Carbon dots-based nanocarrier system with intrinsic tumor targeting ability for cancer treatment

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Abstract

Doxorubicin (DOX) is a traditional broad-spectrum antitumor drug, which has a wide range of clinical applications, but has no tumor non-specificity. Nanoparticles have been explored as drug delivery agents to enhance the therapeutic efficacy and reduce toxic and side effects. Carbon dots (CDs), a carbon-based nanomaterial, has many unique advantages such as easy synthesis, good biocompatibility, and low toxicity. In this study, folic acid was used as raw material to prepare new CDs, and DOX was loaded on the surface of CDs through electrostatic interaction. The prepared nano-drugs CDs/DOX could effectively release DOX under mild acidic pH stimulation. Cell imaging showed that CDs/DOX could transport doxorubicin (DOX) to cancer cells and make them accumulated in nucleus freely. Flow cytometry tests and cellular toxicity assay together confirmed that CDs/DOX could target tumor cells with high expression of folate receptor and increase anti-tumor activity. The therapeutic effect on 4T1 tumor-bearing mice model indicated that CDs/DOX could alleviate DOX-induced toxicity, effectively inhibit tumor growth, and prolong the survival time. Hence, such a targeting nanocarrier is likely to be a candidate for cancer treatment.

1. Introduction

Cancer has become a major public health problem in the world, threatening human health. At present, chemotherapy is an effective method for the treatment of cancer [1, 2]. However, many chemotherapy drugs such as doxorubicin, oxaliplatin, camptothecin, and paclitaxel cannot distinguish between cancer cells and normal cells. Chemo drugs kill cancer cells, but they also damages some normal cells. The resulting serious side effects have limited their widespread use in cancer treatment. In recent years, drug delivery systems have developed rapidly. Nanocarriers such as liposomes, micelles, inorganic nanoparticles, and carbon nanotubes have been widely used in cancer treatment [3–6]. An important factor in nanocarriers is their ability to target cancer cells. The establishment of tumor-targeting nanocarrier is an effective strategy to improve the efficacy of chemotherapy drugs.

Carbon dots (CDs) have been reported as fluorescent markers exhibiting excellent optical properties, low toxicity, good chemical stability, rich surface groups and favourable biocompatibility [7–9], which appear to be promising candidates for drug delivery [10–13]. According to recent reports, various methods have been conducted to synthesize CDs through diverse approaches, such as microwave methods [14–16], teflon hydrothermal reactor [17] and electrochemistry [18]. In order to achieve different functions, the CDs’ surface requires coupling of different functional reagents, including targeting ligands and anticancer drugs. Recently, it has been reported that nuclear localization signal peptide (NLS) was used as a target ligand to chemically conjugate with CDs. The resulting complex NLS-CDs could deliver DOX to the cancer cell nucleus, effectively inhibiting the growth of A549 tumor in mice [19]. In addition, folic acid (FA) was used as a target ligand to covalently link onto the surface of CDs [20]. After DOX was loaded, the resulting FC-dots–FA–DOX complex
showed a higher cancer cell killing rate and less toxicity to normal cells compared with free DOX. FA has been exploited as a navigational molecule because many cancer cells tend to overexpress folate receptors (FR). Recent research has demonstrated that some CDs have intrinsic tumor-targeting ability and can be directly used as carriers for anti-cancer drugs [21, 22]. It inspired us to develop a simple strategy to fabricate new nano-drugs with tumor-targeting capabilities.

Doxorubicin (DOX) is anthracycline-based drugs with a broad spectrum of antitumor activity, it can cross the cell membrane, reach the nucleus, and eventually lead to DNA damage and inhibition of nucleic acid synthesis [23]. It is well known that the inhibitory effect of DOX on cancer cells lacks specificity. Thus, it is quite meaningful to synthesize tumor-targeting nanocarriers to achieve the loading, delivery, and release of chemotherapy drugs.

In this research, we synthesized blue fluorescent CDs by a facile one-step hydrothermal approach using FA as the raw material. Positively charged DOX was adsorbed to the surface of the negative zeta potential CDs via electrostatic interaction, resulting in the formation of nano-drugs CDs/DOX. CDs/DOX could selectively enter tumor cells with high expression of FR, and deliver DOX to the nucleus. We investigated the pH-dependent release of CDs/DOX, in vitro cytotoxicity, and cell uptake by flow cytometry and confocal microscopy. Furthermore, the breast cancer 4T1 tumor-bearing mouse model was established to evaluate the therapeutic effect of CDs/DOX in vivo. This paper proposed a simple strategy for constructing nano-drugs with tumor-targeting ability, which is expected to efficiently deliver DOX specifically to cancer cells for medical effects.

2. Experimental

2.1. Materials and methods
FA was obtained from Energy Chemica. Doxorubicin (DOX) was purchased from Aladdin. All the chemicals and reagents were used without further purification. Transmission electron microscopy (TEM) images of CDs and CDs/DOX were collected using a transmission electron microscope (JEM-2100). The chemical composition of the fluorescent CDs was characterized by x-ray photoelectron spectroscopy (XPS, Thermo ESCALAB 250Xi). Ultraviolet visible absorption spectra were measured on an UV–vis spectrophotometer (UV-3600 Plus). Fluorescence spectra were recorded on a Perkin Elmer LS-55 fluorimeter. Zeta potential and Dynamic Light Scattering (DLS) were determined using a ZETASIZER Nano ZS (Malvern, U.K.). Fourier transform infrared spectra (FT-IR) were recorded on a VERTEX 70 (Bruker, Germany) ranging from 375 to 8000 cm$^{-1}$. Flow cytometry tests were measured on a BD Facsverse (BD, America). Cells morphological changes were taken using an ArrayScan VTI HCS 600 (TFS, America). Confocal microscope images were performed with Ziss 880. Images of biological tissue sections were acquired using a fluorescent inverted microscope Ti2-U (NIKON, Japan).

2.2. Synthesis of carbon dots
CDs were synthesized by hydrothermal reaction under high temperature and high pressure referring to relevant literature [24]. Detail synthesis procedure was implemented as follows: 100 mg FA was dispersed in 100 ml of pure water, and mixed by ultrasonic method. The mixture was then sealed in a Teflon-equipped stainless autoclave and heated 200 °C for 6 h. The autoclave was cooled at room temperature. The obtained yellow mixture was centrifuged to remove large particles. The dialysis bag (MWCO 1000 Da) was used for purification and equilibrated for 24 h against pure water. Then, the solution was filtered through a 0.22 μm microporous membrane. Finally, the yellow solid CDs were obtained by freeze-drying CDs aqueous solution.

2.3. DOX loading and pH dependently releasing
A series of CDs-DOX conjugates were prepared with various weight proportions of CDs and DOX. In short, different concentrations of DOX were added into CDs (4 mg ml$^{-1}$), and the mixture was oscillated in the dark for 4 h before being stored in 4 °C.

To reveal the impact of tumor microenvironment to the drug release, 6 ml of PBS solution with different pH values (7.4 or 5.5) containing 50 mg of 0.15 eq. CDs/DOX was put into a dialysis bag (MWCO 1000), which was immersed in 400 ml of PBS and stirred at 37 °C. Then, 1 ml of solution was taken out and replaced with the same volume of PBS at different time points. The amount of DOX in the obtained supernatant was calculated by UV absorbance at 488 nm.

2.4. Cytotoxicity
The MTT assay was carried out to assess cytotoxicity of CDs and CDs/DOX complexes to HeLa cells. Cells were suspended in a 96-well plate with a density of 4500 cells per well for 24 h incubation. DOX or CDs/DOX complex at different DOX concentrations (0, 1, 1.5, 2.5 and 3 μg ml$^{-1}$) were added to each well and cultured
for 24 h or 48 h, respectively. Then, MTT solution was added to each well for further 4 h incubation, and the formazan crystals were dissolved by DMSO. The cells viabilities were determined by the absorption values at 570 nm. Each sample needed five parallel experiments under identical condition. A similar MTT method was used to detect the cytotoxicity of CDs (0, 20, 50, 100, 200 and 500 $\mu$gm l$^{-1}$) to HL-7702 cells and HeLa cells.

2.5. Cells fluorescent imaging
In order to observe the uptake process and fluorescence changes of CDs/DOX in HeLa cells, 10 $\mu$gm l$^{-1}$ CDs/DOX was added into HeLa cells and cultured for a period of time. Confocal laser scanning microscopy was used to track the cellular uptake of CDs/DOX at 1, 4, 6 and 8 h, respectively.

In the other group, HeLa cells were pretreated with Hoechst 33342 for cell nuclei staining, and then incubated with the same amount of DOX for 6 h. The fluorescence signals of Hoechst 33342, CDs and DOX were excited at 405, 380 and 480 nm and detected at around 450, 450 and 580 nm, respectively.

2.6. Flow cytometry study
The extent of difference cells’ uptake of free DOX or CDs/DOX was determined using flow cytometry by measuring the fluorescence intensity of intracellular free DOX. Briefly, Cells (HL-7702, A549 and HeLa) suspended in 96-well plates were added with free DOX or CDs/DOX for 6 h incubation at 37 °C separately, in which the DOX content was 1.2 $\mu$g ml$^{-1}$. Then the intracellular fluorescence was measured by flow cytometry in the PE channel under 488 nm excitation wavelengths.

2.7. Antitumor activity in vivo
The antitumor activity of CDs/DOX complexes were observed in 4T1 tumor-bearing mice. Male BALB/c mice (6–8 weeks old) were purchased from the experimental animal center of Zhengzhou University. The 4T1 cells ($2 \times 10^7$) were subcutaneously injected into the right anterior armpit of each mouse. The tumor volume reached about 100 mm$^3$ after feeding for 7 days. Then, the tumor-bearing mice were randomly divided into 3 groups (16 mice per group), and each group was intravenously injected with normal saline, free DOX and CDs/DOX via the tail vein every two days at a dose of 5 mg DOX per kg of body weight, respectively. The tumor volume and body weight were periodically recorded. Tumor volume was calculated as the following formula: tumor volume = length $\times$ width $\times$ width/2. At the end of the experiments, the mice were sacrificed, and the tumors were collected and analyzed.

Mice were injected with saline, CDs and CDs/DOX, and sacrificed after 10 days. The tumors and main organs (heart, liver, spleen, lung and kidney) were taken, fixed with formalin solution, embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E). Finally, the stained sections were observed with a microscope.

3. Results and discussion

3.1. Characterization
The nanocarrier CDs and the nanodrugs CDs/DOX were synthesized as shown in scheme 1. CDs were prepared using folic acid through hydrothermal reaction, and DOX was loaded onto the surface of CDs through electrostatic interaction to construct the drug delivery system CDs/DOX. The morphology of CDs and the complexes CDs/DOX were analyzed by TEM as presented in figure 1. The carbon dots were well-dispersed with relatively uniform size. The embedded columns plots showed that CDs/DOX possessed an average diameter of 6.1 nm, which was larger than CDs’ average diameter of 4.7 nm, implying that DOX molecules were successfully adsorbed on the CDs surface.

The chemical structure of the CDs was characterized using FTIR and XPS. The FTIR spectra of CDs were depicted in figure S3. The broad absorption centered at 3420 cm$^{-1}$ was attributed to the stretching vibrations of NH$_2$ and OH groups. The multiple peaks around 1650 cm$^{-1}$ corresponded to the C–O bond stretching vibration of carboxyl groups and N–H bending vibration, respectively. XPS analysis was adopted to further
The elemental composition of CDs shown in Figure S2. The XPS spectra showed the CDs had elemental compositions including carbon, nitrogen and oxygen, and the corresponding content was 63.62%, 17.8% and 18.58%, respectively. The pronounced C1s XPS spectrum was deconvoluted into four peaks at 285.8 eV, 288.1 eV, 284.4 eV and 286.5 eV, which were assigned to C–N, C–O/COOH, C–C=C and C–O, separately. The N1s XPS spectrum had two deconvoluted peaks at 399.2 eV and 400.1 eV belonging to N–C=N=C and N–H groups, respectively. Two peaks were deconvoluted at O1s XPS spectrum, the binding energy at 531.3 eV and 532.4 eV were attributed to C=O and C–OH, separately. The obtained CDs possessed high water solubility, probably due to the various groups. The result indicated that the existence of COOH on the surface of the CDs, which increased the electronegativity of CDs as mentioned below.

3.2. Photophysical properties

Figure 2(c) displayed the zeta potential measurements of CDs, DOX and CDs/DOX complexes. The CDs had a zeta potential value of −16.7 mV, which was due to the abundance of negatively charged carboxyl group functions on the surface. The electronegativity tended to be weaker with the DOX loading increased. The mass ratio of 0.05 DOX/CDs conjugation was still far away from being saturated, which was selected in the consequent experiments.

The UV–vis absorption of CDs, DOX and CDs/DOX were displayed in figure S1 is available online at stacks.iop.org/NANOX/1/030007/mmedia. CDs exhibited an absorption peak at around 350 nm, and DOX presented a characteristic absorption peak at 480 nm. While the absorption spectrum of CDs/DOX displayed two broad bands at nearly 350 and 500 nm. The typical absorption peak of DOX at 480 nm red-shifted, indicating the conjugation of DOX to CDs. The emission peaks of CDs gradually red-shifted with excitation wavelength increasing from 300 to 440 nm (figure 2(a)). We chose 380 nm/450 nm as excitation/emission wavelengths for the following experiments. The fluorescence intensity of CDs/DOX decreased with the increasement of DOX content (figure 2(b)). The fluorescence quenching of CDs by DOX proved that DOX was
successfully loaded onto the surface of CDs. Besides, CDs/DOX displayed a new fluorescence peak at 600 nm attributed to DOX, which further confirmed the successful fabrication of CDs/DOX complex. The fluorescence intensity of CDs exhibited dependence on the pH values. As shown in figure S4, although the fluorescence intensity of CDs significantly enhanced in the alkaline solution at pH of 10, it showed stable emission in the pH range of 2–8, which was suitable for biological imaging.

To investigate the release behavior of DOX from the complex CDs/DOX, the kinetics of drug release was investigated at pH 7.4, 6.5 and 5.5, respectively. As can be seen from figure 2(d), CDs/DOX displayed significant pH-responsive drug release profiles. The release rate of CDs/DOX at both pH 6.5 and pH 5.5 was notably higher than that at pH 7.4. In mildly acidic environments (pH 5.5–6.5), drug release was significantly increased (up to 85%), much higher than that in the pH 7.4 (51.5%). Considering the slightly acidic pH values of the tumor microenvironment, the pH-responsive nanodrugs would help chemotherapy drugs to fully exert antitumor effects and alleviate the toxicity to normal tissues.

3.3. Cell viability studies
The cytotoxic effects of CDs on two type cells were investigated using MTT assay. As shown in figure 3(a), it can be seen that after incubation for 24 h, the cytotoxicity of CDs to HeLa cells is stronger than that of normal cell HL–7702 cells. Due to the overexpression of FR in HeLa cells and the low expression of FR in HL–7702 cells, it was speculated that the folic acid residues on the surface of CDs still had the ability to recognize FR on the surface of tumor cells [25], so that more CDs entered HeLa cells and increased cytotoxicity. The IC50 value of HL–7702 cells was 4280 µg ml−1, demonstrating that CDs had low toxicity.

The cytotoxicity of CDs/DOX on HeLa cells was further evaluated as displayed in figure 3(b). After incubation for 24 h, the survival rate of HeLa cells in the CDs/DOX group was lower than that of DOX group, indicating that CDs, as a nanocarrier, could increase the tumor cellular uptake of DOX and enhanced cytotoxicity. Moreover, after 48 h incubation, a considerable reduction in cell viability was observed in the CDs/DOX group due to the increasing total cumulative dose of DOX from the complex CDs/DOX. Thus, CDs, as a targeted drug carrier, could increase the toxicity of DOX to tumor cells.

In order to further confirm that CDs/DOX had the function of targeting tumor cells with high expression of FR, flow cytometry was used to detect the uptake of CDs/DOX by normal cells (HL–7702) and cancer cells (HeLa and A549). As shown in figure 4, the corresponding fluorescence intensity of these cells revealed a remarkably difference after incubation for 6 h. The fluorescence intensity of A549 cells and HL–7702 cells was relatively low. In comparison, the fluorescence intensity of CDs/DOX entering in HeLa cells was obviously stronger than that entering HL–7702 cells and A549 cells, indicating that more CDs/DOX significantly entering HeLa cells. The results may be ascribed to different mechanisms of cellular uptake [26, 27]. It is well known that free DOX enters cells by passive diffusion, and previous literature demonstrated that CDs enter cells through endocytosis pathway and passive diffusion [28, 29]. In this work, the folate-mediated endocytosis pathway played an important role in the uptake of CDs/DOX by HeLa cells. Thus, the CDs with tumor targeting ability could significantly improve the cellular uptake of CDs/DOX.

The cellular uptake behavior and sequential drug release of CDs/DOX in HeLa cells were investigated by confocal laser scanning microscope. As shown in figure S4, HeLa cells incubated with CDs/DOX for the indicated times were monitored. With the extension of culture time, intracellular fluorescence of CDs and DOX gradually increased, exhibiting the release process of DOX in living cells. By monitoring co-localization with nuclear dye Hoechst 33342, it was determined that the red fluorescence signals of free DOX group were mainly localized in nucleus (figure 5). At the same time, the red fluorescence in CDs/DOX group also showed obvious nuclear accumulation, with blue fluorescence of CDs almost distributing throughout the cell. The results demonstrated that CDs could deliver drugs into the nucleus.

Figure 3. (a) Cytotoxicity study of CDs on HL–7702 and HeLa cells for 24 h; (b) Cell viabilities of HeLa cells incubated with free DOX or CDs/DOX at different concentrations for 24 or 48 h.
Annexin V-FITC/PI assay were performed to investigate the cell apoptosis, which were common methods in previous studies \[30\]. As shown in figure 6, compared with the DOX group, CDs/DOX induced higher apoptosis rate. The improvement of apoptosis rate may be due to the increased internalization of CDs/DOX complex, which is conducive to improving the therapeutic effect of the chemotherapy drug.

AO/EB staining was used to investigate the cells morphological changes. In figure S7, No obvious apoptosis was observed in the control group, while the cell morphology changed significantly in the presence of DOX or CDs/DOX. Cells displayed fragmented and condensed chromatin and appeared yellow to orange, which is characteristic of apoptotic cells. As the content of CDs/DOX increased, the number of apoptotic cells increased, and necrotic cells accounted for a tiny fraction. The results were consistent with the flow cytometric analysis.

### 3.4. In vivo antitumor efficacy

Previous literature demonstrated that FR receptor was also highly expressed in 4T1 cells \[31, 32\]. Thus, 4T1 tumor-bearing BALB/c mice were used to evaluate the antitumor activity of CDs/DOX. CDs/DOX was intravenously injected into mice with 4T1 tumor every 2 days. It is worth noting that the CD/DOX group had a significant inhibitory effect on tumor size, and the tumor volume began to decrease from the fourth day of administration (figure 7). In contrast, the tumor volume of mice in the saline group and the DOX group increased significantly. Moreover, mice in CDs/DOX group didn’t experience any weight loss, while those in the DOX group displayed significant weight loss due to the toxic side effects of free DOX that spread through the
bloodstream. In addition, CDs/DOX had a significant effect on prolonging the survival time of tumor-bearing mice compared with both the saline and free DOX groups (figure 7(d)). The results indicate that CDs/DOX could effectively deliver DOX to tumor, reduced cytotoxicity, and increased anti-tumor effect.
To further reveal the anti-tumor effect of CDs/DOX, mice in the three groups were sacrificed after 10 days of treatment, and the heart, liver, spleen, lung, kidney, and tumor were collected. H&E staining images of tumor slides showed that the cancer cells in the CDs/DOX group were obviously damaged (figure 7(c)), further providing an additional evidence of the effective antitumor activity of CDs/DOX in vivo. In addition, histological analysis of the main organs including heart, liver, spleen, lung and kidney was shown in figure 8. No obvious organ damage was detected in the DOX group, CDs/DOX group, and saline group. Overall, CDs with tumor-targeting function could improve therapeutic efficiency and reduce systemic toxicity, which may be a promising carrier for DOX delivery.

4. Conclusions

In summary, CDs with tumor-targeting ability were prepared to effectively deliver drug to tumor. Flow cytometry tests as well as cell imaging confirmed CDs/DOX could selectively accumulate in the tumor cells with high expression of FR, and delivery doxorubicin (DOX) into the nucleus. 4T1 tumor-bearing mice were used to assess the efficacy of CDs/DOX. CDs/DOX displayed superior tumor growth suppression, less toxicity and longer survival time compared with the DOX group. These properties make CDs/DOX have the potential to become nano-drugs for tumor targeted delivery and cancer treatment.

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Conflict of interest

The authors declare no competing financial interest.

Data availability statement

The data that support the findings of this study are openly available.

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