5], [6].

To achieve mitigation of the adverse effects of chemotherapeutic agents, more research has focused on precise targeting, enhanced penetration and retention, and controlled drug release [7]. Targeted nano-delivery systems can be constructed by combining biocompatible carriers with chemotherapeutic drugs and modifying ligands that bind specifically to receptors overexpressed on the surface of cancer cells. Through receptor-ligand interactions, the drug is delivered precisely to the tumor, reducing side effects such as cytotoxicity to normal cells. In recent years, researchers have invested a lot of effort in the synthesis of nanomaterial-based drug delivery systems. GO is considered to be an effective drug delivery material due to its high specific surface area, dispersibility and hydrophilicity, and has received a lot of attention in the biomedical field. However, GO tends to agglomerate in physiological environments, which hinders its drug-carrying capacity and drug delivery applications. Both CO and γ -PGA have high water solubility, biocompatibility, bioactivity and are easily absorbed and utilized by living organisms, and can be used to increase the solubility and stability of GO. AS1411 is a nucleic acid aptamer that specifically binds to C23 overexpressed on the surface of cervical cancer cells, and the nano modification of AS1411 on the vector enhances targeting to HeLa cells.

However, the clinical efficacy of nanodrug delivery systems alone is limited and studies have found that combination therapy has better therapeutic efficacy, one of which is the combination of chemotherapy and photothermal therapy [8], [9]. Photothermal therapy (PTT) under near-infrared (NIR) irradiation is attracting increasing interest in tumor suppression due to its low negative impact and low invasiveness [6]. Near-infrared light (700-1100 nm) penetrates deep into human tissue without being absorbed in large quantities [10], [11], is absorbed by photothermal converters and converted into local heat therapy, and the slight increase in tumor temperature makes cancer cells more susceptible to radiation and treatment [12]. The applied photothermal agents require good biocompatibility, photothermal conversion efficiency and stability [13]. GO is widely used for tumor inhibition in combination therapy modalities due to its good drug delivery capacity and photothermal properties [14], [15]. In order to achieve optimal therapeutic effects, this study aims to develop a novel pH and photothermal dual-responsive nano-drug delivery system A-G-C-P-D with high photothermal conversion, excellent drug delivery capacity and controlled drug release capability. It showed that in Scheme 1, The system uses A-G-C-P as the targeting nanocarrier and DOX as the model drug, which can be specifically targeted to tumors and responds rapidly to low pH and near-infrared stimuli to promote DOX release, realizing the advantageous combination of chemotherapy and thermotherapy and enhancing synergistic efficacy; reducing DOX release during blood transport of the nano system and alleviating the toxic side effects caused by excessive doses of chemotherapeutic drugs to the body.

2 Methods/experimental

2.1 Materials

Graphene oxide (GO) was supplied by Suzhou Hengqiu Graphene Technology Co. Chitosan oligosaccharide (CO) and DOX were provided by Dalian Meilun Biotechnology Co. γ -polyglutamic acid (γ -PGA) were purchased from Xi'an Wanfang Biotechnology Co. The nucleic acid aptamer APT (5'-NH₂-GGTGGTG GTTGTGGTG-3') was synthesized by Sangon Biotech. HeLa cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology. BALB/c nude mice were purchased from Jinan Pengyue Experimental Animal Breeding Co. Hematoxylin, and Eosin Staining Kit Dalian from Meilun Biotechnology Co. Ki67 and Caspase-3 Rabbit Monoclonal Antibody were purchased from Beyotime Biotech Inc.

2.2 Preparation of A-G-C-P

The nano-drug delivery system was synthesized by amidation, with reference to our previous work [16], [17]. 160 mg of monolayer GO was weighed and dissolved in 40 mL of ultrapure water, sonicated for 1.5 h in an ultrasonic disintegrator and centrifuged to obtain a well-dispersed GO suspension. The pH of the GO suspension was adjusted to 4.5-6 with MES buffer, the cross-linking agents EDC and NHS were added and the reaction was carried out for 20 min at 37°C. The supernatant was removed by centrifugation. The GO precipitate was resuspended with 40 mL of CO solution (4 mg/mL) and the pH of the mixture was adjusted to neutral. After 12 h of reaction the GO-CO solution was dialyzed using a dialysis bag (MW=8-14 KDa) and freeze-dried to obtain GO-CO powder. G-C-P was obtained by ligating 40 mg of γ -PGA in the same way. 40 mg of G-C-P was weighed and dissolved in 40 mL of 20 mM Tris-HCl buffer containing 0.1 M KCl, then 40 μ L of APT was added to react for 12 h. A-G-C-P was obtained by dialysis using a dialysis bag. The synthesized samples were characterized using FTIR, UV-Vis, XRD, TEM, DLS and Zeta potential.

2.3 Photothermal effect

Photothermal properties can be measured with a near-infrared laser device and a thermocouple pyrometer [18]. The photothermal properties of A-G-C-P were measured under 808 nm laser irradiation [19]. In order to investigate the photothermal properties of A-G-C-P, the effect of carrier concentration on temperature rise, the effect of NIR laser power density on temperature rise and photothermal stability, four different sets of experiments were designed, namely: (1) 1 mL of ultrapure water, GO, γ -PGA and A-G-C-P suspensions were placed in tubes and irradiated at a power density of 1.5 W/cm² for 10 min; (2) 1 mL of different concentrations of A-G-C-P suspensions (0.05, 0.25, 0.5, 1 and 2 mg/mL) were irradiated for 10 min; (3) 1 mL of A-G-C-P suspensions (1 mg/mL) irradiated at different power densities (0.8, 1.5 and 2 W/cm²) for 10 min; (4) 1 mL of A-G-C-P (2 mg/mL) suspension irradiated (2 W/cm²) for 5 on/off cycles.

2.4 DOX loading and release

DOX was selected as a model drug to study the drug loading and controlled release properties of A-G-C-P nanocarriers. The DOX solution (1.5 mg/mL) was added to A-G-C-P for 24 h and centrifuged to collect the A-G-C-P-D nanocarrier system loaded with DOX, and then washed with ultrapure water to remove the unloaded DOX. The absorbance value of the supernatant at 480 nm was measured to analyze the

loading and release of DOX. The drug loading content (LC) is expressed by the following equation. $LC = (\text{weight of initial drug} - \text{weight of unloaded drug}) / (\text{total weight of nanoparticles}) \times 100\%$. In vitro release studies were carried out in PBS buffer (pH 7.4 and 5.6) with and without NIR light irradiation. A-G-C-P-D were divided into four groups for different treatments. (1) dispersed in PBS solution at pH=7.4; (2) dispersed in PBS solution at pH=5.6; (3) dispersed in PBS solution at pH=7.4 and irradiated with NIR laser; (4) dispersed in PBS solution at pH=5.6 and irradiated with NIR laser. The nanoparticles were placed in a dialysis bag and then placed in a PBS solution containing different pH values and the beaker was placed on a shaker at 37°C, 120 rpm. The 4 mL PBS was removed at regular intervals and the amount of released DOX was measured by UV-vis spectrophotometer. To keep the volume of the release medium constant, 4 mL of fresh PBS buffer (pH 7.4 or 5.6) was added to the corresponding beaker. In addition, the nanoparticles (in PBS buffer, pH 7.4 or 5.6) were exposed to a NIR laser (2 W/cm²) for 5 min. The cumulative release of DOX under different conditions (pH 7.4 or 5.6, with or without NIR laser irradiation) was then calculated.

2.5 In vitro cell experiments

The synergetic chemo-photothermal capacity of A-G-C-P in vitro was investigated by toxicity assay with MTT [20]. 3×10^3 cells/well were seeded in a 96-well plate and incubated overnight. HeLa cells were treated with different concentrations of sample. After 4 h, the NIR group was irradiated with a 2 W/cm² 808 nm laser for 2 min and incubated continuously for 20 h. The remaining groups were incubated directly with the samples for 24 h. 15 μ L MTT was added to each well and the relative viability of the cells in each group was calculated after 24 h.

Qualitative and quantitative analysis of internalized A-G-C-P-D in HeLa cells. HeLa cells were inoculated in six-well plates and cultured for 24 h. In each well, 3 μ g/mL of the corresponding sample was added. After 4 hours, the NIR group was irradiated with a 2 W/cm² 808 nm laser for 3 minutes. The distribution of DOX in HeLa cells was examined by two-photon laser confocal microscopy. The excitation wavelength of DAPI was 405 nm and the emission wavelength was 410-510 nm. The excitation wavelength of DOX was 488 nm and the emission wavelength was 550-600 nm. Meanwhile, the fluorescence values of the internalized DOX of the cells were measured by flow cytometry.

Hemolysis test can be used for preliminary biosafety assessment [21]. The hemolysis test was used to assess the biocompatibility of A-G-C-P. The erythrocyte suspension was diluted to 2% with physiological saline. 0.2 mL of erythrocyte suspension mixed with 0.8 mL of deionized water as a positive control, 0.8 mL of saline as a negative control, and 0.8 mL of different nanoparticles were mixed as the experimental group. Each group was centrifuged after 30 min of 37°C water bath. The absorbance value of the supernatant was recorded at 540 nm using an enzyme marker. Hemolysis rate (HR) was calculated according to the formula below:

$$HR = \frac{OD_{\text{sample}} - OD_{\text{negativecontrol}}}{OD_{\text{positivecontrol}} - OD_{\text{negativecontrol}}} \times 100\%$$

Where OD_{sample} represents the measured absorbance of ultraviolet-visible of different nanoparticles. $OD_{\text{positive control}}$ and $OD_{\text{negative control}}$ represent the measured absorbance of ultraviolet-visible with ultrapure water and saline, respectively.

2.6 In vivo antitumor research

3~4-week-old female BALB/c nude mice, weighing 12~16 g, were purchased from Jinan Ponyue Experimental Animal Breeding Co. (License number: SCXK(LU)20190003)

BALB/c nude mice were acclimatized and fed for one week according to the standard protocol of the Animal Feeding Center, while the resuscitated HeLa cells were passaged and cultured three times to collect cells in log phase. Collected HeLa cells were resuspended in PBS solution (5×10^6 cells/200 μL) [22] and injected subcutaneously into the left axilla of each nude mouse [23]–[25].

When the tumor volume of nude mice grew to approximately 100 mm^3 , the nude mice were randomly divided into five groups ($n = 3$). Saline was injected intraperitoneally as a control group, and DOX, G-C-P-D, A-G-C-P-D and A-G-C-P-D+NIR were injected through the intraperitoneal injection every 3 days with a DOX level of 5 mg/kg. Tumors in the fifth group were additionally exposed to NIR (2 W/cm^2) irradiation for 5 min. Tumor size was measured with calipers and calculated as:

$$\text{Volume of tumor (mm}^3\text{)} = \frac{1}{2} \times \text{length} \times \text{width}^2.$$

Nude mice were anesthetized at the end of drug administration. The distribution of the drug in the nude mice was observed by a mouse imaging system [26]. At the end of 24 h imaging, nude mice were killed and dissected. Tumor tissues and organs were excised [27], [28] and subjected to quantitative fluorescence analysis.

After 21 days of treatment, normal tissues and tumors were fixed in 4% paraformaldehyde, then embedded in paraffin, 8 μm sections were made, stained with hematoxylin and eosin (H&E), and the tissue morphology was observed by light microscopy to evaluate the in vivo safety of the nano-drug delivery system. The expression of Ki67 and Caspase-3 in the tumor tissues of each experimental group was detected by immunohistochemistry. After dewaxing of the sections, they were subjected to microwave antigen repair, 3% H_2O_2 treatment, serum closure, incubation with primary antibody and secondary antibody, and color development by SABC method, and then sealed with neutral gum and observed by light microscopy. The antitumor effect of combined chemotherapy and photothermal treatment was investigated.

3 Results

3.1 Characterization of nano drug delivery system

A-G-C-P-D was prepared by amidation method. Novel pH and photothermal dual-responsive nanodrug delivery systems with high biocompatibility and tumor targeting were prepared by enhancing the biocompatibility of GO using CO and γ -PGA and targeting cancer cells by APT. This multifunctional drug delivery system has the following advantages over other nano systems. Firstly, A-G-C-P can precisely target cancer cells and reduce the damage of DOX to normal tissues. Second, A-G-C-P exhibits superior photothermal properties under NIR irradiation and promotes drug release, which can increase tumor sensitivity to DOX and reduce the use of DOX, reducing the possibility of drug-resistant cancer cells. We verified the success of the A-G-C-P construction using FTIR, UV-Vis, XRD, TEM, DLS and Zeta potential characterization methods. Figure 1a shows the FTIR spectrum, where GO has O-H stretching vibration peak at 3407 cm^{-1} , C=O stretching vibration peak at 1724 cm^{-1} , C=C stretching vibration peak at 1624 cm^{-1} , and C-O stretching vibration peak at 1053 cm^{-1} . Compared with GO, G-C-P showed C-H stretching vibration peak at 2932 cm^{-1} , C=O stretching vibration peak at 1641 cm^{-1} (amide I band), and N-H bending vibration peak at 1558 cm^{-1} (amide II band), but the -COOH absorption peak at 1724 cm^{-1} disappeared, which proved that GO was successfully connected with CO and γ -PGA by amide bond. Compared with G-C-P, A-G-C-P showed C=O stretching vibrational peak (keto group) at 1730 cm^{-1} , which originated from guanine and thymine of AS1411 aptamer, and P=O stretching vibrational peak at 1166 cm^{-1} , which originated from AS1411.

Figure 1b shows the UV-Vis spectrum, the C=C bond in GO undergoes an electron $\pi \rightarrow \pi^*$ leap and shows a characteristic absorption peak at 230 nm, and G-C-P has obvious absorption peaks at 204 nm and 234 nm. Compared with GO, its absorption peak was blue-shifted, indicating that GO-CO was successfully connected with γ -PGA. Because of the absorption peak of APT at 254 nm, the characteristic peak of A-G-C-P was right-shifted. Figure 1c shows the XRD pattern, where the characteristic peak of GO is 10.12° . After CO and γ -PGA modification, the characteristic peak of G-C-P is shifted to 8.50° . After APT modification, the characteristic peak of A-G-C-P continued to be shifted to 8.26° . The accuracy of the FTIR and UV-Vis results was proved from the side.

The morphology of GO and A-G-C-P was observed by TEM (Figure 1d and Figure 1e), and the GO, G-C-P and A-G-C-P dimensions were determined by DLS (Figure 1f). GO showed lamellar and folded surface with an average diameter of 309.2 nm. Compared with GO, the modified G-C-P and A-G-C-P remained lamellar and granular material appeared on its surface, and the average diameter was 319.3 nm and 321.1 nm, respectively. The zeta potential results for GO, G-C-P and A-G-C-P are shown in Figure 1g. The potential of GO is $-26.64 \pm 0.41\text{ mV}$. Because of the presence of a large number of carboxyl and epoxy groups on the GO surface, GO is negatively charged. CO with a large number of amino groups and γ -PGA with a large number of carboxyl groups. After modification by CO and γ -PGA, the potential of G-C-P slightly increased to $-23.14 \pm 0.31\text{ mV}$, which proved that CO and γ -PGA were successfully connected with GO. These results proved that A-G-C-P was prepared successfully.

3.2 Photothermal Properties Measurement

Evaluation of the photothermal properties of composite materials using 808 nm near infrared light. Figure 2a shows the temperature rise curves of different materials at the same concentration, in which A-G-C-P increases the temperature from 25°C to 55.8°C in 10 min under 808 nm NIR light irradiation, retaining the superior photothermal conversion ability of GO. Figure 2b and Figure 2c show the temperature rise effect under NIR irradiation with different concentrations of A-G-C-P and different power densities, respectively. The temperature could rise to 53.6°C at A-G-C-P concentration of 0.5 mg/mL. The temperature can rise to 62.1°C at a power of 2.0 W/cm². All of them reached the temperature to destroy the damaged cells. Photothermal stability is also an important parameter of photothermal reagents, and the photothermal stability of A-G-C-P is shown in Figure 2d. After several cycles, the warming effect of A-G-C-P was stable and the photothermal conversion efficiency did not change. A-G-C-P has good photothermal properties in the near-infrared region and can be used as a photothermal agent for cancer therapy.

3.2 Drug Release Test

DOX was chosen as a model drug to confirm the potential ability of A-G-C-P composite nanoparticles to release encapsulated drugs in a dual pH/photothermal response. Figure 3a shows the comparison of the FTIR spectra of A-G-C-P before and after loading DOX. Compared with A-G-C-P, A-G-C-P-D has the characteristic peaks at 1618 cm⁻¹, 1582 cm⁻¹ of DOX. Figure 3b shows the UV-Vis absorption spectra. A-G-C-P-D has characteristic peaks at 232 nm and 488 nm. Both proved that A-G-C-P successfully loaded DOX. Figure 3c shows the concentration-absorbance standard curve of DOX. There was a linear relationship between the concentration and absorbance of DOX in the concentration range of 0.045~187.5 µg/mL. The calculated drug loading of A-G-C-P was 37.0 ± 0.74%. In order to investigate the pH and photothermal effects on the drug-controlled release process of drug delivery systems. PBS with pH 7.4 and 5.6 was used to simulate the physiological environment of normal and tumor tissues, respectively, to investigate the effects of pH and photothermal on drug release from the drug delivery system. Figure 3d demonstrates that drug release was significantly faster at low pH and NIR irradiation. The cumulative release of A-G-C-P-D in the acidic environment was 53.1% at 60 hours. The release rate increased to 61.6% after the administration of NIR light irradiation. It was demonstrated that A-G-C-P-D has pH/photothermal sensitivity, and NIR light irradiation greatly stimulated drug release, which is conducive to controlling drug concentration in tumors and exerting synergistic chemotherapy-photothermal therapeutic effects.

3.3 Cellular assays

The cytotoxicity and photothermal toxicity of nanocarriers on HeLa cells were examined by MTT assay. Figure 4a shows that the cell survival rate was at 86.7% when the vector concentration reached 200 µg/mL, which indicates that the vector concentration in the range of 0~200 µg/mL has almost no killing effect on cells and shows low toxicity. In contrast, cell survival was significantly reduced after NIR laser

irradiation compared with no NIR light irradiation applied. Cell survival rate was less than 60% when the vector concentration was only 25 $\mu\text{g/mL}$. Figure 4b shows that cell survival was 66.30%, 42.61%, 25.74%, and 20.62% at DOX concentrations of 1.25, 2.5, 5, and 10 $\mu\text{g/mL}$, respectively. The cell survival rates in the A-G-C-P-D group were 62.25%, 38.24%, 20.45%, and 12.59%, demonstrating that the nanosystem with targeting had superior anti-tumor effects than free DOX. The cell survival rates were 53.32%, 29.19%, 11.41%, and 5.62% when laser irradiation was applied again, showing that the synergistic effect of chemotherapy and photothermal treatment was significantly better than that of single treatment.

Cellular uptake and intracellular drug release of DOX-loaded A-G-C-P nanoparticles were further investigated for HeLa cells. Figure 5 shows that the fluorescence signal intensity of G-C-P-D, A-G-C-P-D and A-G-C-P-D+NIR-treated cells increased sequentially to 1352, 3013, 9503 and 21655, respectively. This demonstrates that A-G-C-P can target cancer cells and release the drug, and that NIR irradiation stimulated the drug release. The internalization properties of G-C-P-D, A-G-C-P-D and A-G-C-P-D+NIR in HeLa cells were studied by two-photon confocal microscopy. As shown in Figure 6, intracellular fluorescence was observed at 4 h, indicating that the nanocarriers effectively entered the interior of the cells. More fluorescence was found in cells irradiated with NIR light for 3 minutes, implying that the increase in temperature accelerated the release of DOX inside the cancer cells, which is consistent with the results of the previous experiment. As shown in Figure 6, the rapid internalization of the targeted nanodrug delivery system proved its good targeting effect on tumor cells. More intense fluorescence was found in the cells after NIR light irradiation, which implies that more nanoparticles were endocytosed by the cells and accelerated the release of DOX by the increase of temperature.

3.4 Biocompatibility

The erythrocyte hemolysis test is often used to test the biocompatibility of biological materials. As can be seen in Figure 7, the hemolysis rate is below 5% at A-G-C-P concentrations of 1-100 $\mu\text{g/mL}$, indicating that the hemolytic toxicity of A-G-C-P nanocomposites is very low and suitable for biological experiments. Hemolysis has been reported at GO concentrations of 80 $\mu\text{g/mL}$ [29]. The upper limit of safe concentration of A-G-C-P in this study has exceeded 80 $\mu\text{g/mL}$, indicating its good biocompatibility.

3.5 In vivo pharmacodynamic evaluation

The in vivo chemotherapy-photothermal synergistic treatment effect of the nano-drug delivery system was verified by the anti-tumor efficacy in transplanted tumor-bearing nude mice. As shown in Figure 8a, the tumor volume of the nude mice treated with A-G-C-P-D injection and NIR irradiation decreased significantly, while the tumors of the control group kept increasing. The inhibition of tumor growth was very limited in the free DOX-treated and G-C-P-D groups, with tumor inhibition rates of 29.7% and 60.6%. The tumor inhibition rates were 95.6% and 90.7% in the A-G-C-P-D group with and without combined photothermal therapy, respectively. It can be concluded that photothermal therapy combined with chemotherapy can be effective in improving the treatment of cervical cancer. In addition, the body weight of the nude mice increased throughout the treatment, demonstrating laterally that the nanodrug delivery system did not exhibit serious side effects at the doses used (Figure 8b).

The distribution and accumulation sites of the drug in nude mice were visualized by an in vivo imaging system. As shown in Figure 9a, the fluorescence intensity at the tumor site of nude mice showed an increasing trend over time and reached a maximum at 24 h after injection. Fluorescence at the tumor site was observed in the A-G-C-P-D group at 2 hours, while it was not observed in the DOX group at 4 hours after administration. The fluorescence intensity of the A-G-C-P-D group was significantly higher than that of the G-C-P-D group, which indicates that the former has good targeting in vivo and can be effectively accumulated at the tumor site. In addition, the DOX content in each organ (Figure 9b) verified this result. Compared with the free DOX group, cardiotoxicity was significantly reduced in the G-C-P-D group, and DOX accumulation in the liver was also reduced. The effect of A-G-C-P-D was more pronounced than that of the G-C-P-D group. In conclusion, A-G-C-P-D could target the tumor site and reduce the toxic effects of DOX on normal tissues.

The biosafety and antitumor effects of the nano-drug delivery system were further confirmed by histopathological analysis and immunohistochemical experiments on major organs of mice. As shown in Figure 10a, the H&E staining results showed that DOX had a significant toxic effect on the heart compared to the control group, while the nude mice in the nano-drug delivery system group showed normal morphological characteristics of the major organ tissues. The tumor sites in the A-G-C-P-D group had significant damage compared with the DOX group, which was further aggravated by NIR light irradiation. It shows that the nano-drug delivery system has some biosafety in nude mice, reduces the toxic side effects of DOX, and shows better synergistic treatment effect of chemotherapy and photothermal. Immunohistochemical assay to further investigate the tumor suppression effect of nano-drug delivery system. As shown in Figure 10b, treatment in the A-G-C-P-D group significantly decreased Ki67-positive cancer cells and increased Caspase-3 staining positivity compared with other experimental groups. The administration of NIR irradiation further inhibited the proliferation of tumor cells by suppressing the expression of Ki67 and promoted the expression of Caspase-3 to promote apoptosis of cancer cells. Once again, the combination of A-G-C-P chemotherapy and photothermal therapy proved to have good in vivo anti-tumor effects.

4 Discussion

Nanoparticle-based synergistic therapy has emerged as a promising strategy for cancer treatment. Among them, combined chemotherapy and photothermal treatment has efficient antitumor effects. For example, Y. Zhang et al. synthesized laser-triggered gold nanoparticles with synergistic chemotherapy-photothermal effect for targeted colon cancer therapy [30]. Rong Ma et al. constructed a therapeutic indocyanine green-based nanoplatfrom for near-infrared fluorescent image-guided chemotherapy/photothermal treatment of cervical cancer [31]. J. Huang et al. developed metal organic framework-coated gold nanorod as an on-demand drug delivery platform for chemo-photothermal therapy [32]. However, previously constructed nanocarriers mainly focus on anticancer research in vitro, and not many in-depth studies conducted on the antitumor mechanism of nanocarriers in vivo. In this study, a targeted nano-drug delivery system was also synthesized, and the biosafety and synergistic therapeutic effects of this drug delivery system were investigated to provide a theoretical basis for clinical application.

The nanocarriers synthesized in this paper were modified with AS1411 to bind to C23 overexpressed on the surface of cervical cancer cells and have the ability to target cervical cancer cells. The application of near-infrared light irradiation improved the ability of anti-tumor effect. We evaluated the targeting ability, cellular uptake, cytotoxicity and in vivo antitumor effects of this nanodrug delivery system. The photothermal effect and drug release of A-G-C-P were examined with 808 nm NIR laser irradiation. It has been demonstrated that 41-43°C can inhibit tumor cells with minimal adverse effects on normal cells [33]. The prepared A-G-C-P reaches this temperature within 3 min to damage cancer cells. The composite also has a pH/photothermal dual response to release DOX. Electron exchange during photothermal conversion and ionization of amino groups at low pH values both accelerate the release of the drug. On the other hand, the good solubility of DOX at low pH conditions may also improve drug delivery [34]. In addition, the local temperature increase under NIR laser irradiation can change the cell membrane permeability, which means that laser irradiation can promote nanoparticle internalization. They both enhance the antitumor effects of targeted nanodrug delivery systems and predict that A-G-C-P has great potential for clinical applications.

5 Conclusion

In this study, we designed a novel pH and photothermal dual-responsive nanodrug delivery system with high biocompatibility and tumor targeting, and further investigated the effect of synergistic chemotherapy and photothermal therapy in vivo and in vitro. The multifunctional nano-drug delivery system was synthesized by amidation method and exhibited excellent photothermal conversion effect and biocompatibility. The temperature of A-G-C-P can rise to 53.6°C at a concentration of 0.5 mg/mL, which can ablate the cells. The nano-drug delivery system can accelerate the release of DOX under low pH and NIR light irradiation, and its combined chemotherapy and photothermal treatment is better than the same dose of chemotherapy or photothermal treatment. This relatively non-invasive method may provide a good direction for targeted drug delivery and chemotherapeutic photothermal treatment of tumors, which greatly reduces the side effects of chemotherapy. This nano-drug delivery system provides new ideas for clinical treatment strategies with potential applications and reference values, offering a broad prospect for biological applications.

Abbreviations

GO: graphene oxide

CO: chitosan

γ-PGA: γ-polyglutamic acid

MES: MES monohydrate

EDC: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

NHS: N-Hydroxy succinimide

APT: NH₂-AS1411

DOX: doxorubicin hydrochloride

G-C-P: graphene oxide-chitosan-γ-polyglutamic acid

G-C-P-D: graphene oxide-chitosan-γ-polyglutamic acid- doxorubicin hydrochloride

A-G-C-P: NH₂-AS1411-graphene oxide-chitosan-γ-polyglutamic acid

A-G-C-P-D: NH₂-AS1411-graphene oxide-chitosan-γ-polyglutamic acid- doxorubicin hydrochloride

PTT: Photothermal therapy

NIR: Near-infrared

FTIR: Fourier-transform infrared

UV-Vis: Ultraviolet-visible spectrophotometer

XRD: X-ray diffraction

TEM: Transmission electron microscopy

DLS: Dynamic light scattering

Declarations

Availability of Data and Materials

Data for the current study period are available from the corresponding authors upon request.

Credit authorship contribution statement

Ruixia Gao: Acquisition of data, Writing - Review & Editing, Analysis and interpretation of data, Investigation. Zhaoyi Liang: Methodology, Validation, Data curation. Chengchuan Che: Visualization, Supervision. Jinfeng Liu: Investigation. Meiru Si: Formal analysis. Ge Yang: Project administration, Funding acquisition, Conceptualization, Supervision, Resources, Validation

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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Scheme

Schemes 1 is available in the Supplemental Files Section.

Figures

Figure 1

a FTIR spectra of GO, G-C-P and A-G-C-P. **b** UV-Vis absorption spectra of GO, G-C-P, APT and A-G-C-P. **c** XRD spectra of GO, G-C-P and A-G-C-P. **d** TEM image of GO. **e** TEM image of A-G-C-P. **f** DLS spectra of GO, G-C-P and A-G-C-P. **g** Zeta potential of GO, G-C-P and A-G-C-P.

Figure 2

Photothermal property **a** Temperature variation of ultrapure water, GO, γ -PGA and A-G-C-P (1 mg/mL) under 1.5 W/cm². **b** Temperature changes of A-G-C-P with different concentrations under 2 W/cm². **c** Temperature changes of A-G-C-P under different power 808 nm laser irradiation (0.8, 1.5 and 2 W/cm²). **d** Temperature variation of A-G-C-P over 5 on/off cycles of 808 nm laser irradiation (2 W/cm²).

Figure 3

a FTIR spectra of DOX and APT-GO-CO- γ -PGA-DOX. **b** UV-Vis spectra of DOX and APT-GO-CO- γ -PGA-DOX. **c** Standard curve of concentration-absorbance of DOX. **d** Drug release profiles of A-G-C-P-D under different conditions.

Figure 4

a Cytotoxicity assay of A-G-C-P with and without NIR light irradiation. **b** Cytotoxicity assay of A-G-C-P-D with and without NIR light irradiation.

Figure 5

Flow cytometry analyses of DOX uptake in HeLa cells treated with G-C-P-D, A-G-C-P-D, and A-G-C-P-D+NIR.

Figure 6

Intracellular distribution of DOX in **a** G-C-P-D, **b** A-G-C-P-D and **c** A-G-C-P-D+NIR treated HeLa cells after 4 h.

Figure 7

Hemolysis rate of nanoparticles with different concentrations.

Figure 8

a Tumor growth curves of nude mice injected intraperitoneally with saline (control group) and different nano-drug delivery systems. **b** Body weight change of nude mice during 21 days administration.

a **b**

Figure 9

a Drug distribution in nude mice after injection of DOX, G-C-P-D, and A-G-C-P-D. **b** Drug content of organs (heart, liver, spleen, lung, kidney, tumor).

a **b** **Figure 10**

a H&E staining of heart, liver, spleen, lung, kidney and tumor tissue sections of nude mice. **b** Immunohistochemical detection by Ki67 and Caspase-3 staining of tumors treated with saline or different nano-drug delivery systems.

Supplementary Files

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- [Scheme1.png](#)