Tumor Microenvironment Stimuli-Responsive Macrophage Cell Membrane-Decorated Multi-Functional Self-Assembled Gold-Quantum Dots Nanomaterials for Tumor Theranostic

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Abstract

Tumor microenvironment (TME) is intently related to tumor growth, progression and invasion, leading to drug resistance and insufficient therapeutic efficacy. However, remodelling TME and utilizing TME for exploring intelligent nanomaterials that can realize tumor theranostic is still challenging. Nowadays, the theranostic based on chemotherapy exposes some deficiencies, such as low targeting, weak permeability and premature clearance. Furthermore, it is challenging to cure drug-resistant tumors effectively. For the sake of solving these problems, a biomimetic decomposable nano-theranostic (MMV-Au-CDs-DOX) was well-established in this work. The Au-CDs are coated with macrophage-derived microvesicle to realize drug release accurately and enhance the biocompatibility of internal nanoparticles. Furthermore, MMV-Au-CDs-DOX would locate in the inflammation position of tumor, and disintegrate correspondingly into pieces with certain different functions stimulated by TME. Subsequently, the released anti-tumor nanodrugs were used for multimodal therapy, including chemotherapy and chemodynamic therapy. In addition, combined with the ability of Au-CDs to recognize GSH specifically, the off-on fluorescent probe was constructed to monitor the GSH of patients and provided information on chemotherapy resistance.

Introduction

Tumor microenvironment (TME), presenting hypoxia, reducing conditions, acidic pH and hydrogen peroxide (H$_2$O$_2$) overexpression characteristics and so on [1], reduce a breeding ground for carcinoma cells origination and residence, which could induce malignant tumor progression, high drug resistance and aggressive metastasis [2, 3], and pose a severe challenge for the development of novel anticancer strategies. However, from another perspective, the TME distinct from normal cells and tissues provides effective therapeutic targets for cancer treatment [4]. To achieve precise treatment of tumor, the adoption of intelligent nanomaterials that exhibit TME stimuli-responsive theranostic capacities has been expected to be one of the ideal ways to improve therapeutic efficiency while minimizing side effects [5]. Although broad researches have been conducted on the development and construction of the TME-triggered theranostic platform, endowing such a system with satisfactory characteristics of programmed control is still highly desirable.

Assisting by the fast development of nanotechnology, engineered nanomaterials have become an ideal way to construct a programmed control system under the extracellular and intracellular TME activation [9, 10, 11]. Among them, the procedural disintegration of nanocomposites is a valuable method for producing size-tunable nanomaterials. Generally, the hierarchical self-assembly of complex nanocomposites is the prerequisite for achieving procedural decomposition. Meantime, the process of self-assembly is mediated by structurally different building blocks organic macromolecules between inorganic nanoparticles using synergetic non-covalent interactions, such as electrostatic interactions, hydrophobic interactions and hydrogen bond interactions [12, 13]. Of note, the excellent availability of building block units, the simplicity of synthesis and synergistic emerging properties make self-assembly work as a powerful technique for constructing functional hierarchical materials [14, 15]. More importantly, on the concept of theranostic, fabricating nanomaterials using assembly strategies by integrating with
diverse inorganic nanoparticles could also endow nanomaterials with multifunction besides improved enhanced-permeation-and-retention (EPR) effect, including drug loading, magnetic, heating, and fluorescence effects; while efficient self-assembly might be used to control the size, surface charge, and water-solubility of the nanocomposites, and resulting in new applications, ranging from optical and electronic devices to cellular imaging, biological sensing and disease treatment [16, 17, 18].

Carbon dots (CDs) and gold nanoparticles (AuNPs), the versatile and functional materials, usually served as the “star” building block units, gained increasing interest due to their extensive application to delivery or act as the theranostic agent to lesion location, especially for tumor [19, 20]. Basically, CDs is a potential fluorescence carbon-based material with uniform size, remarkable modifiability and biocompatibility [21, 22]. However, the size of traditional CDs is usually less than 10 nm which restrict its accumulation on the tumor site effectively because of the poor effect of EPR, leading to reduced theranostic outcomes [23, 24]. Furthermore, the uncontrolled size and miscellaneous surface ligands are double-edged, limiting the flexibility of application, while provided multiply dentate N/O donor atoms contained ligands for integrating metal nanoparticles. Meantime, the non-therapeutic of CDs restricted its application in drug therapy [25]. As AuNPs were widely used for tumor photothermal therapy, the catalytic activity, such as dynamic therapy, was rarely investigated [26]. Interestingly, AuNPs could be successfully dispersed on the carbon-based substrate, and the catalytic activities of the nanomaterials were appropriately applied by controlling their nanostructures and compositions [27, 28]. What's more, electron transfer between CDs and AuNPs would induce the dynamic change of fluorescence during assembling, which is a benefit for constructing the off-on sensing interfaces.

Herein, in this paper, we fabricated an intelligent nanomaterial, called Au-CDs, via “step by step” inter-particle assembled, which exhibited glutathione (GSH) and H2O2 sensitive procedural disintegration and fluorescence enhancement, besides size controllability and variability; and be used as the specific anti-tumor adjuvants after being biomimetic modified by macrophage-derived micro-vesicle (MMV) for the treatment of the lung metastatic breast cancer, as well as for the test of tumor drug resistance. The biomimetic MMV-Au-CDs-DOX nanocomposite would locate in the inflammation position of tumor driven by the chemotactic cell membrane and disintegrate correspondingly into pieces with certain different functions stimulated by TME, such as GSH and H2O2, which exhibited selective tumor targeting and precisely controlled drug release. Subsequently, the released anti-tumor nanodrugs were used for multimodal tumor therapy, including chemotherapy (DOX) and chemodynamic therapy. In addition, combined with the ability of Au-CDs to recognize GSH specifically, the off-on fluorescent probe was constructed to monitor the GSH in the tumor lesion of cancer patients and provide the information on chemotherapy resistance (as shown in Scheme 1).

Materials And Methods

Materials
Cetylpyridinium chloride monohydrate (CPC, 98%), sodium hydroxide (NaOH, 97%), gold chloride hydrate (HAuCl$_4$, 45.5%), sodium borohydride (NaBH$_4$, 98%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrochloric acid (HCl, 37%), dichloromethane (DCM, 99.9%) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd.

The human umbilical vein endothelial cells (HUVEC) and murine breast cancer 4T1 cell line (4T1 cells) were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Healthy male ICR mice (18-20 g) were purchased from Laboratory Animal Center, Chongqing Medical University, Chongqing, China.

All the animal procedures in this work were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Chongqing Medical University, and approved by the Animal Ethics Committee of Chongqing Medical University (SCXK2018-0003).

**Apparatus**

UV-Vis absorption spectra were recorded with a Model Ultra-6600A (Rigol, China). PL spectra were recorded by RF-5301 spectrofluorophotometer (Shimadzu, Japan). All optical measurements were performed at room temperature. The size and ζ-potentials measurements of Au-CDs-based nanocomposites were characterized by Mastersizer 2000 Laser Particle Size Analyzer (LPSA, Malvern, British). In addition, the TEM images of the samples were taken on a JEOL JEM-2100 transmission electron microscope with an acceleration voltage of 200 kV. Carbon coated copper grids were dipped in sample solution to deposit them on the film. The absorbance for the MTT assay was measured with a Tecan’s Infinite M200 microplate reader at a wavelength of 490 nm. Finally, the fluorescence images of cells were obtained by confocal laser scanning system (Leica, Germany).

**Synthesis of cpc-CDs cluster**

The water-soluble cpc-CDs cluster was prepared according to our previous reports [29]. In brief, 2.0 M NaOH was added into a CPC aqueous solution (15 mM) and left to stand at room temperature without applying any external energy. The color of the mixture solution turned from colorless to light yellow and then to wine red as the reaction time proceeded, indicating the carbonisation of CPC. The reaction was terminated 24 h later by adjusting to neutral (pH = 7) with HCl. After the reaction, oil-soluble CDs could be separated by adding DCM to extract the oil-soluble CDs into the bottom organic layer. Subsequently, the oil-soluble CDs were transferred into water by simply ultrasound assistant self-assembly. Typically, 50 μL of the oil-soluble CDs (1 mg/mL) in DCM were mixed with 1 mL water by sonication in a cold-water bath. The dichloromethane was gradually evaporated, yielding a slightly turbid solution. After centrifugation, the aqueous CDs-clusters were obtained and kept at room temperature for further use.

**Preparation of Au-CDs**
Au-CDs was fabricated by the inter-particle assembly of gold nanoparticles and cpc-CDs. Firstly, 200 μL of the cpc-CDs were mixed in 1.9 mL NaOH solution (pH = 8.0). Subsequently, 1% HAuCl₄ solution (300 μL) dropped slowly into the above reaction solution with slightly stirring at 30 °C. Dripping finished, a little superfluous NaBH₄ solution (1 mg/mL) was slowly added into the reaction system until the color of the mixture solution turned from light yellow to dark grey. Then, the obtained aqueous solution was further centrifuged at 5,000 rpm for 10 minutes, separated the supernatant to obtain light brown Au-CDs aqueous solution, which was changed into solid grey powder after freeze-drying.

**Drug loading**

To construct Au-CDs-DOX and Au-CDs-ICG, 1mg DOX or 1mg ICG was first dissolved in dichloromethane (1 mL) and then added into 4 mL prepared Au-CDs solution under sonication in a cold-water bath. After the dichloromethane was evaporated, the limpid Au-CDs-DOX or Au-CDs-ICG nanoparticles were yielded in a water solution. Followed by stirring at room temperature for 24 h, the crude product was purification by dialysis (MWCO 1000 D) in distilled water for another 24 h. Furthermore, the standard curve of DOX was settled by the UV absorption peak (λ=550 nm) to calculate the concentration of DOX in Au-CDs-DOX.

**Preparation and characterization of MMV-Au-CDs-DOX**

The MMV was prepared according to our previous work [30]. Then, MMV was added to the Au-CDs-DOX solution and sonicated for 5 min at a power of 100 W to collect crude MMV-Au-CDs-DOX. The free MMV was removed by centrifugation at 4000 rpm for 20 min. After centrifuging at 15000 g for another 30 min, the purified MMV-Au-CDs-DOX was collected and lysed for western blot and SDS-PAGE electrophoresis analysis (total macrophages were used as a control for analysis).

**Drug release**

To investigate the DOX release behavior under different condition, 1 mL of each formulation encapsulated 0.7996 mg DOX was conveyed into a dialysis bag (MWCO 1000 D) and then put in 10 mL PBS with the simulated TME condition (pH=5, GSH=10 mM) or the neutral condition (pH=7). Finally, 1 mL release medium was collected at predetermined time points, followed by replenishing with an equal volume of fresh medium, and the UV-vis absorption quantified the released DOX.

**In vitro and in vivo GSH detection**

To examine whether the synthesized Au-CDs have the potential ability to detect endogenous and exogenous GSH, firstly, 100 μL GSH (10 mM) was added into 1 mL Au-CDs solution under different pH (5, 8 and 12), and the UV-vis collected the absorption change. Next, 100 μL GSH (10 mM) was added into 1 mL Au-CDs solution (pH=8) in different incubation times (0, 5, 10, 20 and 30 min, respectively), and UV-vis also observed the absorption change. Furthermore, the GSH sensing sensitivity of Au-CDs was evaluated by range concentration detection. In brief, different concentrations of GSH (0 to 40mM) were added into 1 mL Au-CDs solution for incubating 5 min followed by UV irradiation. Digital photos took the properties
change of solution. Finally, we examined the intracellular GSH detection ability of Au-CDs, 10 mM GSH was added into the prepared 4T1 cells in 12-well plates, after incubating 30 min, the Au-CDs was added and incubated for another 90 min, followed by washing with sterilization PBS, CLSM acquired the fluorescence image of intracellular GSH.

**In Vitro Cell Study**

Cell Culture: The cells (4T1 and HUVEC) were cultured in DMEM medium supplemented with 10% (v/v) calf serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity Evaluation: The in vitro cytotoxicity of nanocomposites was assessed preliminarily by the colorimetric MTT assay using 4T1 and HUVEC cell lines. Typically, the cells were seeded onto 96-well plates (1 × 10⁴ cells per well) and cultured at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the cells were incubated with sample solutions at a wide concentration range from 10 μg/mL to 300 μg/mL for another 24 h at 37 °C. The cells were rinsed using fresh DMEM medium, followed by incubation with 20% MTT (10 μL) at 37 °C for 4 h. After the medium was discarded, DMSO (150 μL) was used to lyse the cells. The microplate reader recorded the absorbance at 570 nm. Every experiment was conducted quintic, and the cell viability was calculated by referring to the control group without the sample treatment.

In Vitro Cell Imaging: The cell imaging experiments were performed with 4T1 cells. In brief, these cells were seeded in laser confocal scanning microscope (LCSM) culture dishes were incubated at 37 °C in a humidified incubator with 5% CO₂ until the whole cells occupied about 70% of the dish bottom space. Subsequently, Au-CDs-based nanocomposites DMEM solution (200 μL) was added to cells for co-incubation 2 h to stain. Before being imaged by LSCM, the cells were co-incubated with Hoechst 33342 for another 30 min to stain the nucleus. Then, the cells were washed by fresh DMEM three times, and the samples were measured under laser light (λ = 405, 488 nm) excitation.

**In Vivo Study**

Establishment of 4T1 breast cancer lung metastasis model: Tumor migration models were built up by tail vein injection of 4T1 cells (obtained from the mouse cancerous ascites) into each ICR mouse (4−6 weeks old, 18−20 g). After growing for 2 weeks, all of the mice were sacrificed to evaluate the area of necrotic tissues on the lung surface.

In Vivo Fluorescence Imaging: Normal saline injection (200 μL) containing Au-CDs-based nanocomposites (100 μg) was injected into the 4T1 lung metastasis mice (n ≥ 3) through the vena caudalis. Then, the mice were imaged at predetermined time intervals (0-72 h) post-injection with a homemade in vivo small animal NIR imaging system, and the background image was taken before injection.
Histology Examination

Histology analysis was carried out on the 15th day after the treatment. Typical heart, liver, spleen, lung, kidney and tumor tissues of the mice in the control and best treatment groups were isolated. Then, the organs were dehydrated using buffered formalin, ethanol with different concentrations, and xylene. After that, they were embedded in liquid paraffin. The sliced organs and tumor tissues (3-5 mm) were stained with hematoxylin and eosin (H&E) and examined by a microscope.

Results And Discussion

Fabrication of the “off-on” Fluorescent Au-CDs

Well-designed procedural-disintegrated nanomaterials were an effective way to fulfil nanocomposite with specific deformation to adapt to the complex tumor microenvironment for effective targeting and permeability. However, poor functionality and non-monitoring greatly limited its bio-application. Herein, a promising “off-on” fluorescent procedural-disintegrated nanomaterials with the therapeutic function was designed due to carbon dots -supported gold.

As shown in Figure 1A, the conception is based on the fact that (i) cpc-CDs was a potential imaging-guided drug carrier with excellent self-assembly performance discovered by our team, which could self-regulate size by adjusting amphiphilic precursor. In addition, the widespread N/O donor dentate atoms on the surface also provided the binding sites for AuNPs, while the weaker Au-N bond would disintegrate and rebind into Au-S once encountered GSH for realizing the construction and disintegration of Au-CDs comprehensively, and the release of the upload cargo in fixed space and time. (ii) individual Au atoms or small Au clusters on the carbon-based substrate could catalyze H2O2 into ROS for inducing the process of tumor apoptosis. (iii) electron transfer between cpc-CDs and AuNPs went through the whole process of assembling and dissociation, even catalysis, which achieved the “off-on” fluorescence.

Compared with the initial cpc-CDs, the absorption spectrum (Figure S1) of as-prepared Au-CDs emerged at a novel peak around 500 nm, which belonged to AuNPs, indicating the successful upload of AuNPs. Interestingly, during the assembling between cpc-CDs and AuNPs, the bright yellow fluorescence of cpc-CDs was disappeared gradually with the increase of HAuCl4 concentration (Figure 1B). And the photoluminescence (PL) spectra (Figure 1C) intuitively exhibited the process of fluorescent quenching accompanied by adding HAuCl4 solution. As expected, the size of the assembled cpc-CDs-AuNPs was also depended on the concentration of HAuCl4 (Figure S2). This phenomenon could be attributed to the intermolecular or intramolecular Au-NH bond (Figure 1G). We also observed, during the process of assembling, the ζ-potential of the complex in PBS overturned from 28 (cpc-CDs) to -58 (Au-CDs) (Figure 1D), indicating the assembled nanocomposite was stable in the dispersion medium. More importantly, at the intermediate assembled state, the morphology of the as-prepared composite was trending to aggregate (Figure 1E). Essentially, the driving force of aggregation was inter/intra-molecular interactions. As cpc-CDs and AuNPs constructed Au-CDs, Au-NH bond was rife with the surface of cpc-CDs and the
early (or smaller) assembled cpc-CDs-AuNPs, as well as the whole process of formation. And subsequently, Au-CDs were assembled into the spherical complexes, of which the diameter was ~200 nm (Figure 1F).

**Disintegration and mimetic enzyme properties of Au-CDs**

As the weak Au-NH binding force among AuNPs and cpc-CDs tended to fracture or be replaced by stronger chemical bonds (e.g. Au-S bond), which caused the disintegration of the system eventually. [31, 32]. As we know, the rich complexity of the TME is now recognized not only as an integral factor contributing to carcinogenesis but also as playing critical roles modulating cancer therapy, and the mild acid TME with the high level of \( \text{H}_2\text{O}_2 \) and GSH was most recognized by researchers. Herein, the different morphologies of Au-CDs were captured by TEM before and after incubation with the simulated TME (Figure 2B-2D).

Essentially, the fracture or formation of chemical bonds belonged to electron transfer, and the surface electron flow further affected the catalysis of metal nanoparticles, especially for AuNPs. [33]. Then we discovered that the different appearance of Au-CDs had a certain influence on the fluorescence and peroxidase property by studying PL and catalytic activity of Au-CDs before and after incubation with GSH and \( \text{H}_2\text{O}_2 \), which was the similar physiological concentration with TME. As shown in Figure 2E, the change of emission intensity was reversible after mixing with \( \text{H}_2\text{O}_2 \). During the initial 20 min incubating, the fluorescence of Au-CDs was restored gradually, and the rate of PL increase was most significant in the early stage. Accompanied with decreasing in increment, even reversal (30 to 60 min), the fluorescence intensity of Au-CDs reduced to the origin. Alternately, this phenomenon could also be explained by the electron flowing as reductive AuNPs catalyzing \( \text{H}_2\text{O}_2 \), causing the disintegration and reorganization of the Au-NH bond. At the beginning of the reaction, the weak Au-NH bond between cpc-CDs and AuNPs was destroyed by the electron flux, and the fluorescence was restored. With the response and consumption of \( \text{H}_2\text{O}_2 \), Au-CDs disintegrated into a smaller spherical particle (~12 nm, Figure 2C) compared with the integral Au-CDs (Figure 2B). As Au-S bonds are more potent than Au-N bonds, the assembled Au-CDs would completely dissociate when they coexist with sulfur compounds, like GSH and cysteine, which was proved by TEM (Figure 2D). Figure 2C and 2D showed the morphologies of Au-CDs after reacting with \( \text{H}_2\text{O}_2 \) and GSH, respectively, appeared with three existing forms of disaggregation, included incompletely dissociated Au-CDs (the agglomerates with voids) and dissociative cpc-CDs (low contrast ~ 10 nm particle) and AuNPs (the darker ~2 nm nanodot). The alterations of fluorescence intensity (Figure 2G) and oxidase activity (Figure 2F) were further verified above conjecture. The fluorescence was irreversibly and permanently illuminated after AuNPs were stripped. The peroxidase property weakened due to the more vital binding ability to surface electrons of new Au-S bonds and the larger size of gold nanoparticles.

**Detection of Glutathione in vitro and in vivo**
Chemotherapy is the conventional approach to curing tumor diseases. However, tumor micro-environment is characterised by high glutathione (GSH) expression, which seriously limits its clinical application, especially for drug resistance of chemotherapeutic hydroquinone agents. [34, 35]. Research shows that glutathione content related with multidrug resistance-associated protein, even interfered with the efficacy of radiotherapy [36]. Grasping the concentration of GSH as soon as possible, simplicity and speed are necessary for patients to acquire a more reasonable medical plan. As mentioned above, Au-CDs disintegrated after incubation with the GSH (equivalent to the TEM concentration), accompanied by the fluorescence emission resuming, which was similar to the “off-on sensor” extremely.

As shown in Figure 3A, the “off-on” sensor test paper was obtained by dropping Au-CDs into polyvinylidene fluoride membrane (PVDF membrane, the self-fluorescence was lower than that of ordinary paper). In order to get the optimal detection conditions and inspection time, the solution containing Au-CDs was co-incubated with 10 mM GSH in different pH within a certain time range (Figure 3B-3E). As shown in Figure 3B, the characteristic fluorescence emission of Au-CDs fluctuated within a narrow range under different pH conditions (the pH value was 5, 8 and 12), along with the slight fluorescence variation amplitude after reacting with GSH at pH 5 and 12. However, significantly, the fluorescence was obviously enhanced when pH was equal to 8. And the real sample photos (Figure 3C) captured under the irradiation of ultraviolet lamp ($\lambda_{\text{ex}} = 365$ nm) could visually reflect the different degrees of fluorescence increase. This phenomenon might be explained by the protonation of amino groups on the surface of Au-CDs. After that, the selection of optimum detection time was also performed in GSH solution (pH = 8). As shown in Figure 3D, the fluorescence intensity of the original wholly quenched while Au-CDs rapidly enhanced, which could be visible to the naked eyes (Figure 3E), during the first 5 min incubation, and with the stabilization of the enhanced trend, the time of maximum recovery of fluorescence was 30 min. Compared with these fluorescence enhancement values, the optimal co-incubation time was considered to be 5 min, which considerably shortened the detection time and cost.

In order to verify the sensing range for GSH, Au-CDs aqueous solution (pH = 8) was reacted with different concentrations of GSH for 5 min. Figure 3F intuitively reflected the dynamic change of fluorescence during detection and displayed that with the increase of GSH concentration (from 0 to 40 mM). The amplitude of fluorescence recovery was gradually enhanced and could be directly photographed to record as long as the concentration increased to 10 mM (equivalent to the lowest concentration of GSH in TME). Inspired by the sensitive GSH (an indicator of chemoresistance) response, Au-CDs was further developed as the guiding test paper for the clinical selection of tumor chemotherapy drugs. As shown in Figure 3G, PVDF membrane-covered Au-CDs was imaged under the light and UV lamp, and it was apparent that the “Au-CDs + GSH” group exhibited the similar fluorescence as initial cpc-CDs, while showing a significant color difference compared with Au-CDs group (the blue PL emission in H2O and CDs-AuNPs groups was the auto-fluorescence of background, and the more suitable paper will be selected for improvement in the future), which indicated Au-CDs infiltrative paper could be detected GSH in vitro. To further evaluate the ability to detect GSH in multi-level, 4T1 cell was selected as a model. Topically, an MTT assay was carried out before detecting intracellular glucose levels to assess the biocompatibility safety of Au-CDs (Figure S3). As shown in Figure 3H, the bright fluorescence of cpc-CDs was recovered as Au-CDs entered into
cytoplasm fulling of glutathione. And the fluorescence is scattered outside 4T1 cell (white arrow in figure 3H) as the redundant GSH is added into the medium. The above results indicated that Au-CDs was an exciting material that acted as an “off-on” probe for glutathione detection in multi-level.

**Drug loading, biomimetic construction and drug-sensitivity test of Au-CDs**

Previous researches showed that cpc-based CDs was an ideal carrier for hydrophobic drugs, such as loading doxorubicin (DOX), owing to the inherent amphiphilicity of CPC, and the subsequent self-assembly and accompanying water transfer [37,38,39]. Furthermore, the secondary assembly of cpc-CDs mediated by AuNPs was beneficial for enhancing the EPR effect as the accurate size control of the resultant aqueous Au-CDs clusters. Accordingly, in this work, DOX was selected as the antitumor agent and was uploaded into the Au-CDs to fabricate the drug-loaded nanocomposites (Au-CDs-DOX) to study the subsequent tumor therapy and alleviate its side effects (Figure 4).

To acquire the maximum upload volume, the oil-soluble DOX was prepared through a desalination reaction due to a previous study with a minor modification, and the obtained oil-soluble DOX was then mixed with Au-CDs to form co-assembled Au-CDs-DOX particles. As shown in Figure 4A, Au-CDs-DOX contained the intrinsic UV-vis absorption of CDs, and characteristic absorption peaks of DOX (~ 495 nm) indicated the successful loading of DOX. Additionally, the following visible color change also manifested the practical construction. At 365 nm excitation, the fluorescence peak of DOX and cpc-CDs-AuNPs appear at 598 nm and 450 nm, and they both appear in the fluorescence spectra of Au-CDs-DOX (Figure 4B), which further demonstrated the successful loaded of DOX. The uploaded amount was ~ 0.7996 mg/mL (Figure S4).

Most studies have emphasized that the macrophages micro-vesicles (MMVs) derived from cells have the ability of organ- or cell-specific tropism, so it is likely to be an ideal natural drug carrier with low immunogenicity to enhance the targeting of the action site [30, 40, 41]. For the purpose of making Au-CDs-DOX turned into a higher targeted vehicle for tumor therapy not simply through passive targeting of EPR, we next impelled biomimetic construction by using the MMVs derived from Raw 264.7 after treating with cytochalasin B (a cell-permeable mycotoxin which can block the formation of contractile microfilaments, Figure S5). TEM examinations defined the successful construction of MMV-Au-CDs-DOX as the presence of a transparent outer membranous structure around it (Figure 4D). To verify the correctness of formation, SDSS-PAGE and western blot analysis experiments were performed to prove the existence of α4 and β1 integrin, which was the tumor-targeted ligands of macrophages, of the series of sub-unit of MMV-Au-CDs-DOX. Figure 4E showed that MMV-Au-CDs-DOX had the same total protein expression spectrum as MMVs, extracted from the integral Raw 246.7 cells, while no protein signal was presented in the Au-CDs-DOX group. WB analysis (Figure 4F) was further performed to prove the existence of α4 and β1 integrin. Regarded β-action signal as the control, α4 and β1 integrin (represent as Mac-1) signal appeared in macrophage, MMV and MMV-Au-CDs-DOX group, while no protein signal was presented in the Au-CDs-DOX group. The above result showed that most of α4 and β1 integrins have remained after the micro-vesicles was separated and isolated from Raw 264.7 cell lines and cloaked drug
carriers. After extruded by polycarbonate film, the average hydrated particle size of the obtained MMV-Au-CDs-DOX was about 180 nm, and its zeta potential was found to be -49.12 mV while Au-CDs-DOX was -53.67 (Figure S6).

The drug release behaviors (Figure 4C) and cell uptake (Figure 5B) were further investigated by monitoring the fluorescence changes based on the PL signals of Au-CDs and DOX, during the incubation with simulated solution (pH = 6.5 or 7.4 with/without GSH were represented TME and body fluid respectively) and real 4T1 cell. In the simulated TME condition with the addition of GSH, the release of DOX was ~ 71%, while only ~ 8% in the condition of natural plasma imitator. Compared with Au-CDs, the distinctive green (Au-CDs) and red (DOX) fluorescence were simultaneously observed during Au-CDs-DOX and MMV-Au-CDs-DOX incubation with 4T1 cell for 4 h. Whereas, the additive intensity value of the different channels signals in MMV-Au-CDs-DOX was dramatically higher than cpc-CDs-AuNPs-DOX, which meant the MMV packaging might enhance cellular uptake.

For further confirming the antitumor activity and targeting of different nano-drugs, MTT assay was used to evaluate (Figure 5A). After being co-incubated with tumor (4T1) and normal (HUVEC) cells for 24 h, Au-CDs and Au-CDs-DOX exhibited similar trends as the survival rate of HUVEC was slightly lower than 4T1. However, the inhibitory effect of MMV-Au-CDs-DOX flipped due to the more uptake of 4T1, indicating clearly that the drug delivery system encapsulated by macrophage membranes could enhance the tumor targeting.

**In vivo anti-tumor activity of MMV-Au-CDs-DOX nanocomposite**

Inspired by the above excellent tumor targeting and antitumor activity at the cellular level, the breast cancer lung metastasis model was further used to verify the relative therapeutic indexes in vivo. Herein, ICG, having the NIR fluorescence at ~805 nm and similar polarity to DOX, was chosen to replace DOX (the absorption and PL spectra were shown in Figure S7) for tracing the nanocomposites in vivo, revealing the internal metabolism and distribution. MMV-Au-CDs-ICG (0.1 mg ICG/kg body weight) was injected intravenously into the pulmonary metastasis of 4T1 breast cancer in mice. The NIR small animal imaging system observed the PL signal of bio-distribution. As displayed in Figure 6B, the signal of MMV-Au-CDs-ICG was found in tumor at 30 min after intravenous injection and extensively accumulated at tumor site over the next 2 h.

Profiting by the favorable location targeting and the subsequent effective release of MMV-Au-CDs-ICG, we intentionally investigated MMV-Au-CDs-DOX feasibility and curative effect for treating ICR mice with breast cancer lung metastasis. Herein, mice were randomly divided into five groups (n=5) receiving specific treatments with different remedies via tail vein, and the grouping was as follows: saline alone (Group I), DOX alone (Group II), Au-CDs (Group III), Au-CDs-DOX (Group IV) and MMV-Au-CDs-DOX (Group V). As shown in Figure 6E, the mice weight of the positive DOX group gradually decreased during all the period of treatment, while the weight of negative control lost slightly relatively, which should be attributed to the intrinsic bio-toxicity of antitumor chemotherapeutic drugs for Group II, and might be the growth of tumors affected the food intake of mice and the excessive consumption of nutrients by the tumor tissues.
for Group I. Contrarily, the weight of mice in Group III to V did not change significantly, which seemed no apparent toxicity, and improve/even eliminate the side effects of DOX, indicating that transported by nano-vectors would be a beneficial delivery platform for therapeutic agents with some toxicity.

After two weeks of administration, the mice were sacrificed and separated the lungs ex vivo to observe the metastatic nodules. As shown in Figure 6C-D, a large area of tumor necrosis tissue was found in the lungs of mice treated with saline, and slightly inhibition of lung metastasis was observed in mice treated with DOX (~13% decrease compared with Group I), Au-CDs showed better inhibition of tumor growth than those of two former intervention groups. Remarkably, the metastatic nodules of mice decreased notably in Au-CDs-DOX and MMV-Au-CDs-DOX groups. Furthermore, from the results of sections of the lungs stained by H&E (Figure 6G), tumor lesions were significantly reduced after Au-CDs-DOX and MMV-Au-CDs-DOX injection compared with these in negative, positive and materials control groups, indicating that Au-CDs could increase the drug accumulation at the tumor sites via EPR effect and inhibiting metastatic nodules. After biomimetic construction, the MMV-Au-CDs-DOX would inherit the superiority of Au-CDs and improve the active target to the lung metastasis site.

As the bio-toxicity, especially the strong cardiotoxicity, of free DOX, the toxicity to various organs was further investigated. The pathomorphology analysis of the main organs, including heart, liver, spleen, lung, and kidney was shown in Figure S8. The heart section of the DOX treating mice exhibited significant lesion and lymphocytic invasion for the positive drug group, indicating high cardiotoxicity. By contrast, there were no apparent damages in the detected organs from other groups, implying that Au-CDs-DOX-based nanocomposites have good biocompatibility in vivo. 3D tumor spheroids model was used for further testing the tumor permeability of Au-CDs-DOX. As shown in Figure 6F, the red fluorescence of DOX at the free DOX group was primarily observed on the outer layer of the tumor spheroids, while that at the Au-CDs-DOX group could be observed throughout the tumor spheroids. These results indicated that Au-CDs could advance the permeability of DOX into tumor spheroids effectively causing by disintegrating into the smaller nanoparticles by the distinctive micro-environment of solid tumor

**Conclusion**

In summary, a well-designed self-assembly Au-CDs, which exhibited GSH and H$_2$O$_2$ sensitive procedural disintegration and fluorescence enhancement was fabricated via “step by step” inter-particle assembled for tumor development stage detection and treatment. During the preparation, AuNPs was exploited as a mediation for in situ aggregation of cpc-CDs through Au-NH bonds, and the bonds could further completely dissociate when coexistence with sulfur compounds, which included the sensitivity tumor drug resistance detection for GSH and even the rational targeting drug release for oncotherapy. After artificially coated with macrophage-derived microvesicle, the obtained MMV-Au-CDs-DOX would effectively target in tumor site, combing with the response disintegration of nanocomposites, higher curative effect and less side-effect was achieved. This study is interesting for exploiting a stragetegy to fabricate biocompatible CDs-based nanomaterials to improve the clinical applications of the combination
of metal nanoparticles and carbon dots, and also provides an intriguing bio-application scenarios for the inorganic nanoparticles with similar structure.

**Declarations**

**Availability of data and materials**

All data generated or analyzed during this work are included in this published article.

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**Contributions**

FW, QHY and JL carried out experiments, analyzed data and wrote the paper. TD and CY designed this study and supervised the project. Junhao Jiang assisted in the data analysis, figure preparation and discussion. All authors check and approved the final manuscript.

**Conflict of Interest**

The authors declare no conflict of interest.

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

Figure 1

(A) Schematic illustration of Au-CDs preparation. (B) The true-color photographs of as-prepared Au-CDs captured under room light and UV light ($\lambda_{\text{max}} = 365$ nm) excitation. (C) PL spectra ($\lambda_{\text{ex}} = 365$ nm) of different concentrations of Au mediated carbon dots nanocomposites. (D) \(\zeta\)-potential variation trend of cpc-CDs-AuNPs during assemble. (E) the TEM image of the intermediate assembled state and (F) the morphology of Au-CDs. (G) Cartoon model of the self-assembled procedure of Au-CDs.

Figure 2

(A) Schematic diagram of Au-CDs disintegrated by H2O2 and GSH. TEM image of (B) integral Au-CDs and dis-assemble by (C) H2O2 and (D) GSH. (E) PL spectra of Au-CDs during co-incubation with H2O2. (F) i-t curve of Au-CDs modified electrodes in PBS solution containing Gly (Glycine), Lcy (L-Cysteine) and GSH. (G) PL spectra of Au-CDs during co-incubation with GSH.

Figure 3

(A) Schematic illustration of the construction and operation of GSH test paper. (B) PL spectrum and (C) Digital photo of Au-CDs excited under 365 nm laser during co-incubation with GSH in various pH. (D) PL spectrum and (E) Digital photo at different times of Au-CDs excited under 365 nm laser during 30 min co-incubation. (F) The true-color images of Au-CDs solution with a range concentration of GSH under room light (up) and UV lamp (down) with excitation wavelength at 365 nm. (G) Test paper reaction with or without GSH under room light (up) and UV lamp (down) with excitation wavelength at 365 nm ($\blacklozenge$: H2O, $\blacklozenge$: cpc-CDs, $\blacklozenge$: Au-CDs, $\blacklozenge$: Au-CDs+GSH). (H) CLSM image of intracellular GSH levels using Au-CDs probe.
Figure 4

(A) Absorbance and (B) PL spectra of Au-CDs before and after DOX uploading, and the inserts were the digital photo of these samples captured under room light and 365 nm laser. (C) The curve of DOX release from Au-CDs-DOX and MMV-Au-CDs-DOX in different body fluid simulation, such as the fluid in peritumor tissue and blood. (D) TEM images of MMV-Au-CDs-DOX. The images of (E) SDS-PAGE and (F) western blot of Raw 264.7 cell, macrophages micro-vesicles and MMV-Au-CDs-DOX.

Figure 5

(A) Drug targeting experiment of MMV-Au-CDs-DOX via MTT assay. (B) CLSM image of the intracellular DOX release in 4T1 cells incubated with DOX, Au-CDs and MMV-Au-CDs-DOX.

Figure 6

(A) Schematic diagram of therapeutic mechanism and the specific treatment process of MMV-Au-CDs-DOX. (B) In vivo distribution of ICR mice with breast cancer lung metastasis treatment with MMV-Au-CDs-ICG. (C) Typical photographs of lungs from mice and (D) the quantitative analysis of tumor necrosis area after treated with saline alone (Group I), DOX alone (Group II), Au-CDs (Group III), Au-CDs-DOX (Group IV) and MMV-Au-CDs-DOX (Group V). (E) The weight changes of mice during the treatment for 15 days. (F) Tumor permeability verification of Au-CDs-DOX and MMV-Au-CDs-DOX via FIM images of 3D 4T1 tumor model. (G) Histological analysis of the lung lesion after treatment with different agents.

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